1 **Division and adaptation to host nutritional environment of apicomplexan**

2 **parasites depend on apicoplast lipid metabolic plasticity and host organelles**

3 **remodelling**

4 Running Title: Apicomplexa lipid synthesis controls division and metabolic adaptation

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24   phosphatidic acid, host-parasite interaction, lipidomics, host nutritional environment,

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27   **Abstract:**

28   Apicomplexan parasites are unicellular eukaryotes responsible for major human

29   diseases including malaria and toxoplasmosis. Apicomplexan parasites must obtain

30   and combine lipids both from host cell scavenging and *de novo* synthesis to maintain

31   parasite propagation and survival within their human host. Major questions on the

32   actual role for each lipid source or how these are regulated upon fluctuating host

33   nutritional conditions remain unanswered. Characterization of an apicoplast

34   acyltransferase TgATS2, shows that the apicoplast provides local (lyso)phosphatidic

35   acid balance, which is required for the recruitment of a novel dynamin (TgDrpC)

36   critical during parasite cytokinesis. Disruption of TgATS2 led parasites to shift

37   metabolic lipid acquisition from *de novo* synthesis towards host scavenging.

38   Importantly, both lipid scavenging and *de novo* synthesis pathways exhibit major

39   metabolic and cellular plasticity upon sensing host lipid-deprived environments

40   through concomitant (i) up-regulation of *de novo* fatty acid synthesis capacities in the

41   apicoplast, and (ii) parasite-driven host cell remodelling to generate multi-membrane-

42   bound structures from host organelles that are imported towards the parasite.

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Introduction:

Apicomplexa are protozoa, most of which are intracellular parasites that cause serious infectious and chronic diseases in humans including malaria, and toxoplasmosis. Most Apicomplexa harbour a relict non-photosynthetic plastid, the apicoplast, acquired by the secondary endosymbiosis of a red alga. The apicoplast lost photosynthetic capability during the conversion to a parasitic lifestyle (Botté et al., 2011; Botte et al., 2013). However, it still contains plant-like pathways including a prokaryotic type II fatty acid synthesis pathway (FASII) (Waller et al., 1998). The apicoplast is essential for parasite development and survival in both *T. gondii* and *P. falciparum* (MacRae et al., 2012).

The FASII pathway is thought to be essential for parasite survival only during specific life stages (Waller et al., 1998). Indeed in *Plasmodium,* disruption of FASII demonstrated that it is dispensable in asexual blood stages but essential for late liver stage in rodent malaria parasites, and for sporozoite schizogony during mosquito stages (Vaughan et al., 2009). Nevertheless, changes in *P. falciparum* blood stage growth conditions—such as lipid starvation during *in vitro* growth, or physiological stress in human patients—induced re-activation of apicoplast FASII (Botte et al., 2013; Daily et al., 2007), suggesting plasticity of the FASII in response to nutritional environment. In *T. gondii* FASII is essential during tachyzoite development, including for apicoplast biogenesis and parasite proliferation (Mazumdar et al., 2006; Ramakrishnan et al., 2015). Taken together, these data suggest that fatty acids (FA)/phospholipids (PL) produced by apicoplast FASII are a vital contribution to parasite membranes generated during intense stages of parasite replication and division in their life cycle.

70 Apicomplexan parasite membranes comprise up to 80% PL as a proportion of total

71 lipid classes, primarily phosphatidylcholine (PC), phosphatidylethanolamine (PE),

72 phosphatidylserine (PS) and phosphatidylinositol (PI, (Gulati et al., 2015; Welti et al.,

73 2007). *T. gondii* can readily scavenge PL, and triacylglycerols (TAG) from the host

74 but is also capable of-, and dependent on- *de novo* synthesis of several PL classes

75 (Amiar et al., 2016; Charron and Sibley, 2002; Hu et al., 2017; Nolan et al., 2017).

76 Like other eukaryotes, apicomplexan *de novo* PL synthesis is initiated by the

77 assembly of FA (i.e. esterification onto a glycerol-phosphate backbone) into specific

78 PL precursors. In *T. gondii*, FAs to be integrated into PLs derive from three not

79 exclusive sources: (i) apicoplast FASII generating FA chains (C12:0, C14:0, and

80 C16:0) (Amiar et al., 2016; Ramakrishnan et al., 2012), (ii) FA elongases located on

81 the parasite endoplasmic reticulum (ER; C16:1, C18:1, C20:1, C22:1 and C26:1)

82 (Dubois et al., 2018; Ramakrishnan et al., 2015), and (iii) FAs scavenged from the

83 host cell (Bisanz et al., 2006; Charron and Sibley, 2002).

84 Typically, phosphatidic acid (PA) is the central precursor for the *de novo* synthesis of

85 all phospholipid classes by the two-step esterification of FAs onto a glycerol-3-

86 phosphate backbone; first by glycerol-3-phosphate acyltransferases (GPAT) to form

87 lysophosphatidic acid (LPA), and then by acyl-glycerol-3-phopsphate

88 acyltransferases (AGPAT) to convert LPA to PA. In eukaryotic cells, GPATs and

89 AGPATs of diverse origins (prokaryotic, eukaryotic, plant/algal-like) work as a pair at

90 several locations within the cell. Apicomplexan seem have two pairs of

91 acyltransferases: one plastid-like pair putatively in the apicoplast, and another pair

92 predicted to work in the ER (Amiar et al., 2016; Lindner et al., 2014; Shears et al.,

93 2017). In *T. gondii* the apicoplast GPAT, *Tg*ATS1, is essential for tachyzoite

94 development where it generates LPA from apicoplast-FA for the bulk synthesis of PL

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95 (Amiar et al., 2016). Recently, PA was shown to act as an essential signal transducer

96 for microneme secretion in *T. gondii* (Bullen et al., 2016). Beyond their roles as lipid

97 precursors and signal transducers, PA (and LPA) also have important biophysical

98 properties by controlling the formation of positive or negative membrane curvatures,

99 and thereby influence the recruitment of proteins involved in membrane fusion/fission

100 events in other eukaryotic models (Brown et al., 2008; Kooijman et al., 2005; Schmidt

101 et al., 1999).

102 Lipidomics suggests that most *T. gondii* PLs are hybrids, comprising one FA moiety

103 from the apicoplast *de novo* synthesis pathway and a second one scavenged from

104 the host (Amiar et al., 2016). Thus, both scavenging and *de novo* synthesis of FA are

105 critical for intracellular development. Recent studies suggest that host nutritional

106 changes can impact metabolism, intracellular development, and thus parasite

107 virulence or stage differentiation, especially in *P. falciparum* (Brancucci et al., 2017;

108 Costa et al., 2018; Mancio-Silva et al., 2017; Zuzarte-Luis et al., 2017). However,

109 regulation of these pathways is poorly understood and nothing is known on the

110 sensing of host environmental/nutritional changes in *T. gondii.*

111 Here we characterise *T. gondii* AGPATs, focusing on one (*Tg*ATS2) that we localize

112 in the apicoplast. We show the function of *Tg*ATS2 as an acyltransferase by

113 complementing a bacterial mutant, and we construct a knockdown mutant in which

114 we observe the impact on parasite division, and the parasite lipid profile. Particularly

115 the impact of PA/LPA changes on the localization of a dynamin-related protein

116 (*Tg*DrpC) in the *Tg*ATS2 mutant is described and provides a rationale for cytokinesis

117 defects associated with drug inhibition of apicoplast FASII (Martins-Duarte et al.,

118 2015). Finally, changes in parasite lipid composition and lipid fluxes led us to subject

119 parasites to lipid starvation to explore how host nutritional environment impacts

120   parasite growth. Analysis of lipid fluxes and growth screening under adverse lipid

121   conditions shows that parasites can sense the environment and respond by (i)

122   upregulation of *de novo* lipid synthesis in the apicoplast and (ii) manipulate the host

123   through vesiculation from host organelles and import of such material to the

124   parasitophorous vacuole (PVM) mediated by export of parasite effectors to improve

125   their lipid scavenging.

126   **Results**

127   **Deletion of the *Toxoplasma gondii* apicoplast acyltransferase *Tg*ATS2 results**

128   **in replication and division defects due to aberrant cytokinesis and residual**

129   **body formation**

130   To explore *de novo* PA synthesis in *T. gondii*, we searched the genome for AGPATs

131   potentially able to catalyse the esterification of an activated FA (i.e acyl-CoA or acyl-

132   ACP) onto LPA to make PA. We found two AGPAT candidates with conserved

133   motifs: TGME49_297640, and TGME49_240860. Phylogenetic analyses reveal that

134   TGME49_297640 clusters with a prokaryotic clade with plant and algal sequence and

135   that TGME49_240860 clusters with a eukaryotic clade (**Fig. 1AB**). So we termed

136   these enzymes *Tg*ATS2, and *Tg*AGPAT based on the plant and eukaryotic

137   terminology, respectively. We generated parasite lines expressing *Tg*ATS2 and

138   TgAGPAT endogenously tagged at the C-terminus with a triple HA tag, under control

139   of their respective promoters (**Fig. S1**). Immunofluorescence assays (IFAs) confirmed

140   that *Tg*ATS2 targets to the apicoplast (**Fig. 2A**), and that transient expression of

141   TgAGAPT-HA showed a perinuclear structure that corresponds to the parasite ER

142   (**Fig. 2B**).

143   To test if *TgATS2* and *TgAGPAT* have predicted 1-acylglycerol-3-phosphate

144   acyltransferase activity, we complemented an *E. coli* temperature sensitive mutant

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145   SM2-1 Δ*plsC* lacking the AGPAT activity (Coleman, 1990), with recombinant *TgATS2*

146   *and TgAGPAT*. All transformants grew at the permissive temperature of 30°C (**Fig.**

147   **2C**). Only those complemented with bacterial *Ec*PlsC and *Tg*ATS2 grew at the non-

148   permissive 42°C **(Fig. 2C)**. The constructs with *TgAGPAT*, with or without its long

149   hydrophobic N-Ter extension*,* did not grow at 42°C likely because of *Tg*AGPAT

150   eukaryotic origin (**Fig. 1AB**). Indeed, eukaryotic AGPATs favour acyl-CoA substrates

151   over acyl-ACP substrates used in bacterial and plastid systems. Thus, it is unclear

152   from our results if *Tg*AGPAT has acyltransferase activity, but *Tg*ATS2 complements

153   PA defective *E.coli* SM2-1 AGPAT enzymatic activity *in vivo,* confirming PA synthetic

154   capability.

155   To investigate the importance of apicoplast *Tg*ATS2 during tachyzoite life stages, the

156   *TgATS2* locus was disrupted to generate a knock-in (KI) and a knock-out (KO)

157   mutant of *Tg*ATS2 using CRISPR-Cas9 strategies (**Fig. 2D**). Loss of the protein

158   product was confirmed by western blot (**Fig. 2EF**) and IFA (**Fig. 2G)**. We attempted

159   to generate a Tet inducible mutant of *Tg*AGPAT but were unsuccessful. We then

160   attempted to knock out *Tg*AGPAT-HA using CRISPR-Cas9 (**Fig. S1**) in either wild

161   type (Δ*Tg*AGPAT) or Δ*Tg*ATS2 (Δ*Tg*AGPAT-Δ*Tg*ATS2) genetic backgrounds, but the

162   parasites were not viable suggesting that *Tg*AGPAT is indispensable, consistent with

163   a genome wide knock-out screen (Sidik et al., 2016). Both Δ*Tg*ATS2 mutants were

164   viable but plaque and replication assays revealed that Δ*Tg*ATS2 had a mild yet

165   significant growth defect with significantly more small (2-4 parasites) vacuoles, and

166   significantly fewer large vacuoles (especially >10parasites that were halved) (**Fig. 2I-**

167   **K**). Parasite egress was significantly affected in the Δ*Tg*ATS2 mutant (**Fig. S1**) but

168   invasion ability showed no difference with the parental line (**Fig. S1**). Morphology of

169　different intracellular tachyzoite organelles showed no obvious defects except the

170　apicoplast displaying a biogenesis defects (**Fig. S1**).

171　We further explored parasite replication by assessing Inner Membrane Complex

172　(IMC) formation in ∆*Tg*ATS2 by expressing *Tg*MORN1-mCherry as a marker of the

173　basal complex (Lorestani et al., 2010). The expression of *Tg*MORN1-mCherry in both

174　parental and ∆*Tg*ATS2 lines seemed to show correct localization of *Tg*MORN1 to the

175　ring structure at the basal complex and centrocone (Lorestani et al., 2010).　However,

176　careful observations showed an enlargement of residual body (**Fig. 3A)**. We further

177　performed IFAs using GAP45 antibody as a marker for the IMC. Indeed, ∆*Tg*ATS2

178　parasites often displayed a larger area at the centre of big vacuoles (>4 parasites),

179　corresponding to an enlarged residual body (**Fig. 3B**). Statistical analysis confirmed

180　that the size of residual body area significantly increased in ∆*Tg*ATS2 mutant

181　compared to the parental line, especially in large vacuoles (**Fig. 3C**). Interestingly,

182　egressed extracellular parasites often remained tethered at their basal pole via a

183　plasma membrane (PM) structure (**Fig. 3D**). Electron microscopy (EM) revealed that

184　the ∆*Tg*ATS2 parasites, despite normal organelle morphology, incur numerous

185　segregation defects. Most strikingly, ∆*Tg*ATS2 cells frequently showed a late

186　cytokinesis defect in which the dividing cells were tethered to each other at their

187　basal ends (**Fig. 3E**). Parasites were attached through their PM, which was found

188　stretched, forming cytosolic bags likely explaining the enlargement of residual body

189　sizes. The IMC of the affected parasites was fully formed in each daughter cell

190　except at the basal site of daughter cell separation (**Fig. 3E[1,2]**). Furthermore, affected

191　vacuoles displayed enlarged residual bodies that often contained various

192　organelles—including the nucleus, mitochondrion, acidocalcisome vesicles, and other

193　cytosolic material—that appeared to be ejected from the dividing cells due to

194   improper segregation (**Fig. 3E**[3,4]). Pieces of mitochondria were a particular feature

195   within enlarged residual bodies (**Fig. 3E**[5,6]).

