

1 **Lipoic acid biosynthesis is essential for *Plasmodium falciparum* transmission and**
2 **influences redox response and carbon metabolism of parasite asexual blood stages**

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26 Abstract

27 Malaria is still one of the most important global infectious diseases. Emergence of drug
28 resistance and a shortage of new efficient anti-malarials continue to hamper a malaria
29 eradication agenda. Malaria parasites are highly sensitive to changes in redox environment.
30 Understanding the mechanisms regulating parasite redox could contribute to the design of
31 new drugs. Malaria parasites have a complex network of redox regulatory systems housed in
32 their cytosol, in their mitochondrion and in their plastid (apicoplast). While the roles of enzymes
33 of the thioredoxin and glutathione pathways in parasite survival have been explored, the
34 antioxidant role of α -lipoic acid (LA) produced in the apicoplast has not been tested. We
35 analysed the effects of LA depletion on mutant *Plasmodium falciparum* lacking the apicoplast
36 lipoic acid protein ligase B (*lipB*). Our results showed a change in expression of redox
37 regulators in the apicoplast and the cytosol. We further detected a change in parasite central
38 carbon metabolism, with LA depletion influencing glycolysis and tricarboxylic acid cycle
39 activity. Importantly, abrogation of LipB impacted *P. falciparum* mosquito development,
40 preventing oocyst maturation and production of infectious sporozoite stages, thus flagging LA
41 biosynthesis as a potential target for the development of new transmission drugs.

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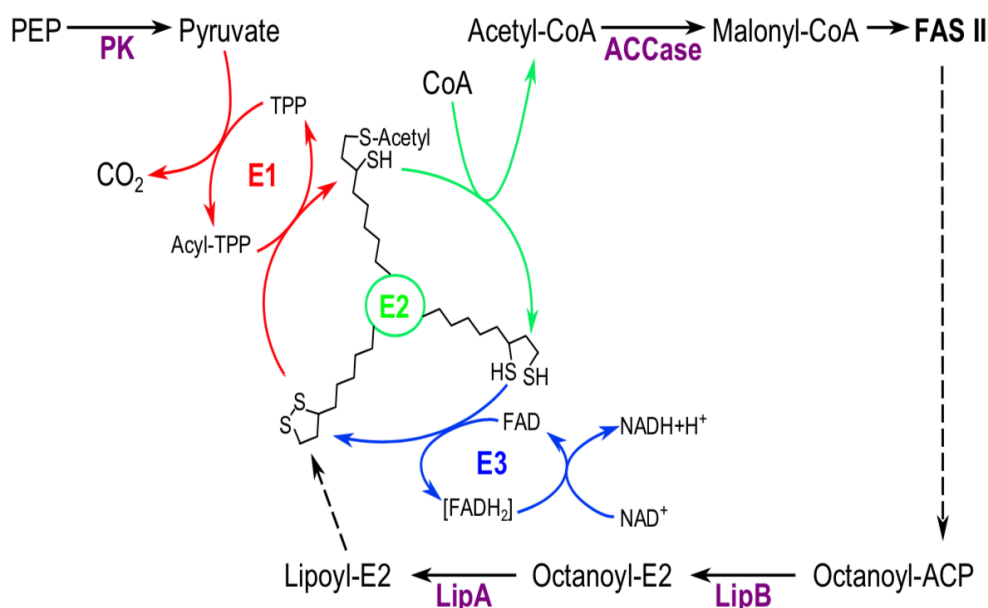
43 INTRODUCTION