196   ***Tg*ATS2 disruption dramatically reduces C14:0 FA incorporation into *T. gondii***

197   **lipids, skews the LPA/PA ratio, and alters phospholipid abundance and**

198   **composition**

199   To investigate the role of *Tg*ATS2 in lipid metabolism, we performed lipidomic

200   analysis on the ∆*Tg*ATS2 mutant. Disruption of *Tg*ATS2 resulted in a large and

201   significant reduction of the relative amount of C14:0 (14 carbon FA chain with no

202   desaturation), which is mainly produced via the apicoplast FASII (**Fig. 4AB**). Smaller

203   but significant decreases in C18:1 and C20:1 were also observed (**Fig. 4AC**). In

204   contrast, there was a clear increase in the abundance of C18:0, C18:1(trans), C22:1,

205   C24:1, C20:4, C20:5 and C22:6 (**Fig. 4A-C**), which are most likely scavenged from

206   the host, especially C20:4 and C20:5 as confirmed by analysis of the HFF/FBS

207   content (Welti et al., 2007) (**Fig. S2**). Comparison of the relative FA abundance

208   between ∆*Tg*ATS2 and its parental line showed significant decrease of C14:0,

209   C18:1(cis), C20:0, C20:1 and C22:2 (**Fig. 4C**). These results prove that in addition to

210   the abovementioned cytokinesis defect, the ∆*Tg*ATS2 mutant has a highly modified

211   lipid content that relies less on short-chain, apicoplast-generated FAs and more on

212   long chain fatty acids scavenged from the host (**Fig. 4A-C**).

213   To further investigate ∆*Tg*ATS2 lipid defects, we analysed and quantified each PL

214   class and its FA content. The ∆*Tg*ATS2 mutant accumulates significantly more LPA

215   compared to the control parental line and slightly yet significantly less PA (**Fig. 4DE**),

216   consistent given that LPA and PA are the substrate and product of ATS2 respectively

217   (**Fig. 2C)**. This slight difference in PA likely indicates that *Tg*ATS2 is not responsible

218   for the bulk PA synthesis but rather for a specialist function. Importantly, the LPA/PA

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219   ratio was drastically affected in ΔTgATS2 (**Fig. 4E**). We investigated other related PL,

220   namely diacylglycerol (DAG), PC, PE, PI, PS, phosphatidylglycerol (PG) and

221   cardiolipin (CL, **Fig. 4D**). The relative abundance of both DAG and PG significantly

222   decreased in ΔTgATS2 (**Fig. 4D**). This is relevant since DAG is a direct product of

223   PA, and PG is the sole phospholipid made from PA in plant chloroplasts (Ohlrogge

224   and Browse, 1995). In contrast, the relative abundance of PS, PI and PE increased in

225   the mutant (**Fig. 4D**).

226   We then examined the FA profiles of each of these lipid classes. LPA had small but

227   significant increases in the amounts of C16:0 and C18:0 in ΔTgATS2 parasites,

228   whereas small, but significant, decreases in the apicoplast-specific FA, C12:0 and

229   C14:0 were observed in the mutant (**Fig. 4F**). No major difference was observed in

230   PA composition in ΔTgATS2 parasites (**Fig. 4G**). Strikingly though, DAG, PC, PI and

231   PE all had significantly reduced C14:0 content (**Fig. 4D, Fig. S2**), which is the main

232   product of FASII, and is used by TgATS1 for bulk *de novo* synthesis of PC, PI and

233   PE (Amiar et al., 2016). This indicates that TgATS2 likely uses apicoplast generated

234   C14:0 as its major substrate to make these lipids. In contrast, the levels of two long,

235   polyunsaturated FAs (PUFA), C20:4 and C20:5, in all three major PL (PC, PI and PE)

236   were significantly increased in the ΔTgATS2 line (**Fig. S2**), which is again consistent

237   with mutant parasites compensating for the lack of *de novo* made FAs by increasing

238   scavenging long chain FAs from the host. The FA composition of foetal bovine serum

239   (FBS) and host cell (human foreskin fibroblast, HFF; **Fig. S2**) confirmed that C18:0

240   and PUFA (such as C20:4, C20:5) were all found in FBS and HFF cells. This is

241   consistent with the ΔTgATS2 mutants increasing scavenging of these PUFAs to

242   make PC, PI and PE and likely not fully synthesising them *de novo*. We

243   complemented ΔTgATS2 and wild type parasites (**Fig. S2)**, using exogenous

244   PA(14:0;14:0) the putative product of *Tg*ATS2 and PA(16:0;18:1) made of host

245   derived FAs. Proliferation assays showed that both exogenous PA sources could

246   significantly boost parasite growth (**Fig. S2**), but could not rescue Δ*Tg*ATS2 growth

247   phenotype. This indicates that the PA source needs to be made *de novo* via *Tg*ATS2

248   for proper division. Since parasites are capable of scavenging lipids from the host

249   and medium, we determined whether the Δ*Tg*ATS2 imported more PA, and used PC

250   as a control. Δ*Tg*ATS2 imported significantly more PA and PC than the parental

251   control line (**Fig. S2**). Together these data on extracellular Δ*Tg*ATS2 corroborate our

252   lipidomic analyses (**Fig. 4A**) indicating that the mutant scavenges more lipids to

253   compensate for reduced *de novo* synthesis.

254   **Disruption of *Tg*ATS2 induces a mislocalisation of the parasite DrpC which**

255   **perturbs parasite cytokinesis, IMC formation and plasma membrane stability**

256   Lipidomic analyses revealed a drastic LPA/PA imbalance in the Δ*Tg*ATS2 mutant

257   (**Fig. 4E**). LPA and PA have important structural influences on membrane

258   architecture by inducing strong local membrane curvatures, which can impact the

259   recruitment and functions of specific dynamins at precise membrane domains for

260   organelle/vesicle fission: synaptic vesicles transport between neurons requires a

261   protein complex composed of a dynamin and an endophilin that exerts an ATS2

262   activity to create the proper membrane groove where the dynamin can pinch and

263   release the synaptic vesicle; or even in human mitochondrial fission by the protein

264   Dynamin-like 1, HsDrp1, which require insertion, recruitment and regulation through

265   PA (Adachi et al., 2016; Schmidt et al., 1999). In *T. gondii*, there are three known

266   Drps: *Tg*DrpA, *Tg*DrpB, and *Tg*DrpC. TgDrpA and TgDrpB have roles in apicoplast

267   fission, and secretory organelle biogenesis, respectively (Breinich et al., 2009; van

268   Dooren et al., 2009). *Tg*DrpC was recently localized to the basal poles of dividing

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269   daughter cells (Heredero-Bermejo et al., 2017). We generated a parasite line

270   expressing *Tg*DrpC fused to a 3xHA tag under the control of its endogenous

271   promoter using CrispR-Cas9 (**Fig. S3**) and localized *Tg*DrpC-HA during the

272   tachyzoite intracellular division cycle in Δ*Tg*ATS2 and its parental line (**Fig. 5A**). In

273   parental line parasites *Tg*DrpC-HA clustered in small punctate-like compartments in

274   the apical post-Golgi area during interphase (**Fig. 5A**). During daughter budding,

275   *Tg*DrpC re-localized to form two distinct ring-like structures coinciding with the

276   growing ends of the IMC from the budding daughter cells which constricted at the

277   base of the mother cell during cytokinesis and eventually formed basal caps on the

278   each newly divided parasite (**Fig. 5AB**).

279   In Δ*Tg*ATS2, localization of *Tg*DrpC-HA was drastically affected only during division

280   (**Fig. 5B**). Whilst interphase punctate localisation was unchanged, *Tg*DrpC-HA failed

281   to form the typical ring structures at the initiation and during division. Instead, it

282   scattered in the cytosol, or formed rings pushing on the side of mother IMC, or not

283   constricted at the daughter basal pole. To investigate the reason of the specific

284   mislocalisation of TgDrpC after TgATS2 disruption, we performed *in silico* analyses,

285   which showed that (i) TgDrpC is the closest homolog to the human Dynamin-like 1,

286   HsDrp1, which allows mitochondrial fission through its interaction with PA via its Stalk

287   domain including a loop with specific hydrophobic residues (Adachi et al., 2016;

288   Adachi et al. 2017), (ii) that both the Stalk domain and this PA binding loop are

289   conserved in TgDrC, (iii) but are absent in the other T. gondii TgDrpA, and TgDrpB

290   (**Fig. S3**). To confirm this, we tagged and monitored the localization of other TgDrps

291   in the Δ*Tg*ATS2 background. No obvious change in localization of *Tg*Drp*A* was

292   observed in Δ*Tg*ATS2 parasites, even during the fission of the apicoplast (**Fig. S4**).

293   Similarly, no change in the localization of *Tg*DrpB was observed upon *Tg*ATS2

294 disruption as (not shown), as expected from both IFAs and EM , showing no defect in

295 rhoptries biogenesis, where TgDrpB is involved. confirming protein sequence and

296 domain analysis. Taken together, our data strongly suggest that perturbation of the

297 LPA/PA content in ΔTgATS2 corrupts the orderly processes of cell division by

298 inducing mis-localization of TgDrpC.

299 In dividing parasites, it is commonly seen that the PM is kept connected between two

300 recently divided cells so that the cells are stuck together and distributed to daughter

301 cells during cytokinesis. Very little is known on the molecular mechanisms of the PM

302 segregation/biogenesis during cytokinesis. In the parental line parasites, initial steps

303 of endodyogeny showed the formation of the daughter cell apical pole along with

304 organelle division before the formation of the daughter cells within the mother cell

305 (**Fig. 5C**). Emergence of the daughter cells initiates the apical-to-basal biogenesis of

306 their PM, partly recycled from the mother (**Fig. 5C[1-5]**), and ends by a constriction of

307 both IMC and PM at the basal poles, leaving a small basal residual body (**Fig. 5C[6]**).

308 In contrast, there were many division and cytokinesis defects in ΔTgATS2, which

309 were unable to separate although new round of daughter formation could be initiated

310 (**Fig. 5D[1]**). Daughter cells were found tightly apposed at normal emergence sites and

311 their PMs were often missing between daughter IMCs. Instead, interconnection of

312 PM, vesicles or cisternae could be observed at these apposition sites and at the

313 basal end of dividing cells (**Fig. 5D[2]**). These defects suggested issues at the PM

314 composition and/or problems in membrane fusion/fission sites. Furthermore, there

315 was no constriction of both IMC and PM from daughter cells resulting in enlarged

316 residual bodies containing organelles and cytosol portions (**Fig. 5D[34]**). Altogether,

317 these changes in ΔTgATS2 likely results from the imbalance of the LPA/PA ratio

318 impacting membrane curvature required during cytokinesis (**Fig. 5EF**)

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319 **Nutrient starvation enhances the synthesis of FA by apicoplast FASII in *T.***

320 ***gondii* and blocks intracellular proliferation of *P. falciparum* blood stages**

321 **lacking a functional FASII.**

322 Since *Tg*ATS2 has a role in maintaining parasite lipid homeostasis, we set out to

323 determine the balance of *de novo* synthesized versus scavenged lipids in Δ*Tg*ATS2

324 using a stable isotope precursor of apicoplast synthesised fatty acids, U-^{13}C-glucose.

325 (Amiar et al., 2016; Dubois et al., 2018; Ramakrishnan et al., 2012). Incorporation of

326 ^{13}C within each FA species is detected by increase of mass and determined in

327 relation to non-labelled FA. Distribution of ^{13}C incorporation to each FA isotopologue

328 is shown as its own mass (M) plus number of ^{13}C carbon incorporation, *i.e.* M+x. In

329 both parental and Δ*Tg*ATS2 mutant lines, we observed significant differences of ^{13}C

330 incorporation in C14:0, C16:1, C18:0 and C18:1 (**Fig. 6A**). Isotopologue distribution

331 of apicoplast-signature C14:0 showed that Δ*Tg*ATS2 had ^{13}C incorporation up to

332 M+14 but major incorporation occurred at lower masses (M+8, M+10) than the

333 parental (M+12, M+14, **Fig. 6B**). This indicates that the FASII is active in Δ*Tg*ATS2

334 yet slowed down in the process of making C14:0, thus explaining the C14:0 reduction

335 previously detected (**Fig. 4A**). Similar significant, yet milder, results were observed

336 for C16:0 isotopologue distribution although overall incorporation was similar

337 between parental and Δ*Tg*ATS2 (**Fig. 6C**). C18:0 in Δ*Tg*ATS2 had higher ^{13}C

338 incorporation than the parental and its isotopologue distribution showed more short

339 FA from the apicoplast (**Fig. 6D**).

340 Lipidomic analyses thus indicate that both scavenged and *de novo* lipid fluxes are

341 modified in Δ*Tg*ATS2. To tease out the impact of host nutritional environment on both

342 pathways, we sought to measure parasite lipid fluxes under adverse host

343 nutritional/lipid conditions, through limitations in FBS concentrations in parasite

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344   culture media. Interestingly, GC-MS analysis revealed that ^{13}C incorporation into all

345   FASII-generated and further ER-elongated FA products, i.e. C14:0, C16:0, C16:1,

346   C18:0, C18:1, C20:1, was significantly higher by 5-15% under FBS starvation in the

347   parental line (**Fig. 6EF, S5**). In addition, ^{13}C incorporation into most FAs is increased

348   in the wild type parental line (**Fig. 6F**). These results suggest that apicoplast *de novo*

349   FA/lipid synthesis can be upregulated during FBS starvation to compensate for the

350   lack of nutrients in the external environment. However, in Δ*Tg*ATS2, the ^{13}C

351   incorporation into each FA was decreased by FBS starvation (**Fig. 6F**). No

352   morphological changes could be observed by IFA in the FBS starved Δ*Tg*ATS2

353   mutant (**Fig.5**). Both parental line and Δ*Tg*ATS2 mutant showed a significant

354   reduction in the synthesis of C18:0 in FBS starved conditions, suggesting that C18:0

355   is predominantly obtained by scavenging from the host cell (**Fig. 6F**). Since

356   availability of lipids from the environment is limited, the fatty acid abundance in the

357   parental line was decreased (**Fig. 6G**). Interestingly however, the fatty acid

358   abundance in Δ*Tg*ATS2 was increased in most of its FA species during FBS

359   starvation (**Fig. 6G**).

360   Although we observed a defect in the activation of FASII in Δ*Tg*ATS2, Δ*Tg*ATS2 was

361   nevertheless viable during FBS starvation. This suggests that if FASII is active,

362   regardless of the level of FASII activity, the parasites are viable under FBS

363   starvation, consistent with its essential role in tachyzoites. However, in *P. falciparum*,

364   FASII is not essential during nutrient replete blood stage but is activated under lipid

365   starvation, apparently to compensate for reduced availability of scavenge-able lipids

366   (Botte et al., 2013; Yu et al., 2008). Our results in *T. gondii* led us to re-think the

367   current hypothesis regarding the dispensability of the apicoplast FASII in *P.*

368   *falciparum* blood stages and to test the essentiality of malaria parasite apicoplast

15

369 FASII under nutrient/lipid starved conditions. We grew *P. falciparum* FASII KO,

370 Δ*Pf*FabI (Yu et al., 2008) and its parental line (NF54) in either regular (i.e. lipid rich)

371 culture medium or in "lipid-starved" medium (Botte et al., 2013; Mi-Ichi et al., 2007).

372 Both NF54 and Δ*Pf*FabI grew normally in the regular culture medium (**Fig. 6H**). In the

373 lipid-starved medium, NF54 was viable but grew significantly slower than in lipid

374 replete conditions whereas Δ*Pf*FabI grew only for the first 2 days in lipid starved

375 media, but after 4 days, a sharp decrease in growth occurred, and this led to a

376 complete loss of detectable parasites after 8 days (**Fig. 6H**). This proves that FASII is

377 required for the malaria parasite to adapt its lipid metabolism in response to an

378 adverse host lipid environment, a similar situation to that revealed here for *T. gondii*.

379 Since environmental FBS starvation induces an increase of *de novo* lipid synthesis,

380 we investigated the effect the lipid-nutrient depleted conditions (i.e. 0, 1, 10% of FBS)

381 on various mutants involved defective in lipid metabolism in *T. gondii*. We assessed

382 parasite growth by plaque assay and quantified plaque area. The wild-type and

383 parental parasite lines could grow equally well in DMEM supplemented with 0, 1, or

384 10% FBS (**Fig. 6I-P**), without affecting the integrity of HFF host cells. FBS starvation

385 only affected growth of Δ*Tg*ATS2 mutant under 0% FBS (**Fig. 6I**). FBS availability

386 had a much stronger effect on iΔ*Tg*ATS1-KO (Amiar et al., 2016) **Fig. 6J**). Although

387 *Tg*ATS1-KO already grows much less in the regular culture conditions (*i.e.* 1% FBS),

388 starvation under 0%FBS led to the quasi-absence of plaques whereas an increase

389 up to 10% FBS led to significantly larger plaques than in the 0% and the 1% FBS

390 (**Fig. 6J**). This suggested that in the absence of the major *de novo* phospholipid

391 precursor synthesis pathway, the parasite could compensate the growth defect by

392 accessing more host lipid resources. The acetyl-CoA synthetase, *Tg*ACS (Dubois et

393 al., 2018), was slightly responsive to FBS starvation (**Fig. 6K**). Interestingly, proteins

16

394   not involved in bulk membrane/lipid synthesis, such as *Tg*PKA-iKO, do not display an

395   aggravated phenotype under nutrient starvation (**Fig. 6L**) (Uboldi et al., 2015).

396   Since host fatty acid binding proteins (FABP) are upregulated upon tachyzoite

397   invasion (Hu et al., 2017), we searched the genome of *T. gondii* for homologs of

398   FABP that could be responsible for the transport of FAs in the parasite during

399   starvation but found none. Instead, we found two proteins belonging to the closely

400   related family of acyl-CoA binding protein (ACBP), i.e. *Tg*ACBP1 and *Tg*ACBP2. We

401   identified that *Tg*ACBP1 and *Tg*ACBP2 localised at the parasite cytosol and

402   mitochondrion, respectively (**Fig. S6**). Then we generated inducible knock-down

403   parasite lines for both (**Fig. S6**). However, plaque assays showed that both proteins

404   were dispensable during tachyzoite life stages and neither were responding to FBS

405   starvation (**Fig. 6MN)**, suggesting that neither *Tg*ACBPs are involved as effectors for

406   the adaptation to nutritional environment.

407   We the hypothesized that parasite effectors putatively exported into the PVM or

408   towards the host cell could be used by the parasite to collect putative host membrane

409   material generated during FBS starvation. To test this, we investigated *Tg*ASP5, a

410   Golgi resident aspartyl protease that controls the non-canonical trafficking pathway of

411   parasite effectors towards the parasitophorous vacuole and the host cell, during FBS

412   starvation (Bougdour et al., 2014). Strikingly, FBS starvation significantly exacerbated

413   the growth defect in *Tg*APS5 mutants, whereas there was no change the knockdown

414   of TgMYR1, the canonical system to export effectors towards the host (Franco et al.,

415   2016), the under these conditions (**Fig. 6O, P**). Together, these data provide

416   evidence that parasite effectors are trafficked to the host cell solely thought the

417   TgASP5 export pathway to modulate to nutrient starvation to enhance the ability to

418   scavenge needed resources.

419   **Nutrient depletion/starvation induces the formation of multi-membrane bound**

420   **vesicles in host cells that are taken up by the parasite**

421   FBS starvation induces parasites to activate an adaptation pathway that apparently

422   includes shifts the balance between *de novo* synthesis and scavenging from the host,

423   as well as scavenging from the host/parasite environment. To investigate potential

424   changes to the host cell and hence host/parasite interactions during lipid starvation

425   we performed EM on starved (0, 1 or 10% FBS) HFF host cells infected with either

426   the parental parasite line or ∆*Tg*ATS2. Growth in 10% FBS led to no obvious

427   phenotype changes in the hosts cells or the parental parasite line or the ∆*Tg*ATS2

428   mutant (**Fig. 7AB**), but reduction to 1% and 0% FBS induced striking changes in the

429   host cells, which became massively vesiculated irrespective of whether they were

430   infected with the parental line or ∆*Tg*ATS2 (**Fig. 7AB**). Such vesiculation was not

431   observed in uninfected HFF host cells put under nutrient starvation. Giant multi-

432   vesicular bodies (gMVB), i.e. large membrane bound compartments containing

433   various smaller vesicles, were frequent in 1% FBS grown cells (**Fig. 7A[1-4]**) and very

434   numerous at 0% FBS (**Fig. 7B[1-4]**). The gMVBs could arise from the host ER, as the

435   ER could be seen swelling and forming networks containing large lipid bodies (**Fig.**

436   **7B[3]**). gMVBs were also often seen in close apposition/contact with the mitochondria

437   and/or ER network, indicating that material could also be transferred from

438   mitochondria (**Fig. 7B[3,5]**). However, gMVBs were more often observed arising directly

439   from the host nuclear envelope, likely representing a major contributor for their

440   formation (**Fig. 7B[6-8]**). The gMVB accumulated in close vicinity with the PVM, which

441   houses the parasite during its intracellular development and serves as the exchange

442   interphase between the host and the parasite. The gMVBs were not only close to the

443   PVM but appeared to be interacting with the PVM with host material and vesicles

18

444 from the gMVB apparently "percolating" through the PVM (**Fig. 7B[4])** or directly from

445 their originating organelles (**Fig. 7B[5]**) to eventually be found in the PVM (**Fig. 7A[2]B[1]**).

446 Indeed, these vesicles appeared in both wild-type and Δ*Tg*ATS2, suggesting that the

447 host cell is responding to the nutrient deficiency in a similar manner irrespective of

448 whether it is infected with the parental parasites or Δ*Tg*ATS2. The Δ*Tg*ATS2 parasite

449 cytokinesis phenotype (e.g. **Fig. 3**) was still observed and apparently exacerbated in

450 0% and 1% FBS growth medium (**Fig. 7B**). This vesicle/gMVB formation and

451 trafficking to and within the PVM was not apparent in high (10%) FBS medium,

452 suggesting that host gMVBs somehow allow the parasite to increase its lipid

453 scavenging in the absence of nutrient rich serum. This is first report of gMVB

454 containing multiple vesicles, that (i) are dependent and induced by nutrient

455 availability, and (ii) originate directly from diverse host organelles. The gMVBs are

456 clearly different from the lipid droplets of host cell origin used as a lipid source by *T.*

457 *gondii* (Nolan et al., 2017; Romano et al., 2017).

458 Since lipid droplets from the host can also be used by the parasites as lipid sources,

459 it is possible that FBS starvation leads to increased host cell lipid droplet import. Nile

460 red staining indeed confirmed that FBS starvation induced a significant increase of

461 the amount of lipid droplets into the parasites and its PVM **(Fig. 7C)**, In contrast, low

462 FBS content resulted in a reduced amount of lipid droplets in uninfected host cells

463 whilst high FBS content increased their presence in the host cells alone (**Fig. 7D**).

464 This further indicates that increase of import of lipid droplets to the parasite is

465 upregulated by the parasite during FBS starvation.

466 Since gMVB also seem to arise from host mitochondria, we used an anti-lyso-bi-

467 phosphatidic acid (LBPA) antibody to detect LBPA, i.e. a degradation product of

468 mitochondrial cardiolipin (Kobayashi et al., 1998) that can also be scavenged by the

469   parasite during intracellular development (Romano et al., 2017) (**Fig. 7E**). LBPA was

470   found surrounding the PVM in the host cell, within the PV and the parasite, but its

471   localization and intensity remained unchanged in response to reduced FBS content

472   (**Fig. 7E**). Direct salvage of mitochondrial cardiolipin *per se* might not be the primary

473   up-regulated scavenging pathway during lipid starvation.

474   **Discussion**

475   We have shown that *Tg*ATS2 is an apicoplast acyltransferase able to esterify FAs on

476   LPA to generate PA, a precursor for a wide range of parasite lipids. Perturbation of

477   *Tg*ATS2 resulted in perturbed lipid fluxes, which impacts LPA/PA lipid balance

478   causing mislocalization of *Tg*DrpC and vesiculation during cytokinesis. Furthermore,

479   parasites exhibit considerable metabolic plasticity on both *de novo* synthesis in the

480   apicoplast, and host modification for membrane scavenging critical for adaptation to

481   nutrient limiting conditions in the host (**Fig. 8**).

482   **PA and LPA roles in membrane curvature and cell division**

483   Membrane PLs have different physical shapes according to the relative sizes

484   between the polar head and the fatty acid tails. Most PLs are cylindrical, while PA is

485   cone shaped and LPA adopts an inverted cone shape, thus their insertion into

486   membrane bilayers facilitates curvature and invagination/evagination (Kooijman et

487   al., 2005).

488   In human cells, PA is implicated in membrane cell curvature during exocytosis and

489   synaptic vesicles formation from neuronal cells (Chasserot-Golaz et al., 2010;

490   McMahon and Gallop, 2005; Schmidt et al., 1999). More particularly, the release of

491   synaptic vesicles between neurons is dependent on the pinching exerted by

492   dynamins that require endophilin1, an ATS2 homolog, as partner to create LPA/PA

493   curvatures (Burger et al., 2000; Shin and Loewen, 2011). Presence of PA in

494   membranes creates electrostatic interaction to improve penetration of a larger part of

495   dynamin into the lipid monolayer (Burger et al., 2000; Shin and Loewen, 2011).

496   Although parasites could proceed with division to a partial extent, *Tg*DrpC requires a

497   specific LPA/PA environment—environment that is altered in Δ*Tg*ATS2. Fairly

498   interestingly, *Tg*DrpC is the homolog of the human Drp1, which also requires the

499   proper LPA/PA interaction to exert its pinching role in human cells (Adachi et al.,

500   2016; Kameoka et al., 2018). Our results thus reveal the previously unrecognised

501   importance of apicoplast maintaining internal lipid homeostasis. Furthermore, the

502   functional role of *Tg*ATS2 for PA synthesis during division provides a mechanism for

503   the long-standing question of why drugs targeting the apicoplast display a secondary

504   cytokinesis defect.

505   **Environmental and nutritional conditions drive the adaptation of the apicoplast**

506   **metabolic capacities as well as the scavenging capacities**

507   Importantly, *Toxoplasma* could increase production of FA in the FASII pathway in

508   nutrient-lipid deprived medium similarly to *P. falciparum* (Botté et al., 2011). Hence,

509   apicomplexan parasites show high metabolic flexibility to obtain FA for the major

510   membrane building blocks required for growth as pointed out by recent studies

511   exploring *Plasmodium* survival in nutrient depleted conditions (Brancucci et al., 2017;

512   Mancio-Silva et al., 2017; Zuzarte-Luis et al., 2017). Importantly, our results

513   demonstrate that *P. falciparum* lacking a FASII and grown in lipid-deprived

514   conditions, were unable to properly proliferate, ultimately dying. This suggests that

515   apicoplast FASII is facultative rather than totally dispensable in malaria parasite

516   blood stage and can be activated during lipid starvation to meet needs for

517   phospholipids. This apparent FASII flexibility is consistent with a growing pool of

21

518 evidence such including the upregulation of FASII in malnourished patients and the

519 essentiality of the central acyl-carrier protein, ACP (Sidik et al., 2016; Zhang et al.,

520 2018), all summarized in **Table 1** which together show FASII is requisite depending

521 on the environment. Therefore, environmental conditions could have important

522 consequences in treating patients. Indeed, if patients are under stress/nutrient

523 deprivation/malnourished conditions, then the FASII pathway could become a

524 secondary target of choice to help eradicate the parasites. This approach is

525 supported by transcriptomics analysis in infected patients displaying an up-regulation

526 of the FASII and the apicoplast acyltransferase PfG3apiGPAt (homolog of *Tg*ATS1,

527 **Table1**, (Daily et al., 2007). Interestingly, FASII remains active during artemisinin

528 induced dormancy in *P. falciparum* blood stages, and maintain parasite growth (Chen

529 et al., 2014), also suggesting that FASII gets actively regulated and could thus be

530 considered a target for combination therapies and during the development of

531 artemisin resistance.. Altogether these data questions whether isopentenyl

532 pyrophosphate (IPP) synthesis is the sole essential function of the apicoplast during

533 *Plasmodium* blood stage (Yeh and DeRisi, 2011). Rather they put the parasite back

534 into its physiological context where nutrient availability and environmental conditions

535 drive the requirement and regulation of a given metabolic pathway. This redefines

536 what we call an essential gene, where phenotypes might only be seen under

537 starvation conditions. An interesting avenue on investigating the parasite metabolic

538 plasticity in response to environmental changes would be to systematically

539 investigate the phenotype of known and uncharacterized proteins putatively

540 exacerbated under lipid deprived conditions.