44 Malaria remains a tremendous threat to human health with 200 million infections resulting in
45 405,000 deaths in 2018 (World Health Organization, 2019), so it is imperative that we identify
46 new antimalarial targets. One potential target are the parasite redox regulation systems.
47 *Plasmodium falciparum* is constitutively exposed in all stages of its complex life cycle to
48 molecules that challenge its redox balance. Finding ways to disrupt this delicate balance hold
49 promise for drug development. Indeed, *in vitro* experiments in which parasites were exposed
50 to exogenous H₂O₂-generating systems proved lethal for intra-erythrocytic stages (Dockrell
51 and Playfair, 1984). Likewise, mature gametocytes are sensitive to the oxidative stress
52 generated by exposure to redox-cyclers *in vitro* (Siciliano et al., 2017). Finally, an animal diet
53 that generates an environment rich in reactive oxygen species (ROS) in hepatocytes hosting
54 parasite cells resulted in reduced *Plasmodium* infection *in vivo* (Zuzarte-Luís et al., 2017).
55 Despite this importance, and the fact that redox regulation is a fundamental aspect of cellular
56 functions, our understanding of the parasite redox regulatory networks remains limited (Kehr
57 et al., 2010; Müller, 2015).

58 The apicoplast, a non-photosynthetic plastid acquired via secondary endosymbiosis of a red
59 algal cell, is an active metabolic hub in apicomplexan parasites including *Plasmodium* spp.,
60 (Biddau and Sheiner, 2019; Frohnecke et al., 2015; Kimata-Arigo et al., 2018; Mohring et al.,
61 2014; Sheiner et al., 2013). The apicoplast has key roles in redox balance and hosts
62 components of the thioredoxin and the glutathione systems, which represent the two best
63 characterized cellular antioxidant systems. Apicoplast based redox regulators include the
64 peroxiredoxin antioxidant protein (AOP), the dually-targeted (cytosol and apicoplast) enzymes
65 glutathione reductase (GR), and the glutathione peroxidase-like thioredoxin peroxidase
66 (TPx_G) (Kehr et al., 2010; Laine et al., 2015). Likewise, two glyoxalase system proteins are
67 apicoplast targeted: glyoxalase-1-like protein (GILP), and glyoxalase 2 (tGlolI) (Kehr et al.,
68 2010; Urscher et al., 2010). Glyoxalase 2 is proposed to play a role in detoxification of
69 incomplete triosephosphate-isomerase reaction products, but is apparently dispensable

70 during intra-erythrocytic development (Wezena et al., 2017). Two additional apicoplast
71 thioredoxin-like proteins (ATrx1 and ATrx2) are found in the peripheral compartments (Sheiner
72 et al., 2011). ATrx1 and ATrx2 were recently characterised in *T. gondii* and shown to play an
73 essential role in the control of protein sorting and folding in response to organelle redox status
74 (Biddau et al., 2018). The *Plasmodium* orthologue of ATrx2 (*PfATrx2*; PF3D7_0529100) is
75 likely essential too (Bushell et al., 2017; Zhang et al., 2018). Many other potential redox-active
76 proteins are predicted to be localised in the apicoplast (Boucher et al., 2018), but their roles
77 are uncharacterized.

78 An additional molecule proposed to take part in apicoplast redox regulation is α -lipoic acid
79 (LA) (Frohnecke et al., 2015; Günther et al., 2007; Laine et al., 2015). Due to its reducing
80 properties, LA is known as the 'universal antioxidant' (Goraça et al., 2011; Kagan et al., 1992;
81 Moura et al., 2015; Perham, 2000; Tibullo et al., 2017). The proposed antioxidant role of LA
82 in the apicoplast is based on a link between redox regulation and apicoplast pyruvate
83 metabolism via the pyruvate dehydrogenase enzyme complex (PDC). The three enzymes in
84 the PDC complex are pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and
85 apicoplast dihydrolipoyl dehydrogenase (aE3). Through a series of reactions, PDC transfers
86 an acetyl group from pyruvate to coenzyme A (CoA) to generate acetyl-CoA for the fatty acid
87 biosynthesis pathway (Foth et al., 2005; Mooney et al., 2002) (Fig. 1). This activity depends
88 on LA bound to the E2 lipoyl domain, which is reduced to dihydrolipoic acid (DHLA) during the
89 process. The final reaction of *Plasmodium* PDC is catalysed by aE3, which re-oxidises DHLA
90 back to LA to allow another cycle of PDC activity (Fig. 1). The activity of aE3 is coupled to the
91 reduction of NAD^+ to $\text{NADH}+\text{H}^+$, which in turn takes part in apicoplast redox regulation (Laine,
92 2014; McMillan et al., 2005). The DHLA/LA redox couple has a redox potential of -0.32 V,
93 which is lower than the glutathione/glutathione disulphide (GSH/GSSG) couple potential of -
94 0.24 V, thus making glutathione a potential substrate for DHLA (Packer et al., 1995).



95

96 **Figure 1. Schematic representation of PDC and LA biosynthesis components and**
 97 **function in *P. falciparum* apicoplast.** Each of the PDC enzyme reactions is shown in
 98 different colour (E1, red; E2, green; E3, blue). The three states of E2-conjugated LA are
 99 depicted onto the schematic E2. Abbreviations: ACCase: Acetyl-CoA carboxylase; CoA:
 100 Coenzyme A; FAD: Flavin adenine dinucleotide; FAS II: Fatty acid biosynthesis type II; LipA:
 101 lipoyl synthase; LipB: Octanoyl-ACP:protein *N*-octanoyltransferase; NAD: Nicotinamide
 102 adenine dinucleotide; PK: Pyruvate kinase; TPP: Thiamine pyrophosphate.

103

104 The apicoplast retains its exclusive LA biosynthesis pathway, catalysed by the enzymes
 105 octanoyl-ACP:protein *N*-octanoyltransferase (LipB) and lipoyl synthase (LipA)(Fig. 1), which
 106 operates independently from the mitochondrial LA salvage (Crawford et al., 2006; Günther et
 107 al., 2009). To test the putative role of LA in redox regulation, and its link to parasite metabolism,
 108 we examined the changes in the expression of redox regulation enzymes and the metabolic
 109 changes occurring in a *P. falciparum* mutant (named 3D7^{Δ*Pf*lipB}) where the *lipB* gene is
 110 disrupted (Günther et al., 2007). As a comparison, we also analysed an aE3 deletion mutant
 111 that has only a mild effect on redox balance in the parasite (Laine et al., 2015), potentially
 112 because aE3 function might be compensated by alternative apicoplast enzymatic systems
 113 coupled to NAD(P)⁺ reduction such as the NADP⁺-specific glutamate dehydrogenase (Zocher
 114 et al., 2012). Our results support a link between LA availability and redox regulation.
 115 Additionally, LipB depletion led to changes in central carbon metabolism corroborating a link
 116 between apicoplast redox regulation and cytosolic and mitochondrial metabolic pathways.

117 Importantly, LipB depletion hampers the ability of the parasites to develop in the mosquito,
118 which is in line with the crucial functions of redox regulation and fatty acid biosynthesis in the
119 insect stage of the parasite life cycle (Cobbold et al., 2013; Pastrana-Mena et al., 2010;
120 Siciliano et al., 2017; van Schaijk et al., 2014).