541 The increase of scavenging capacities in the ∆*Tg*ATS2 mutant was confirmed via

542 GC-MS analysis of FAs and phospholipid uptake assays, suggesting another

543 sensing-signalling pathway informing the parasite on its own capacities to produce or

544 not the required lipids necessary for intracellular development. While *Tg*ATS2 is

545 probably not the sensor that modulates this, it is clearly in the chain of response

546 important in accelerating growth of the parasite under nutrient deprived conditions,

547 similar to *Plasmodium*. Moreover, EM observation showed unique vesicle structures,

548 gMVB, induced in the host cells upon lipid deprivation that did not correspond to any

549 of the described cellular structures induced by the invasion of *T. gondii.* This

550 suggests that gMVB are actively manipulated and imported by the parasite by the

551 action of parasite effectors sent out to the PV/PVM, via the non-canonical ASP5

552 pathway. Identification of these effectors warrants future investigations.

553 A major question raised here is the nature the signalling factor(s) responsible for

554 environmental sensing and metabolic adaption of both apicoplast *de novo* synthesis

555 and scavenging pathways. Both *T. gondii* and *P. falciparum* lack the canonical

556 mTOR-based nutrient-sensing pathways present in other eukaryotes but a recent

557 study showed that *P. berghei* is capable of sensing nutrient deprivation by a SNF1-

558 related kinase, KIN1 (Mancio-Silva et al., 2017). *T. gondii* possesses a conserved

559 and uncharacterised homolog of KIN. Determination of protein putatively regulated by

560 KIN in both *Plasmodium* and *T. gondii* may reveal a new set of regulators in

561 adaptation pathways.

562 Since our observations highlight adaptation of the FASII pathway and the utilization

563 of its acyl-CoA products, it is interesting to note that in plants and bacteria the FASII

564 pathway can be repressed though Acyl-CoA by a feedback loop mechanism (Fujita et

565 al., 2007). Furthermore, supplementation of PUFAs in the fatty acid free diet of mice

566 inhibited both ACCase and FASII (Toussant et al., 1981). Such a mechanism where

567 availability of PUFAs, potentially obtained from the host and its environment,

23

568   regulates the activity of the FASII would fit with our data. Furthermore, acetylation of

569   FAs has recently been shown to promote the destabilisation of the FA synthesis

570   complex and to inhibit *de novo* FA synthesis and lipogenesis during tumor cell growth

571   (Lin et al., 2016). Acetylome analysis of mutants utilizing acetyl-CoA may reveal how

572   FASII is regulated in nutrient adaptation.

573   Together, our results reveal the central role of the apicoplast to provide specific

574   precursors for membrane biogenesis during cytokinesis and most importantly to be a

575   central metabolic hub to adapt the parasite metabolic capacities upon nutrient

576   availability and environmental changes. The data also point at major modifications in

577   vesiculation and utilization/scavenging of these membrane structures by the parasite

578   upon such environmental changes. Data also corroborate recent results with the

579   mosquito lipid environment regulates the metabolic activity of transmissible

580   sporozoites (Costa et al., 2018). The fundamental role of these physiological changes

581   induced by the parasite in response to host environment provide novel insights in the

582   parasite biology and offers new avenues to explore in the fight against toxoplasmosis

583   and malaria.

592   **Author contributions**

593   SA and NJK designed and performed experiments, analyzed and interpreted data,

594   and wrote the manuscript. LB performed, analyzed and interpreted data for electron

595   microscopy. SD performed Nile Red/LBPA and related immunofluorescence assays.

596   MJS helped performing and analysing the *P. falciparum* lipid starvation growth assay.

597   CB helped performing E. coli complementation assays. BT performed and analysed

598   *T. gondii* proliferation assays and the related statistical analyses. MAH provided the

599   *T. gondii* CrispR-Cas9 system on *Tg*ATS2 and provided experimental advice and

600   supervision regarding this experiment. GIM supervised the *P. falciparum* lipid

601   starvation growth assay. YYB performed, analysed interpreted and supervised

602   lipidomic analyses data and wrote the manuscript. CYB lead the project, designed

603   and interpreted data and wrote the manuscript.

604   **Declaration of interest:** Authors declare no conflict of interest.

605
606
607   **Figures legends**

608   **Figure1: (A)** Phylogenetic analysis of *Tg*AST2 and *Tg*AGPAT and orthologs from

609   various organisms. Maximum likelihood phylogenies of prokaryotic and eukaryotic

610   acylglycerol acyl transferase activity of 28 enzymes. Branch support values are

611   indicated for each node in different colours (0–25, purple; 25–50, green; 50–75,

612   orange; 75–100, red). (**B**) Multiple protein alignment of the putative orthologues of

613   *Tg*ATS2 from plant chloroplast and *Tg*AGPAT from ER. Green: identical amino acids

614   (aa) residues of active domain (Motif I-IV), grey: aa residues similar among each

615   clade; black aa conserved in Alveolate enzymes in both clades.

616   **Figure 2: (A, B)** IFA of stable *Tg*ATS2-HA expressing parasites **(A)** and transient

617   *Tg*AGPAT-HA expression **(B)**. CPN60: apicoplast marker;TOM40 , mitochondrial

618   marker **.** Scale bars=2 μm **(C) Expression of *Tg*ATS2 and *Tg*AGPAT in LPAAT**

25

619   **deficient *E. coli* strain SM2-1** SM2-1Δ*plsC* E.coli mutant transformedwith *Tg*ATS2

620   (1, 2), *Tg*AGPAT (1, 2), TgAGPAT$_{\Delta Nter1-72}$, *Ec*plsC or empty pQE30Xa expression

621   vector were grown at 30°C (permissive)_ or 42°C (non permissive) for 20 h(n=3). **(D)**

622   CRISPR strategy to generate Δ*Tg*ATS2 mutant lines via integration of HXGPRT

623   cassette within *Tg*ATS2 ORF (knock in; KI) and by *Tg*ATS2 gene replacement by a

624   HXGPRT cassette (knock out; KO) in *Tg*ATS2-HA strain. **(E, F)** Confirmation of

625   *Tg*ATS2-HA loss by Western blot analysis in *Tg*ATS2-KI **(E)** and *Tg*ATS2-KO **(F)**

626   using anti-HA (anti-Gra1: loading control). **(G)** Confirmation of *Tg*ATS2-HA-signal

627   loss in Δ*Tg*ATS2 by IFA using anti-HA (Scale bars=2 μm). **(H)** Plaque assay showing

628   a mild growth defect in Δ*Tg*ATS2 mutants **(I)** Cell-based growth fitness assay

629   confirmed the growth defect in the Δ*Tg*ATS2 mutants 30 h post-infection (n=3). **(J)**

630   Proliferation assay confirmed a replication defect in Δ*Tg*ATS2 mutants (n=3).

631   **Figure 3: (A) IFA** of Δ*Tg*ATS2 and parental line transiently expressing MORN1-

632   mCherry (IMC basal tip) and anti-IMC1 (scale bars=2 μm). Confirmation of enlarged

633   residual bodies in Δ*Tg*ATS2 by IFA using anti-GAP45 (IMC marker) **(B)**, and by

634   statistical analysis of residual body size **(C)** (*:*p value ≤0.05*) **(D)** IFA observation of

635   extracellular parasite using anti-SAG1 (white arrowhead: PM tether). **(E)** Electron

636   microscopy image of Δ*Tg*ATS2 mutants reveals important cytokinesis defects: major

637   enlargement of the PM, defects in mother cell membrane constriction and cell

638   daughter attachment at the basal pole (**1, 2, enlarged in 1', 1'', 2'**, white arrows),

639   IMC fragmentation at the separation sites between dividing parasites (**2''**). Residual

640   bodies containing unevenly separated nuclei (**1, 3, 3', 4, 5**), mitochondria (**4, 5, 6, 6'**)

641   and acidocalcisomes (**5**). **N:** nucleus; **Mt:** mitochondria; **rb:** residual body; **Ac:**

642   acidocalcisome (scale bars= 1 μm)

643 **Figure 4: Lipidomic analysis ∆*Tg*ATS2 mutant. (A, B)** Fatty acid composition of

644 total lipid extracted after 72 h post-infection **(C)** Relative fatty acid abundance of

645 ∆*Tg*ATS2 to the parental line. **(D)** Relative major phospholipid abundance of

646 ∆*Tg*ATS2 to parental line **(E)** LPA/PA ratio. Individual molecular species of LPA **(F)**,

647 PA **(G)**, DAG **(H)**. Fatty acids are shown as Cx:y as x for number of carbons and y for

648 the number of unsaturations. n=4, * indicates *p value ≤0.05*

649 **Figure 5 ∆*Tg*ATS2 induces the mislocalization of *Tg*DrpC and cytokinesis**

650 **defects during tachyzoite division.**

651 **(A**) IFA localization of *Tg*DrpC-HA expressed in parental line shows ring structures at

652 the growing ends of daughter cells during division (top panel) but fails to do so when

653 expressed in ∆*Tg*ATS2 (bottom panel). **(B)** *Tg*DrpC-HA localisation during tachyzoite

654 division cycle in the parental line (top panel) and its mis-localization in ∆*Tg*ATS2

655 mutant. Scale bars= 2 μm **(C, D)** Electron microscopy observation of basal pole of

656 IMC parental line **(C)** and ∆TgATS2 **(D). (C$^{1-3}$)** Endodyogeny starts with the formation

657 of daughter cells (dc) by growth of IMC (white arrows) and organelles segregation.

658 IMC scaffolding then grows towards the basal pole (white arrows) encompassing

659 divided organelles (eg nucleus N). **(C$^{4-5'}$)** Recycling and biogenesis of PM (black

660 arrow) ends daughter cell emergence from mother cell (mc). **(C$^{6-6'}$)** Division ends by

661 cytokinesis through constriction of both IMC and PM at basal pole (black arrows) to

662 form a small residual body (rb)**. (D)** ∆*Tg*ATS2 shows an incomplete separation of

663 daughter cells during cytokinesis with absence of PM biogenesis between closely

664 apposed IMC **(1,3 and insets, white arrows),** presence of vesicle/cisternae inside

665 membrane structures at the inter-IMC space **(2' white arrows),** absence of mother

666 IMC **(3' black arrow),** absence of basal constriction forming large residual bodies

667 leaving floating daughter IMC **(3,4, 4' white. arows).** Scale bar = 1μm. **(E,F)**

27

668   Proposed molecular model for TgDrpC function during endodyogeny and cytokinesis

669   in WT parasite (E) and ΔTgATS2 (F) LPA and PA molecules induce positive and

670   negative curvature creating grooves in biological membranes for TgDrpC to insert at

671   specific sites during division and exert its pinching function during endodyogeny **(E)**.

672   **Figure 6: FASII can adapt its metabolic capacities upon changes in nutritional**

673   **environment in *T. gondii* and is critical during lipid starvation in *P. falciparum***

674   **blood stages.**

675   **(A-G)** U-^{13}C-glucose labelling for 72 h to monitor apicoplast FA synthesis by ^{13}C

676   incorporation to each fatty acids (Blue, parental line, Red, ΔTgATS2) **(A)** ^{13}C

677   incorporation to each fatty acids in 1% FBS **(B-D)** Mass isotopologue distribution in

678   1% FBS for C14:0 **(B)**, C16:0 **(C)** and C18:0 **(D)** The x-axis shown as 'M+X'

679   represents mass with 'X' ^{13}C atoms incorporated during the FA synthesis. **(E)** ^{13}C

680   incorporation to each fatty acids in 0.2% FBS. FASII metabolic activity increased

681   upon FBS starvation in the parental line but not in ΔTgATS2.**(F)** Change in the ^{13}C

682   incorporation between 0.2%FBS and 1% FBS (-FBS/+FBS) **(G)** The relative

683   abundance of each FA (-FBS/+FBS) **(H)** Asexual blood stage growth assay of *P.*

684   *falciparum* FabI-KO and its parental line (NF54) in regular (lipid rich) culture medium

685   and lipid starved medium. **(I)** Plaque assays conducted in 0, 1, or 10% FBS in

686   different *T. gondii* mutants and their reference strain; TgATS2, TgATS1, TgACS,

687   TgPKA, TgACBP1, TgACBP2, TgASP5, TgMyr1. n=3, or more. *, p ≤0.05; **, p≤0.01;

688   ***, p≤0.001; ****, p≤0.0001.

689   **Figure7: Nutrient starvation unveils formation of multi vesicular bodies from**

690   **host cell organelles, which content is imported towards parasites.**

691   (Transmission electron micrographs of intracellular wild types parasites **(A)** and

692   ΔTgATS2 mutant parasites **(B)** grown in 0,1 and 10% FBS. Nutrient starvation (i.e. 0

693   and 1% FBS) induce formation of giant multivesicular bodies (gMVB) in the host cell

694   (hc), containing various vesicles including lipid bodies-like (white stars). In starvation,

695   gMVB localized in the cytosol (cyt) in contact to the parasitophorous vacuole (pv)

696   (**A[1,2],[4]B[1,2]**), and their content was imported through and into the PV (**A[1] black stars,**

697   **B[2,6] black arrows**), gMVB were arising from host endoplasmic reticulum (ER, **A[2]**),

698   mitochondria (mt, **A[2,3]**) and mainly swollen nuclear envelope (N, **B[3,4,5]**). 10% FBS did

699   not induce gMVB formation in both parental and ∆*Tg*ATS2. Scale bar= 1µm. **(C)**

700   Nutrient starvation induces a significant increase of lipid droplets within the parasite

701   and its PV as measured by IFA using Nile red (Nile Red dots were counted for 100 or

702   more parasites n=3, **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). **(D)** Nutrient starvation

703   induces a decrease of lipid bodies in uninfected HFF host cells as measured by IFA

704   using Nile red. **(E)** IFA shows that import into parasites of LBPA (anti-LBPA) is not

705   affected by nutrient starvation.