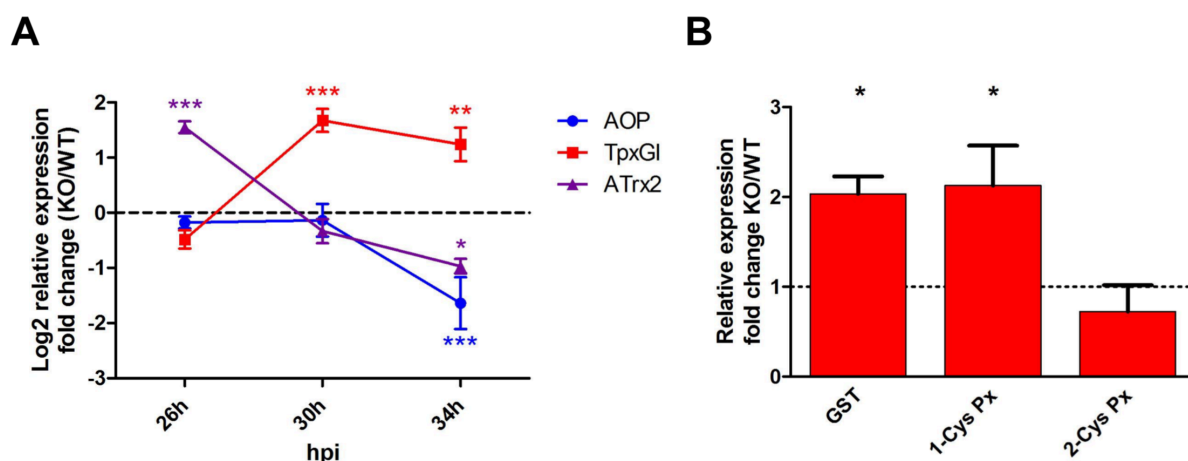
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122 **RESULTS**

123 **Deletion of *lipB* modulates apicoplast and cytosol antioxidant levels**

124 Pronounced LA deficiency was earlier detected in the trophozoite stage in LipB KO line
125 3D7 ^{Δ PflipB} parasites (Günther et al., 2007). To investigate whether this deficiency affected the
126 apicoplast antioxidant composition, we monitored the relative transcription levels of genes
127 encoding apicoplast antioxidants at 26-, 30- and 34-hours post-invasion (hpi) by qPCR for
128 both 3D7 ^{Δ PflipB} and the parental (3D7^{WT}) parasites. We tested the apicoplast redox-active
129 proteins TPX_{GI}, ATrx2 and AOP (Fig. 2), which take part in the thioredoxin redox system, and
130 the apicoplast glyoxalase system proteins GILP and tGlo (Fig. S1). Among these, TPX_{GI} had
131 the most dramatic change in expression levels. Relative transcription levels of TPX_{GI} displayed
132 three to four-fold increases at 30 hpi and 34 hpi when compared to 3D7^{WT}. Similarly, ATrx2
133 relative expression showed a four-fold increase compared to the 3D7^{WT} parasites at 26 hpi
134 followed by a two-fold decrease at 34 hpi, when AOP also showed 2-fold decrease (Fig. 2). In
135 contrast, the apicoplast glyoxalase system enzymes presented no significant differences in
136 relative expression (Fig. S1).

137 In light of this apicoplast antioxidant response, we wanted to test whether the cytosolic
138 antioxidant composition was also affected. Therefore, we used quantitative fluorescent
139 western blotting to monitor the relative levels of glutathione S-transferase (GST;
140 PF3D7_1419300), 1-Cys peroxiredoxin (1CysPx; PF3D7_0802200) and 2-Cys peroxiredoxin
141 (2-CysPx; PF3D7_1438900). These proteins were measured in 3D7 ^{Δ PflipB} and 3D7^{WT} late
142 trophozoites at 34 hpi. We observed a significant two-fold increase in protein levels for GST
143 and 1-CysPx in 3D7 ^{Δ PflipB} mutants (Fig. 2B). Conversely, 2-CysPx levels appeared to remain
144 unchanged between the two lines (Fig. 2B).



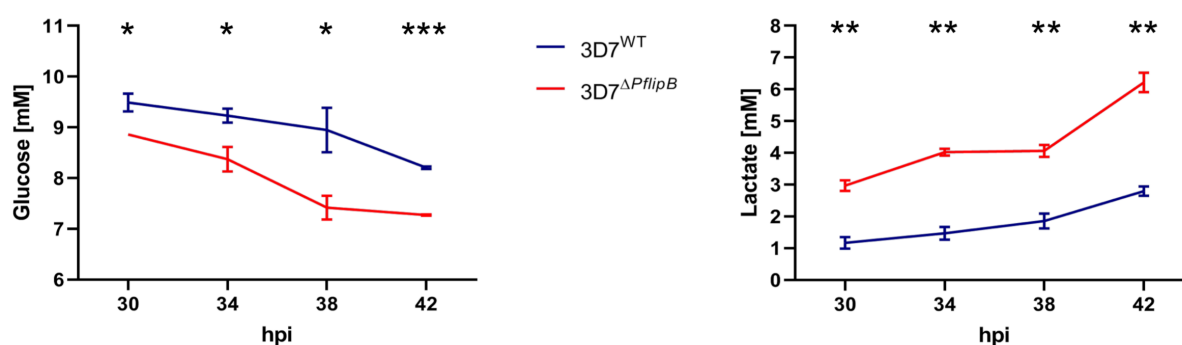
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146 **Figure 2. Analysis of apicoplast antioxidant relative expression levels and cytosolic**
 147 **antioxidant relative protein levels.** (A) Relative expression levels for apicoplast antioxidant
 148 protein (AOP), glutathione peroxidase-like thioredoxin peroxidase (TP_{XGI}), and apicoplast
 149 thioredoxin-like protein 2 (ATrx2), in the LipB mutant compared to wild type. Parasites were
 150 highly synchronised following the sorbitol and MACS protocol (see Materials and Methods)
 151 and harvested at 26, 30 and 34 hours post-invasion (hpi). Differences are expressed as Log₂
 152 of the 3D7^{ΔPflipB}/3D7^{WT} ratio of the mean signals from three experiments performed in biological
 153 triplicates (n=3). Error bars show SD. Variances were analysed using the 2-way ANOVA test
 154 coupled with the Bonferroni test using GraphPad Prism 5. Asterisks and graph lines are colour
 155 coded as shown in the legend; *: P<0.05; **: P<0.01; ***: P<0.001. (B) Relative protein levels
 156 for the cytosolic antioxidant proteins glutathione S-transferase (GST), 1-Cys peroxiredoxin (1-
 157 CysPx) and 2-Cys peroxiredoxin (2-CysPx). Three independent experiments were performed
 158 in biological triplicates and the means (n=3) of actin-normalised fluorescent signals for each
 159 protein were calculated using quantitative fluorescent western blotting. The bars represent the
 160 3D7^{ΔPflipB}/3D7^{WT} average ratio ± SD. The variance was analysed with the Student t-test using
 161 GraphPad Prism 5; *: P<0.05.
 162

163 Deletion of *lipB* affects parasite carbon metabolism

164 In eukaryotic cells, compartmental redox state and the availability of redox conducting and
 165 regulating molecules in different cellular compartments are intertwined with the activity of
 166 metabolic pathways. The redox conditions in a cellular compartment affect the function of its
 167 metabolic enzymes, while in return, the metabolic reactions in a compartment generate
 168 metabolites that impact its redox state. Thus, we proceeded to examine whether the observed
 169 redox changes in 3D7^{ΔPflipB} mutants coincide with changes in central carbon metabolism. We
 170 chose to make this analysis alongside a *P. falciparum* apicoplast dihydrolipoamide
 171 dehydrogenase (aE3) knock-out mutant (3D7^{ΔPfae3}) (Laine et al., 2015). Unlike 3D7^{ΔPflipB}, the
 172 deletion of a PDC component in 3D7^{ΔPfae3} does not result in disruption of LA biosynthesis nor

173 of PDC activity and its effect on the expression of redox regulators is only mild (Laine et al.,
174 2015), which makes $3D7^{\Delta Pfae3}$ an ideal negative control.
175 The levels of D-glucose and L-lactate in spent medium were monitored using commercial
176 enzymatic assays in two independent experiments. As glycolytic activity in *P. falciparum*
177 typically peaks during intra-erythrocytic trophozoite development (Shivapurkar et al., 2018),
178 we collected samples at 30, 34, 38 and 42 hpi to cover this developmental stage. Results
179 showed that spent medium samples from $3D7^{\Delta PflipB}$ contained significantly less D-glucose and
180 more L-lactate than $3D7^{WT}$ at 42 hpi (Fig. 3). Conversely, $3D7^{\Delta Pfae3}$ mutants did not present
181 this trend and the concentrations for these metabolites in spent medium were comparable to
182 the WT controls (Fig. S2).