706   **Figure 8: Proposed model for cytokinesis, lipid acquisition and metabolic**

707   **adaptation under adverse host lipid environment in *T. gondii*.**

708   Under lipid rich environment, *T. gondii* can acquire FA and lipids by host cell

709   scavenging, notably from the sequestration of host mitochondria (mt) and lipid

710   droplets but also from the external host cell environment. Under these conditions, the

711   host cell acquires enough nutrients from its external environment to generate

712   numerous lipid droplets and its own lipids. The parasite also needs to generate DA

713   and lipids de novo, notably via the apicoplast (ap) FASII pathway. The apicoplast

714   acyltransferase ATS2 generates PA and regulates the balance of LPA/PA. Such

715   balance creates local membrane curvature to recruit *Tg*DrpC and form plasma

716   membrane, both essential during cytokinesis and division. Under host lipid starved

717   environment, the parasite adapts its metabolism by up-regulating its apicoplast FASII

718 capacities to produce more fatty acid to compensate their absence from the host cell.

719 Concomittantly, the parasite induces morphological changes in the host cell to

720 increase scavenged ressources. Giant multivesicular bodies (gMVB) are being

721 generated from vesiculation of host cell organelles including nucleus (N), ER and

722 mitochondria. These gMVBs are forming in close vicinity to the parasitophorous

723 vacuole membrane (PVM), and their content imported into the PV.

724 **Table 1: Summary of genomic, transcriptomic and systematic disruption data**

725 **for genes related to fatty acid synthesis in the apicoplast and phosphatidic**

726 **acid de novo synthesis.** (+) and (++) represent important and very important up-

727 regulation, respectively. (-) stands for downregulation. For transcriptomic analysis

728 analysis: X stands for the approximative fold increase. For Late liver stage

729 expression profile, the table depicts the P. yoelii ortholog number, the regulation of

730 late liver stage (LS) time point 40 h compared to the average blood stage expression

731 level, and the late LS at 50 h compared to average blood stage as per (Tarun et al.,

732 2008). The last time point represents the highest LS expression peak when

733 compared to blood stage.

734

735

Table1

| Enzyme name | Full name | Pathway | *T. gondii* | | *P. falciparum* | | | | *P. yoelii* | | Reported essentiality |
			Accession numbers (http://toxodb.org/toxo/)	Phenotype score in10% FBS (Siddick et al. 2016)	Orthologue accession numbers (http://plasmodb.org/plasmo/)	Blood stage mutability in rich culture medium (Zhang et al. 2018)	Blood stage peak expression profile (PlasmoDB)	Blood stage transcriptomic upregulation in starved patients (Daily et al. 2007)	Orthologue accession numbers (http://plasmodb.org/plasmo/)	Late liver stage expression profile (Tarun et al. 2008)	
oTPT	Outer membrane triose phosphate translocator	precursor transporter	TGGT1_261070 *	-4.44	PF3D7_0508300	Non-mutable	Trophozoite (30 h)	(+) 2X	PY00389	(+);(-); 40 h	* Essential (Brooks et al. 2010)
iTPT	inner membrane triose phosphate translocator	precursor transporter			PF3D7_0530200	Mutable	Trophozoite (32 h)	(++) 8X	PY01812	(+);(+); 40 h	
Pyruvate kinase	pyruvate kinase	generation of pyruvate	TGGT1_299070	-2.76	PF3D7_1037100	Non-mutable	Trophozoite (24 h)	(++) 6.5X	PY03879	(+);(+); 40-50 h	
PDH E1 alpha	Pyruvate dehydrogenase E1 alpha subunit	Generation of Acetyl-CoA	TGGT1_245670	0.15	PF3D7_1124500	Non-mutable	no real peak	(++) 6.5	PY00819	(+); (+), 40 h (mid liver stage)	
PDH E1 beta	Pyruvate dehydrogenase E1 beta subunit	Generation of Acetyl-CoA	TGGT1_272290	-0.18	PF3D7_1446400	Mutable	Schizont (40 h)	(++) 6.5X	PY07062	(+);(+); 40 h	
PDH E2	Pyruvate dehydrogenase E2 subunit	Generation of Acetyl-CoA	TGGT1_206610	-2.97	PF3D7_1020800	Non-mutable	no real peak	(++) 6.5X	PY04573	(+); (+); 40 h (mid liver stage)	
PDH E3	Pyruvate dehydrogenase E3 subunit	Generation of Acetyl-CoA	TGGT1_305980	-1.38	PF3D7_0815900	Mutable	no real peak	(++) 9X	PY00573	(+);(+); 40 h	
ACCase	acetyl-CoA carboxylase	Generation of Malonyl-CoA	TGGT1_221320	-3.6	PF3D7_1469600	Mutable	no real peak	(++) 23X	PY01695	(+);(+); 50 h	
ACP	Acyl Carrier protein	FASII	TGGT1_264080 *	-1.43	PF3D7_0208500	Non-mutable	Late trophozoite-Shizont (32-40 h)	(++) 12X	PY04779	(+); (+); 40 h	* Essential (Ramakrishnan et al. 2012)
FabZ	hydroxyacyl-ACP dehydratase	FASII	TGGT1_321570	0.67	PF3D7_1323000	Mutable	Trophozoite-Shizont (26-46 h)	(++) 9X	PY01586	(+); (+); 40 h	
FabB/F	keto acyl-ACP synthase I/II	FASII	TGGT1_293590	-1.21	PF3D7_0626300	Mutable	Ring (0 h)-Shizont (40-48 h)	(++) 9X	PY04452	(+);(+); 40 h	
FabD	Malonyl-CoA transacylase	FASII	TGGT1_225990	-0.98	PF3D7_1312000	Non-mutable	Ring (0 h)-Shizont (40-48 h)	(++) 7X	PY05492	(+); (+); 40 h (mid liver stage)	
FabH	Ketoacyl ACP synthase III	FASII	TGGT1_231890	-1.91	PF3D7_0211400	Mutable	Late trophozoite-Shizont (32-48 h)	(++) 12X	not found in *P. Yoelii*	N/A	
FabG	Ketoacyl ACP reductase	FASII	TGGT1_217740	-1.89	PF3D7_0922900	Non-mutable	Late trophozoite-Shizont (32-48 h)	(++) 14X	PY02416	(+);(+); 40 h	
FabI	Enoyl-ACP reductase	FASII	TGGT1_251930	-1.32	PF3D7_0615100*, **	Non-mutable,	no real peak	(++) 11X	PY03846	(+);(+); 40 h	* Dispensible in red blood stage (Vaughan et al. 2009) ** essential during starvation (this work)
LipA	Lipoic acid synthase	Lipoic acid production	TGGT1_226400	-0.97	PF3D7_1344600	Non-mutable	Late trophozoite-Schizont (32-48 h)	(++) 16X	PY06208	(+);(+); 50 h	
LipB	octanoyl-ACP:protein transferase	Lipoic acid production	TGGT1_315640	-1.74	PF3D7_0823600	Mutable	no real peak	(++) 9X	not found in *P. Yoelii*	N/A	
GpdA	Glycerol-3-	Glycerol-3-	TGGT1_307570	-3.03	PF3D7_1216200	Non-mutable	Trophozoite (24 h),	(-) 2X	PY005585	(+); (=); 50 h	

	phosphate dehydrogenase	phosphate production					Late schizont (48 h)			(late liver stage)	
ATS1	glycerol-3-phosphate acyltransferase	Phospholipid synthesis/ (L)PA production	TGGT1_270910 *	-2.13	PF3D7_1318200 **	Mutable	Late Trophozoite-Schizont (32-48 h)	(++) 25X	not found in *P. Yoelii*	N/A	* Essential (Amiar et al. 2016) ** Dispensable (Shears et al. 2017)
ATS2	acyl-glycerol-3-phosphate acyltransferase	Phospholipid synthesis/ PA production	TGME49_297640	-0.02	PF3D7_0914200	Non-mutable	Schizont (40 h) Rings	(+) 3X	PY02486	N/A	
GPAT (ER)	glycerol-3-phosphate acyltransferase	Phospholipid synthesis/ (L)PA production	TGGT1_256980	-2.36	PF3D7_1212500	Non-mutable	Late Trophozoite (32 h)	not available	PY06015	N/A	
AGPAT (ER)	acylglycerol-3-phosphate acyltransferase	Phospholipid synthesis/ PA production	TGGT1_240860 *	-2.54	PF3D7_1444300	Non-mutable	Late Trophozoite-Schizont (32-48 h)	(++)	PY01678	(+); (+); 50 h (late liver stage)	* Likely essential (this work)

737

738 **Materials and Methods:**

739 *T. gondii culture*

740 *T. gondii* parental lines RH TATi1-*ΔKu80* (56) and RH-*ΔKu80* (57) and derived

741 transgenic cell lines were grown in confluent human foreskin fibroblasts (HFFs) as

742 described (13). Molecular constructs, transfection protocols, growth assays, and

743 egress/invasion assays are described in details in supplemental methods.

744 *Immunofluorescence assay and Microscopy*

745 Parasites were infected HFF cells grown on coverslips. Primary antibodies used:

746 Mouse anti-HA antibody (InvivoGen, 1:1000), rabbit anti-ACP (1:2000), rabbit anti-

747 TOM40 (1:3000), polyclonal rabbit anti-IMC1 and anti-MIC4 antibodies (1:1000),

748 rabbit anti-Sumo21 at (1:500) and mouse anti-Sag1 (1:500). Secondary antibodies:

749 anti-mouse Alexa488-546, anti-rabbit Alexa546- (Life Technologies, 1:10000). Cells

750 were observed using 63x objective and fluorescent images were acquired with a

751 fluorescence microscope (Axio Imager 2_apotome; ZEISS).

752 *Plasmodium falciparum growth assays:*

753 *P. falciparum* NF54 wild type parasites and FabI-KO (5). Parasite lines were

754 maintained as previously described (58) at 2% haematocrit in RPMI-HEPES

755 supplemented with AlbuMAX II (Gibco). Intra-erythrocytic growth assays in standard

756 media were performed by monitoring the replication of tightly synchronous parasites

757 (5% sorbitol) over four asexual cycles as previously described (59, 60). Media was

758 replaced daily, sub-culturing were performed every 48 h when required, and

759 parasitemia monitored by Giemsa stained blood smears. Growth assays in lipid-

760 depleted media were performed by synchronizing parasites, before transferring

761 trophozoites to lipid-depleted media as previously reported (2, 19). Briefly, lipid-rich

762 AlbuMAX II was replaced by complementing culture media with an equivalent amount

763 of fatty acid free bovine serum albumin (Sigma), 30 μM palmitic acid (C16:0; Sigma)

764 and 45 μM oleic acid (C18:1; Sigma). All assays were performed in triplicates on

765 different days.

766 *Activity analysis in LPAAT-deficient E. coli strains*

767 *Escherichia coli* strain deficient in LPAAT/AGPAT activity [MS2-1 Δ*plsC*, Coli Genetic

768 Stock Center #7587, Yale University] (29) was used to confirm LPAAT activity in both

769 *Tg*ATS2 and *Tg*AGPAT (details in Supplemental methods)

770   *Transmission electron microscopy*

771   Parasites were grown for 24 h in Labteks (Nunk, Thermofisher) before fixation in 0.1

772   M cacodylate buffer with 2.5% glutaraldehyde for 2 h. Samples were then kept in

773   fixative. at 4°C until further processing. Sample were then post-fixed 1h with 1%

774   osmium tetroxide in cacodylate buffer followed by overnight in 2% uranyl acetate in

775   distilled water. After dehydration in graded series of acetonitrile, samples were

776   progressively impregnated in Epon812, the wells were then filled with fresh resin and

777   allowed to polymerize 48 h at 60°C. Ultrathin 70 nm section were obtained with a

778   Leica UCT Ultramicrotome and collected on copper grids. Grids were post-stained

779   with uranyl acetate and lead citrate before their observation on a Jeol1200EXII

780   Transmission Electron Microscope. All chemicals were from Electron Micrsoscopy

781   Sciences.

782   *Lipidomic analysis by GCMS extraction from T. gondii tachyzoites*

783   Lipidomic analysis were performed as previously described (13, 15, 16). See details

784   in Supplemental methods.

785   *Stable isotope labelling of T. gondii*

786   Stable isotope labelling using U-^{13}C-glucose (Cambridge Isotope Laboratories, USA),

787   lipid extraction, and GC-MS analysis was performed as previously described (13, 15).

788   Freshly infected HFF were incubated in glucose-free medium supplemented with 8

789   mM U-^{13}C-glucose. For FBS starvation study, 5% FBS was add to U-^{13}C-glucose

790   medium in standard culture conditions and 1% FBS was add to U-^{13}C-glucose

791   medium in starvation culture condition. Parasites were harvested 72 h post-infection

792   and metabolites extracted as above.

793

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1000

1001 **Abbreviations:**

1002 FA, fatty acid; FASII, fatty acid synthesis; PL, phospholipid; PC, phosphatidylcholine;

1003 PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol;

1004 TAG, triacylglycerol; PA, phosphatidic acid; GPAT, glycrol-3-phosphate

1005 acyltransferase; LPA, lysophosphatidic acid; AGPAT, acyl-glycerol3-phosphate

1006 acyltransferase; Drp, dynamin related protein; PVM, parasitophrous vacuole

1007 membrane; KI, knock-in; KO, knock-out; IMC, inner membrane complex; IFA,

1008 immunofluorescent assay; PM, plasma membrane; EM, electron microscopy; DAG,

1009 diacylglycerol; CL, cardiolipin; FBS, foetal bovine serum; HFF, human foreskin

1010   fibroblast; ACBP, acyl-CoA binding protein; gMVB, giant multi vesicular body; LBPA,

1011   lysobisphosphatidic acid; ACP, acyl carrier protein; IPP, isopentenylphosphate

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Supplementary figure legends:

Supplementary information 1: (A) Molecular construct for 3'-insertional tagging of *Tg*ATS2 with a C-terminal HA tag. Plasmid harbouring crossover homologous region of gene was linearized with *BlpI* enzyme and transfected into tachyzoites (RH-*Δku80*), followed by pyrimethamine drug selection and cloned by limiting dilution. Arrow indicated primer set used for clones screen by PCR. **(B)** PCR analysis using primers specific for the *Tg*ATS2 coding region (left), targeting construct integration (right). **(C)** Western Blot analysis, using anti-HA, confirming the presence of *Tg*ATS2-HA as two major bands of ~42kDa and ~60kDa, which correspond to the pre-processed (pATS2, i.e. translated form) and mature (mATS2, i.e. apicoplast located) formsTgATS2. RH-ΔKu80 constitutes the negative control and has no band probed by the anti-HA. GRA1 was used as a loading control. (**D**) Scheme of PCR strategies to isolate a stable △*Tg*ATS2-KI and △*Tg*ATS2-KO. Fragment of 4000 pb (1) is only amplified in △*Tg*ATS2-KI mutants (**E**) but not in RH-*Δku80.* Whereas the fragment of 2360 pb (2) is only amplified in *Tg*ATS2-HA line but not in RH-*Δku80* and △*Tg*ATS2-KO (**F**). **(G)** Microneme secretion assay was conducted by monitoring the secretion of MIC2 inducted by the A23187 compound or by DMSO as a negative induction control, and measured by Western Blot (TOM40 and GRA1 were used as membrane/pellet and soluble/secreted loading controls, respectively). **(H)** Egress assay, as induced by the A23187 compound showed a mild significant egress defect for the △*Tg*ATS2 parasite line (n=3). **(I)** Invasion assay determined via the fluorescent Red-Green assay showed no significant difference between △*Tg*ATS2 and parental lines (n=3). **(J)** Immunofluorescence assays investigating the structure and morphology of organelles in the △*Tg*ATS2 parasite line: Labelling and observation of mitochondrion (TOM40), nucleus (Sumo21), micronemes (MIC2), parasite PM (Sag1) and inner membrane complex (IMC1) showed no obvious defect in the morphology of these organelles. **(K)** Immunoflorescence assay of the apicoplast (CPN60) showed that △*Tg*ATS2 mutants were sometimes lacking their apicoplast or that the organelle was losing integrity (white arrows). Sometimes parasites were attached within a vacuole with one bearing two apicoplasts whilst the other had none (black arrows). Statistical analysis confirmed a statistically significant loss of apicoplast or its compromised integrity between the parental lines and the △*Tg*ATS2 mutants (n=3). Scale bars=2 µm. Statistical analyses (Ttest) with *p values* of ≤ 0.05 (*) were

40

1048 considered significant. **(L)** *Tg*AGPAT-HA construct for localization experiment. **(M)**

1049 Scheme for CRISPR-Cas9 strategy to disrupt *Tg*AGPAT ORF. Disruption of both

1050 *Tg*ATS2 and TgAGPAT using CRISPR-Cas9 affect apicoplast biogenesis.

1051 Immunofluorescence assay performed 48 h post-transfection of RH-Δku80 **(N)** and

1052 ΔTgATS2 parasites **(O)** with pTOXO-Cas9-GFP-CRISPR::gTgAGPAT, using anti-

1053 CPN60 antibody as apicoplast marker. Scale bars=2 μm. Yellow arrows indicate a

1054 correct apicoplast segregation in tachyzoites and white arrows indicate apicoplast

1055 structure loss.

1056 **Supplementary information 2:** Fatty acid composition of PC **(A)**, PI **(B)**, PE **(C)** PG

1057 **(D)**, CL **(E)**, and PS **(F)** molecular species in Δ*Tg*ATS2 (red) and parental line (blue).

1058 Fatty acid composition of HFF cells **(G)** and FBS (**H**). **(I)** Plaque assay performed with

1059 the parental line and Δ*Tg*ATS2 complemented with 20 μM of PA(14:0/14:0) or

1060 PA(16:0/18:1) suggests a increase in parasite proliferation in the parental line

1061 complemented with both PA sources but not in the Δ*Tg*ATS2 mutant line. **(J)** Growth

1062 fitness analysis performed using semi-automated cell-based assay on infected cells

1063 with parental line and Δ*Tg*ATS2 30 h post-infection confirmed a statistical increase in

1064 the intracellular development of the PA-complemented parental line but not in

1065 Δ*Tg*ATS2. Parasite numbers within vacuoles were counted to obtain the proliferative

1066 index, which was evaluated by parasite/vacuole number ratio, in two independent

1067 Δ*Tg*ATS2 lines conducted in triplicate. *, $p \leq 0.05$ were considered significant.

1068 Fluorescent lipid import assay on extracellular parental (*Tg*HX) and Δ*Tg*ATS2

1069 parasites supplied with either PA(C18:1/C12:0-NBD) or PC(C18:1/C12:0-NBD) as a

1070 control. The incorporation of fluorescent NBD-PA **(K)** or NBD-PC **(L)** in the parasite

1071 membrane was quantified by measuring fluorescence intensity. Δ*Tg*ATS2 imported

1072 significantly more PA and PC than the parental control line. Fluorescent PA import

1073 was higher than the PC import in the Δ*Tg*ATS2 mutant **(M)**.

1074

1075 Supplementary information 3: Multiple sequence alignment and protein sequence

1076 analysis of *Tg*DrpC

1077 **(A)** *Tg*DrpC is the closes homolog of the human mitochondrial Dynamin-like 1

1078 (*Hs*Drp1). HsDrp1 function and regulation depends on its interaction with PA within

1079 its Stalk domain (blue insert), and more particularly a variable domain (dark blue

1080 insert) as well as hydrophobic residues in its insertional loop/PAbinding domain
1081 (black insert). *Tg*DrpC posseses a conserver Stalk and hydrophobic PA binding
1082 domain, indicating its is capable of interacting with PA similarly as HsDrp1. None of
1083 *Tg*DrpA, and *Tg*DrpB has a similar conserved variable and PA binding domains **(B,**
1084 **C)**.

1085 **Supplementary information 3:** Scheme depicting the molecular construct for the
1086 endogenous HA tagging of *Tg*DrpC using CrispR-Cas9 approach **(A)** and
1087 endogenous GFP tagging of TgDrpA using CrispR_Cas9 approach **(B).** *Tg*ATS2
1088 disruption does not affect *Tg*DrpA localisation **(C)** Endogenous expression of a
1089 TgDrpA-GFP sequence shows the typical localisation of the protein at the apicoplast
1090 of tachzoite in the parental (wt) line. Similar expression of TgDrpA in the Δ*Tg*ATS2
1091 mutant background did not lead to any mislocalisation of the apicoplast-resident
1092 protein.

1093 **Supplementary information 4**: Mass isotopolog distribution of ^{13}C into C14:0 (A),
1094 C16:0 (B) and C18:0 (C) after U-^{13}C-glucose labelling in FBS starvation (0.2% FBS
1095 final concentration)**.** Experiments were conducted in triplicates. * *P values* of ≤ 0.05
1096 from statistical analyses (Ttests) were considered statistically significant. (D)
1097 Δ*Tg*ATS2 intracellular parasite seem not to be affected by FBS starvation.
1098 Immunofluorescence assay on parental line and Δ*Tg*ATS2 grown 24 h in FBS
1099 starvation conditions 0% and 0.25%, using anti-IMC1/Alexa546 and anti-
1100 Sag1/Alexa488 antibodies. Scale bars=2 μm

1101

1102 **Supplementary information 5**: Scheme depicting the molecular construct to
1103 generate tagged inducible knock-downs of *Tg*ACBP1 (**A**) and *Tg*ACBP2 (**B**) (red
1104 arrows and their respective numbers represent the primers used to probe the
1105 constructs and parental lines). *Tg*ACBP1-iKO and *Tg*ACBP2-iKO clones were
1106 confirmed by PCR (**C,D**) using the primers as indicated **E,F.** Expression of
1107 endogenously tagged *Tg*ACBP1-HA and *Tg*ACBP2-GFP determine their respective
1108 localisation at the cytosol for *Tg*ACBP1 **(G)** and at the mitochondrion for *Tg*ACBP2
1109 **(H)** Western Blots confirm the down-regulation of the inducible knock-downs of
1110 *Tg*ACBP1-HA **(I)** and *Tg*ACBP2-GFP **(J)** by ATc treatment.

1111

1112 **Supplemental methods**

1113 *Gene identification and sequence analysis*

1114 *Arabidopsis thaliana* sequence of ATS2 (GenBank^{TM} and TAIR^{TM} IDs: NP_194787

1115 and AT4G30580 respectively) was used as a query sequences for BLAST searches

1116 against the *Toxoplasma gondii* genome on ToxoDB database (www.toxodb.org).

1117 Phylogenetic analysis of AGPAT related proteins was performed on the Phylogeny.fr

1118 platform (1) using proteins described in **Table1**. Protein sequences were then aligned

1119 by ClustalW software (2) and the maximum likelihood phylogeny was generated

1120 using the PhyML (3). We generated multiple sequence alignment using Clustal

1121 Omega (4).

1122 *Toxoplasma gondii plasmid constructs*

1123 Plasmid LIC-3HA-DHFR was used to generate a 3' endogenous tagging with 3xHA

1124 coding sequence of TGME49_297640 (*TgATS2*) and TGME49_240860 (*TgAGPAT*).

1125 A 2229 bp fragment corresponding to the 3' of *Tg*ATS2 was amplified from genomic

1126 DNA using primer sets 5'-

1127 TCCTCCACTTCCAATTTTAGCGTTCGTCTCGGTGGCGGC-3' and 5'-

1128 TACTTCCAATCCAATGCTTCAGACACTCGGTGCAAA-3. A 5466 bp fragment

1129 corresponding to promoter and gene sequence of *Tg*AGPAT was amplified using

1130 primer sets 5'-TACTTCCAATCCAATGCAGCCAGCAAAGGACGAAAGG-3' and 5'-

1131 TCCTCCACTTCCAATTTTAGCGAGACCGTGGCCTCGGTGGG-3'. These

1132 fragments were cloned into pLIC-3HA-DHFR vector as described previously [Huynh

1133 and Carruthers 2009]. Vectors LIC-*Tg*ATS2-3HA-DHFR and LIC-*Tg*AGPAT-3HA-

1134 DHFR were confirmed by PCR screen using primer sets 5'-

1135 GCATAATCGGGCACATCATA-3' and 5'-ATACGCATAATCGGGCACATCATA-3'and

1136 by sequencing (GATC Biotech^{TM}).

1137 Plasmid pTOXO_Cas9-CRISPR (gift from Hakimi Lab, Grenoble, France) was used

1138 to integrate a gRNA within *BsaI* restriction site as previously described (5). .Briefly,

1139 Crisp-Fwd and Crisp-Rv primer sets were phosphorylated and annealed: *TgATS2-KI*:

1140 5'-AAGTTACGGGTGTGCGCCGCCTTGCG-3' and 5'-

1141 AAAACGCAAGGCGGCGCACACCCGTA-3', *TgATS2-KO*: 5'-

1142 AAGTTGGAGCGCCGACGGGCGACTGG-3' and 5'-

1143 AAAACCAGTCGCCCGTCGGCGCTCCA-3', *TgAGPAT-KO*: 5'-

1144 AAGTTCTCTGCCGAGTTCCAATCGCG-3' and 5'-

1145 AAAACGCGATTGGAACTCGGCAGAGA-3'. The gRNAs were then ligated into

1146   pTOXO_Cas9-CRISPR plasmid linearized with *BsaI*, yielding pTOXO_Cas9-

1147   CRISPR::g*Tg*ATS2-KI, pTOXO_Cas9-CRISPR::g*Tg*ATS2-KO and pTOXO_Cas9-

1148   CRISPR::g*Tg*AGPAT-KO, respectively.

1149   For *Tg*ACBP1iHA KD, the 5' UTR flank was PCR amplified using primers below and

1150   inserted into ApaI/NdeI sites of pPR2-HA3 (6). The 3' flank was then inserted in

1151   frame with a Tet7O/SAG4 promoter using XmaI/NotI sites. Plasmid was linearized

1152   with NotI prior to transfection. For ACBP2, flanks were PCR amplified using

1153   respective primers below into the plasmid pPR2-GFP) or pPR2-mCherry (adapted

1154   from pPR2-HA3, (6). The 5' UTR flank was inserted first using ApaI/NdeI sites. Next,

1155   the 3' UTR flank was inserted using MscI/NotI sites. ACBP2 cDNA sequence was

1156   inserted last into BglII/AvrII sites. ACBP1 pLIC was PCR amplified using pLIC

1157   primers into pLIC-HA3-CAT (7), and linearized prior to transfection and selection on

1158   chloramphenicol. DrpA and DrpC were localized by CRISPR Cas9 strategy. Guides

1159   were inserted into Cas9 U6 universal plasmid (5) by either standard ligation of

1160   annealed primers or Q5 mutagenesis. Cells were transfected together with PCR

1161   product encoding either HA3-CAT and DrpC homology flanks, for DrpC or GFP

1162   sequence without selection and DrpA homology flanks for DrpA.

1163   For DrpC HA3 CAT CRISPR Cas9 tagging, DrpC was tagged at the 3' terminus by

1164   CRISPR Cas9 (5). Primers FOR and REV were annealed together and ligated into

1165   U6 universal plasmid (5). The HA3-CAT cassette was PCR amplified by primers with

1166   50 bp homology flanks corresponding to the 3' end of DrpC and in frame with HA3.

1167   50 µg of both plasmid and PCR product were transfected and placed under

1168   chloramphenicol selection (8). DrpC was also localized by pLIC-HA3-CAT (7) using

1169   For *Tg*ACBP1 iHA KD strain, the 5' UTR flank was PCR amplified using primers

1170   below and inserted into ApaI/NdeI sites of pPR2-HA3 (6). The 3' flank was then

1171   inserted in frame with a Tet7O/SAG4 promoter using XmaI/NotI sites. Plasmid was

1172   linearized with NotI prior to transfection.

1173   For *Tg*ACBP2 iKD GFP strain, flanking regions were PCR amplified using respective

1174   primers below into the plasmid pPR2-GFP) or pPR2-mCherry (adapted from pPR2-

1175   HA3, (6)Katris et al. 2014. The 5' UTR flank was inserted first using ApaI/NdeI sites.

1176   Next, the 3' UTR flank was inserted using MscI/NotI sites. ACBP2 cDNA sequence

1177   was inserted last into BglII/AvrII sites.

1178   For *Tg*ATS2 knockout by CRISPR-CAs9, an appropriate HXGPRT cassette amplified

1179   by PCR from pMini (kind gift from the Hakimi laboratory) using those primer sets,

1180   *TgATS2-KI*:   5'-

1181   GAGGCCCTGCGTCTCCTCAAGCGAAAGGCGCCGCCACAGTCGACGGGTGTGC

1182   GCC-GCCTCAGCACGAAACCTTGCATTCAAACC-3'   and   5'-

1183   GCTACTCCTTCTTCCCCTCTCG-

1184   CGTTGTGTGTCTCCCCGTCGCGTTCTGCGTCGCCAGCAGTGTCACTGTAGCCT

1185   GCCAGAACA-3';   *TgATS2-KO*:   5'-

1186   GACACACAACGCGAGAGGGGAAGAAGGAGTAGCTCTCG-

1187   TCGCCTTTCCAGAAGGTACTCCAGCACGAAACCTTGCATTCAAACC -3' and 5'-

1188   CTTCG-   CTGCTCGTTCGTCTTCATGTGGGGAAGGAGCAGCACGAAACCTTG-

1189   CATTCAAACC-3'.

1190   _Toxoplasma gondii transfection_

1191   RH-*ΔKu80* parasite line was transfected with 100 µg of pLIC-*Tg*ATS2-3HA-DHFR

1192   linearized with *BlpI* for stable integration of HA-tag at C-terminus of *Tg*ATS2. 150 µg

1193   pTOXO_Cas9-CRISPR::g*Tg*ATS2-KO and pTOXO_Cas9-CRISPR::g*Tg*AGPAT-KO

1194   were transfected in *Tg*ATS2-HA line with 10 µg of appropriate HXGPRT cassette for

1195   TgATS2-KI and TGATS2-KO, PCR product as described above. Electroporations

1196   were performed in a 2-mm cuvette in a BTX ECM 630 (Harvard Apparatus, at 1,100

1197   V, 25 Ω, and 25 µF. Stable lines expressing the tagged constructs were selected

1198   selected in media with 1 µM pyrimethamine or 25 µg/ml mycophenolic acid and 50

1199   µg/ml xanthine and cloned by limiting dilution.

1200   RH-*ΔKu80* parasites were also transiently transfected with pLIC-*Tg*AGPAT-3HA-

1201   DHFR. pTOXO_Cas9-CRISPR::g*Tg*AGPAT-KO was transfected in RH-*ΔKu80*

1202   parasites for a simple mutant Δ*TgAGPAT* and in Δ*TgATS2 parasites* to obtain a

1203   double   mutant   Δ*TgATS2*/Δ*TgAGPAT*.   The   plasmid   pMORN1-CherryRFP-

1204   MORN1/SagCAT were transfected in both RH-*ΔKu80* and Δ*TgATS2* parasite lines.

1205   All transfections were performed with 150 µg of DNA and electroporation conditions

1206   were as described above. Transfected parasites were incubated at different

1207   concentration with HFF cell 48 h prior to immunofluorescence assay.

1208   _T. gondii growth assays_

1209   *- Plaque Assay:* HFF monolayers were infected with 500 parasites and allowed to

1210   develop for 10 days before staining with Crystal Violet (Sigma) and cell growth

1211   assessment by light microscopy for the presence of intact HFF.

1212   *- Cell-based assay: T. gondii* growth was determined with an automatic microscope-

1213   based screening (Olympus ScanR, Japan). HFFs were seeded at a density of 10,000

1214   cells per well into 96-well plates and were allowed to grow and equilibrate for 48 h at

1215   37°C. Cells were then infected with 4×10^4 parasites/well. Invasion was

1216   synchronized by briefly centrifugation of plate at 250 *g* and placed at 37°C for 2 h.

1217   The assay was run for 30 h. Hoechst 33342 (Life technologies) stain was then loaded

1218   on live cells/parasites at 5 µg/ml for 20 min. Infected cells were fixed with PFA (3.7%)

1219   for 10 min at 37°C. A mouse anti-GRA1/Alexa488 labeling (dilution 1:500) was used

1220   to identify parasitophorous vacuoles. A total of 20 fields per well were taken using the

1221   20X objective. Images were collected for the distinct fluorescence channels (Hoechst

1222   33342: eg. 360-370 nm, em. 420-460 nm and Alexa488: ex. 460-495, em. 510-550

1223   nm). Images were then analyzed using the ScanR analysis software (Olympus,

1224   Tokyo, Japan). For Alexa488 channels images (vacuoles) an intensity algorithm

1225   module was used where a fixe threshold was defined with a minimum of 100 pixels

1226   size in order to segment the smallest vacuoles (one or two parasite). For Hoechst

1227   channel images (parasites nuclei), image process consists to apply a strong

1228   background correction and detected parasites with an edge algorithm. A minimum

1229   object size of 5 pixels and a maximum object 20 pixel larger one was chosen to

1230   discriminate each parasite. ScanR analysis module interface as in flow cytometry

1231   allow us to extract and display data as scatter plots and histograms. Using a "gating"

1232   procedure we were able to hierarchically filter selected data points with precise

1233   boundaries (e.g. number of vacuoles vs number of parasite/vacuoles). The

1234   proliferative index was evaluated by parasite/vacuole number ratio.

1235

1236   <u>*Activity analysis in LPAAT-deficient E. coli strains*</u>

1237   Coding sequence of *TgATS2* was synthesized (Genscript). *TgAGPAT* coding

1238   sequence was amplified by RT-PCR using primer sets 5'-

1239   ATGGCGTCCACGCCGCTGC-3'/5'-TTAGAGACCGTGGCCTCGGTG-3' and

1240   *TgAGPAT$_{\Delta N\text{-}ter1\text{-}72}$* coding was amplified by RT-PCR using primer sets 5'-

1241   CTCAACCGCCCGCCCAGGAATTA-3'/5'-TTAGAGACCGTGGCCTCGGTG-3'.

1242   These sequences were digested and ligated into *HindIII* restriction site on pQE30Xa

46

1243   vector (Quiagen) to generate expression vectors. Additionally, gene coding for *E. coli*

1244   LPAAT activity *plsC*, was amplified from *E. coli* DH5alpha genomic DNA using primer

1245   sets   5'-CTATATATCTTTCGTCTTATTATTAC-3'/   5'-AACTTTTCCGGCGGCTTC-3'

1246   and ligated into pQE30Xa vector. Then these acyltransferase vectors and empty

1247   pQE30Xa vector as negative control were transfected to eletrocompletent cells of

1248   MS2-1 Δ*plsC* deficient *E.coli.* pREP4 repressor vector to regulate Lac promotor

1249   activity. Transformed bacterial populations were grown at 37°C in order to promote

1250   growth of all isolates. Two independent clones of each bacterial strain that harbours

1251   each plasmid-of-interest were isolated for this study. Rescue of LPAAT activity in

1252   SM2-1Δ*plsC* mutant was measured by the ability to grow at elevated temperature,

1253   42ºC, non-permissive temperature in LB medium as previously described (9).

1254   Bacteria were first grown in LB media at 37°C to stationary phase, then the cultures

1255   were diluted to OD_{600}=0.04 and finally inoculated with several dilutions (at 10^{-1} to 10^{-6})

1256   on LB plates and incubated for 24 h at permissive (30°C) and non-permissive

1257   (42°C) temperatures. All experiments were conducted in triplicate with both

1258   independent clones.

1259

1260   *Parasite egress assay:*

1261   WT or Δ*Tg*ATS2 parasites were incubated on HFF cells for approximately 26 h

1262   before aspirating medium and replacing with DMEM containing 2 µM A23187 or

1263   DMSO. Parasites were incubated for 3 min before addition of an equivalent volume of

1264   2x fixative containing 5% Paraformaldehyde, 0.05% glutaraldehyde in PBS (final

1265   concentration 2.5% Paraformaldehyde, 0.025% glutaraldehyde). Cells were fixed for

1266   15 minutes before permeabilizing with 0.025% TX100 in PBS for 10 min and then

1267   Blocking overnight in blocking solution (2% FBS in PBS). Samples were then probed

1268   by immunofluorescence assay and counted manually for egress.

1269

1270   *Red/Green parasite invasion assay:*

1271   Experiment was performed as per (6). Parasites were grown for 2 days and

1272   harvested intracellular after replacing medium with ENDO buffer (44.7 mM K_2SO_4, 10

1273   mM MgSO4, 106 mM sucrose, 5 mM glucose, 20 mM Tris-H_2SO_4, 3.5 mg/ml BSA,

1274   pH 8.2). Cells were scraped, needle passed, filtered and centrifuged at 1800 rpm for

1275   10 min. Cells were resuspended to a concentration of 2.5×10^7 cells ml^{-1} in ENDO

1276   buffer and settled for 20 min onto host cells. Once settled, medium was aspirated and

47

1277   replaced with Invasion buffer (DMEM, 3% FBS and 10 mM HEPES). Parasites were

1278   allowed to invade for 15 min before fixation with 2.5% Paraformaldehyde and 0.02%

1279   glutaraldehyde. Samples were then blocked in 2% FBS in PBS overnight at 4°C.

1280   Samples were probed with mouse anti-SAG1, before washing with PBS, then

1281   permeabilized with 0.25% Triton-X100 in PBS. Cells were then probed with rabbit

1282   anti-GAP45 and washed in PBS. Samples were then probed with Alexafluor anti-

1283   mouse 546 and anti-rabbit 488 before mounting onto slides. Cells were imaged by

1284   microscopy and invasion rate determined using ImageJ.

1285   *Lipidomic analysis by GC-MS*

1286   Lipid extraction and analysis of tachyzoites was performed as previously described

1287   (10-12). Freshly egressed tachyzoites (1 x 10^{8} cell equivalents) grown in standard

1288   culture or in starvation culture, were metabolically quenched by rapid chilling of the

1289   cell suspension in a dry ice/ethanol bath and lipids were extracted in

1290   chloroform/methanol/water (2:1:0.8, v/v/v containing 25 nmol tridecanoic acid C13:0

1291   as extraction internal standard) for total lipid analysis.

1292   *- For lipid quantification:* Total lipid extraction was performed as described previously

1293   (11). Parasites were prepared as described above except for the addition of 0.1 M of

1294   HCl to promote PA and LPA extraction. Pooled organic phase was subjected to

1295   biphasic separation by adding 0.1 M HCl. In both protocols, the organic phase was

1296   dried with speed vaccum and dissolved in 1-butanol.

1297   *- Total lipids analysis:* An aliquot of the lipid extract was dried in vacuum concentrator

1298   with 1 nmol pentadecanoic acid C15:0 as internal standard. Then the dried lipid was

1299   dissolved in the chloroform/methanol, (2:1, v/v) and derivatised with MethPrep II

1300   (Alltech). The resulting fatty acid methyl esters was analysed by GC-MS as described

1301   previously (11). Fatty acid methyl esters were identified by their mass spectrum and

1302   retention time compared to authentic standards.

1303   *- Lipid quantification:* Total lipid fraction was separated by 2D-HPTLC with 5 µg

1304   PA(C17:0/C17:0)   and   5   µg   LPA(C17:0)   (Avanti   Polar   lipids)   using

1305   chloroform/methanol/28% NH4OH, 60:35:8 (v/v) as the 1st dimension solvent system

1306   and chloroform/acetone/methanol/acetic acid/water, 50:20:10:13:5 (v/v/v/v/v) as the

1307   2nd dimension solvent system (11). For DAG analysis, total lipid fraction was

1308   separated by 1D-HPTLC using hexan/diethlether/formic acid, 80:20:2 (v/v/v) as

1309   solvent system. The spot on the HPTLC corresponding to each lipid was scrapped off

1310   and lipids were directly derivatised with 0.5 M methanoic HCl in the presence of 1

1311   nmol pentadecanoic acid (C15:0) as internal standard. The resulting fatty acid methyl

1312   esters were extracted with hexane and analysed by GC-MS (11). Resulted FAME

1313   and cholesterol-TMS was analyzed by GC-MS (5977A-7890B, Agilent). FAME was

1314   then quantified using Mass Hunter Quantification software (Agilent). All statistical

1315   analyses were conducted using GraphPad Prism software. *P values* of ≤ 0.05 from

1316   statistical analyses (Ttests) were considered statistically significant.

1317

1318   *Phospholipid import assay:*

1319   Freshly lysed cultures of WT or Δ*Tg*ATS2 parasites were harvested, filtered and

1320   resuspended in DMEM to a concentration of approximately 2×10^8 cells ml^{-1}. Cells

1321   were then mixed with a 2x solution containing 10μg ml^{-1} NBD-PA or NBD-PC (5μg ml^{-1}

1322   final) and incubated at 37°C. Parasites were then spun down, resuspended in PBS.

1323   PFA was then added to a final concentration of 2.5%, and cells were fixed for 15 min

1324   before being spun down again and resuspended in 1xPBS. Parasites were smeared

1325   onto polyethyleneimine (PEI) coated coverslips (6), and then probed with anti-SAG1

1326   primary and Alexfluor anti-mouse 546 secondary antibodies by immunofluorescence

1327   microscopy, stained with DAPI and mounted onto slides. Samples were imaged by

1328   microscopy. SAG1 labelling was used to identify parasites using ImageJ and then

1329   estimate the amount of NBD-lipid uptaken by the parasites.

1330

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1363

1364

1365

1366

1367

A

Branch support value
- 🔴 76-100
- 🟡 51-75
- 🟣 26-50
- 🟢 0-25

Eukaryotic type

Vitrella brassicaformis	**Chromerid**
Chromera velia	
Cryptosporidium parvum	
Plasmodium yoelii	**Apicomplexa**
Plasmodium falciparum	
Hammondia hammondi	
Toxoplasma gondii	
Neospora caninum	
Arabidopsis thaliana	
Medicago truncatula	
Brassica napus	**Higher plant**
Brassica napus	
Arabidopsis thaliana	
Medicago truncatula	
Chlamydomonas reinhardtii	**Green algae**
Cyanidioschyzon merolae	**Red algae**
Thalassiosira pseudonana	**Diatoms**
Phaeodactylum tricornutum	
Hammondia hammondi	**Apicomplexa**
Toxoplasma gondii	
Neospora caninum	
Chromera velia	**Chromerid**
Vitrella brassicaformis	
Mycobacterium tuberculosis	**Bacteria**
Escherichia coli	
Synechocystis sp.	**Cyanobacteria**
Plasmodium yoelii	**Apicomplexa**
Plasmodium falciparum	

Prokaryotic type

B

Motif I (Catalysis) · Motif II (Glycerol-3-phosphate binding) · Motif III (Glycerol-3-phosphate binding) · Motif IV (Catalysis) · Accession No.

A Hoechst | αCPN60 | *Tg*ATS2 (αHA) | Merge | Phase | Zoom

B Hoechst | αTOM40 | *Tg*AGPAT (αHA) | Merge | Phase
Hoechst | αCPN60 | *Tg*AGPAT (αHA) | Merge | Phase

C 30°C / 42°C
Bacterial density
SM2-1 ΔplsC
+*Tg*ATS2-1
+*Tg*ATS2-2
+*Tg*AGPAT-1
+gAGPAT-2
+*Tg*AGPATΔNter1-72
+plsC
+empty pQE30Xa

D Δ*Tg*ATS2-HA
GACGGGTGTGCGCCGCCTTGCTGGC
gRNA / PAM
Cas9-HA-GFP / U6-Sag1 / gRNA
Amp[R]
Δ*Tg*ATS2-KI + RH HXGPRT RH → Δ*Tg*ATS2-KO
HXGPRT HA DHFR / HXGPRT DHFR

E kDa M | RH-Δku80 | *Tg*ATS2-HA | Δ*Tg*ATS2-KI
72, 57, 42, 31, 24

F kDa M | Δ*Tg*ATS2-KO | *Tg*ATS2-HA
72 — pATS2
57
42 — mATS2
31
24 — Gra1

G Hoechst | αHA | Merge
Parental line
Δ*Tg*ATS2-KI
Δ*Tg*ATS2-KO

H Parental line | Δ*Tg*ATS2-KI | Δ*Tg*ATS2-KO

I Parasites/vacuole
Parental line | Δ*Tg*ATS2-KI | Δ*Tg*ATS2-KO
**

J Relative abundance (%)
Parental line / *Tg*ATS2-KI / *Tg*ATS2-KO
1 | 2 | 4 (3-6) | 8 (7-10) | 10<
**** / *** / ** / *** / *

A — Hoechst | αIMC1 | MORN1 mCherry | Merge | Overlay — Parental line, ΔTgATS2

B — αGAP45 | Phase | Overlay — Parental line, ΔTgATS2

C — Residual body area (µm): Parental line (blue), ΔTgATS2 (red); groups 8, 16, 32/+; * significance markers

D — αSag1 | Phase | Overlay — Parental line, ΔTgATS2

E — Electron micrographs labeled 1, 1', 1'', 2, 2', 2'', 3, 3', 4, 5, 6, 6' with labels N, rb, RB, Mt, Ac

A

	αIMC1	αHA (DrpC)	αIMC1 αHA	Hoechst	αIMC1 αHA Hoechst Merge	Zoom

Parental line

ΔTgATS2

B   Merge: αIMC1 + αHA (DroC) + Hoechst

Parental line

ΔTgATS2

C Parental line

D ΔTgATS2

E Proposed model for WT

PL
LPA
PA

DrpC proteins

Zoom: Molecular hypothesis
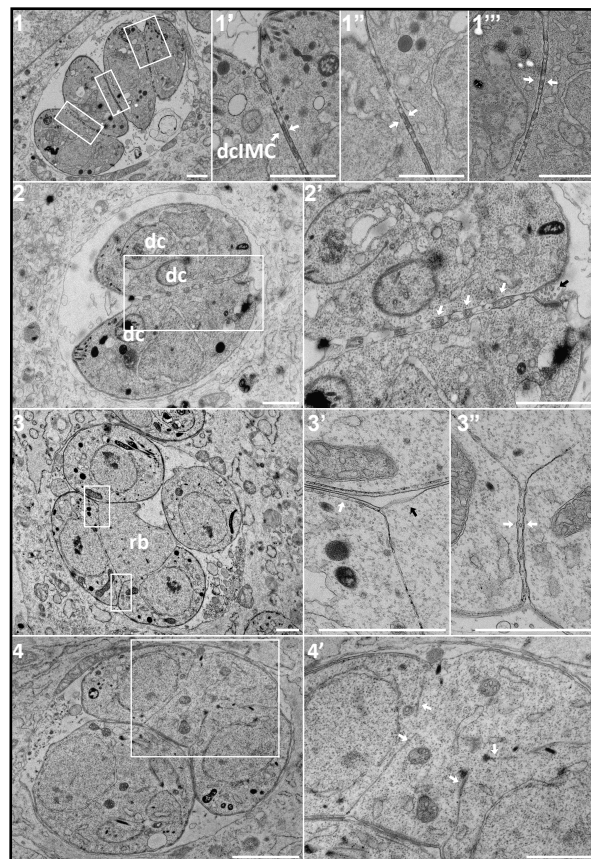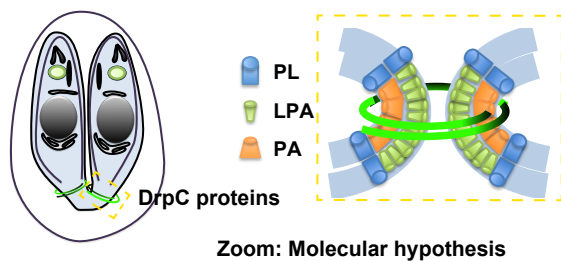
F Proposed model for ΔTgATS2
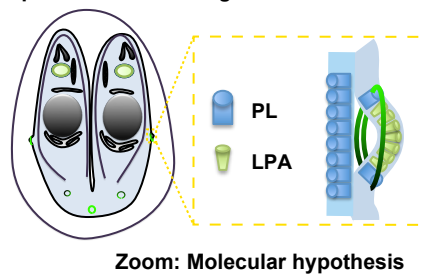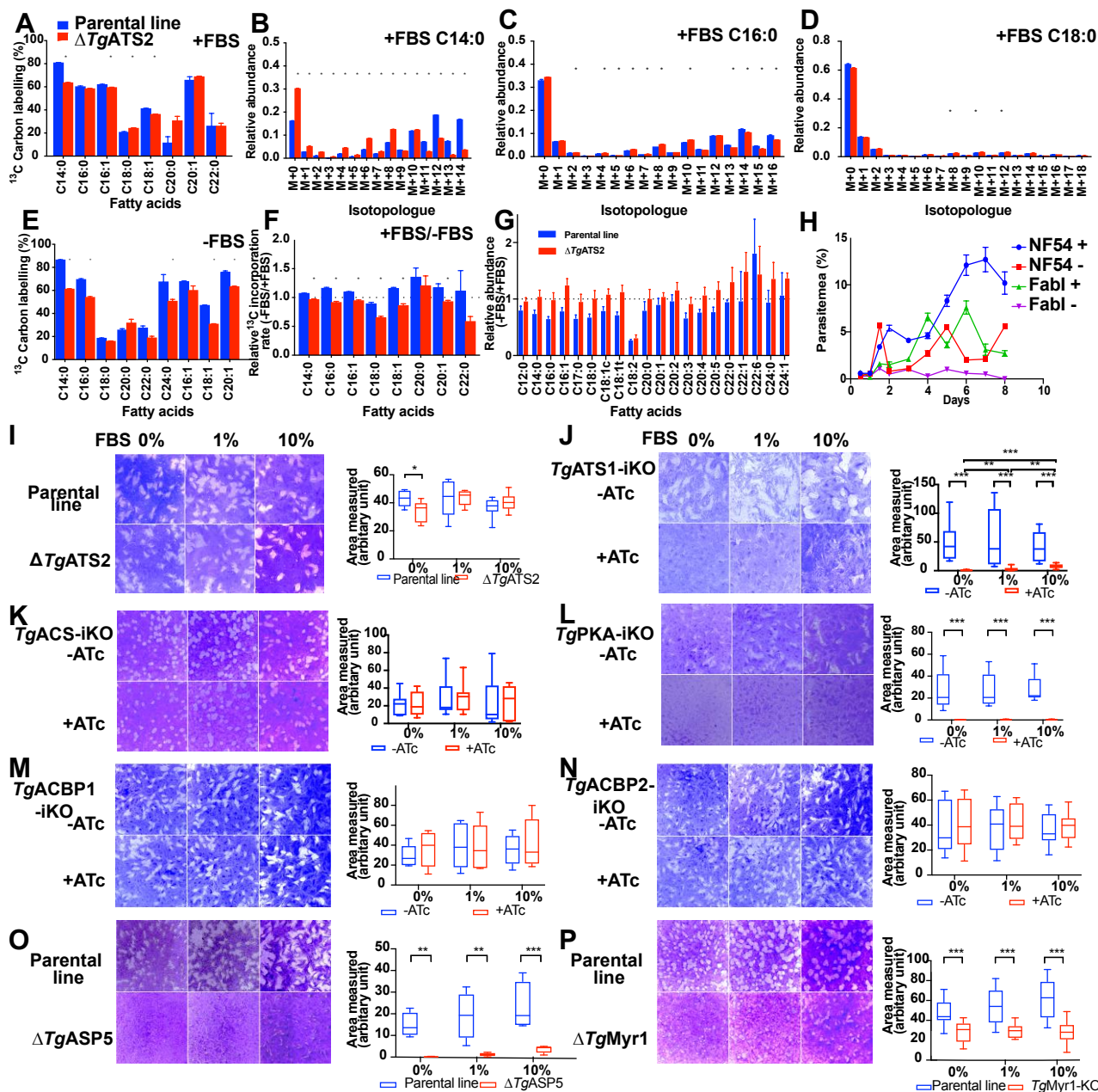
PL
LPA

Zoom: Molecular hypothesis

A Parental line

0% FBS

1 gMVB, PV gMVB, hc, gMVB

2 mt, ER, cyt, mt

3 mt

1% FBS

4 gMVB

10% FBS

5 **6**

B Δ*Tg*ATS2

0% FBS

1 gMVB, pv

2 cyt, pv

3 N, N

4 N **5** N

1% FBS

6

10% FBS

7 **8**

C

	Nile red	Nile red Hoechst	Merge
0% FBS			
1% FBS			
10% FBS			
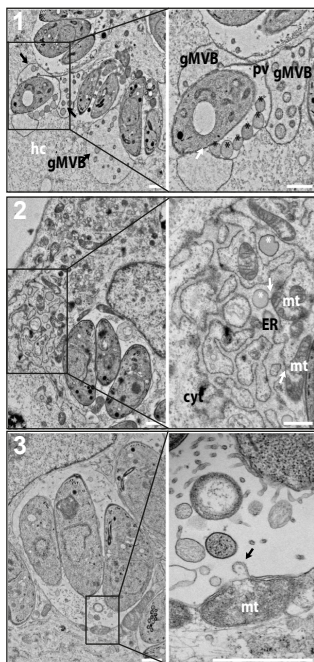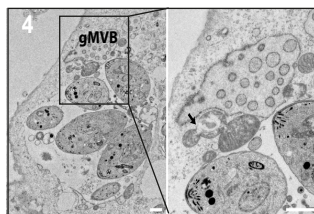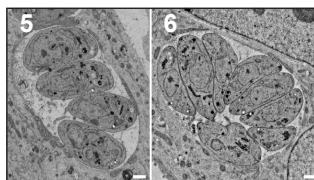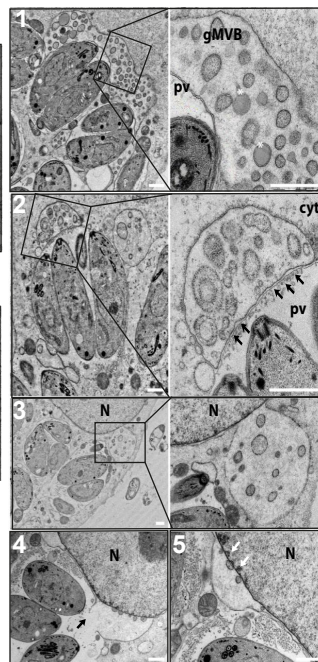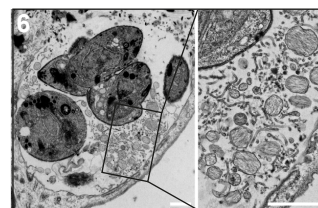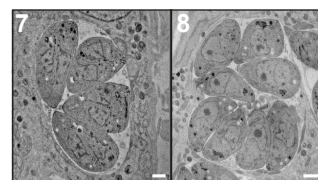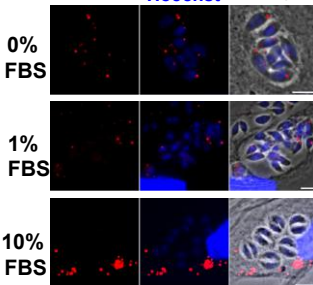
Number of Nile red dot/parasite

0% FBS   1% FBS   10% FBS

**   ****   ****

D

	Nile red	Nile red Hoechst	Merge
0% FBS			
1% FBS			
10% FBS			

E

	αLBPA	αLBPA Hoechst	Merge
0% FBS			
1% FBS			
10% FBS			