183

184 **Figure 3. Analysis of D-glucose (left) and L-lactate (right) in spent medium samples**
185 **from $3D7^{\Delta PflipB}$ mutants and $3D7^{WT}$ parasites cultures.** Spent medium samples were
186 collected at 30, 34, 38 and 42 hpi and analysed using a commercial enzymatic assay for D-
187 glucose and L-lactate. Triplicate cultures at 2% parasitemia were used for each parasite line
188 for this experiment. The mean signals from 2 independent experiments (n=2) are reported with
189 error bars representing SD. The variance between the lines at each time point was analysed
190 with the Student t-test using GraphPad Prism 5; *: P<0.05; **: P<0.01; ***: P<0.001.
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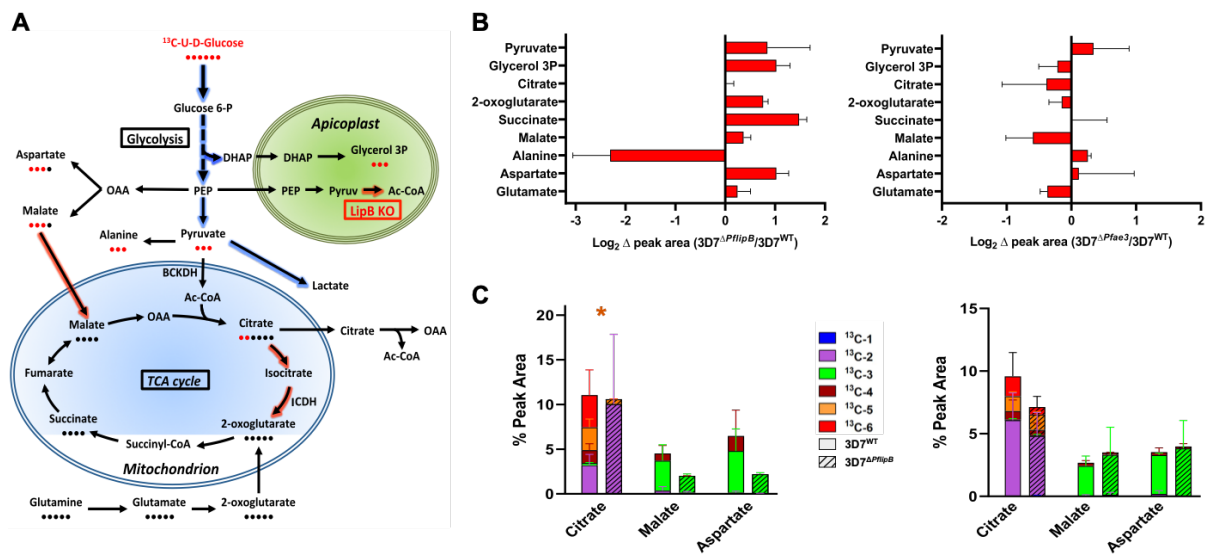
192 We further hypothesised that the up-regulation of glycolysis and the change to antioxidant
193 expression described above might affect downstream metabolism and, especially the
194 tricarboxylic acid (TCA) cycle. Therefore, we proceeded to set up two steady-state targeted
195 metabolomics experiments in biological triplicates, using the isotope-labelled nutrient ^{13}C -U-
196 D-glucose. $3D7^{\Delta PflipB}$ and $3D7^{WT}$ parasites were synchronised and metabolically labelled for
197 28 hours. Parasites at the late trophozoite stage were then rapidly chilled, and the extracted
198 metabolites analysed by liquid chromatography-mass spectrometry (LC-MS) to follow [^{13}C]

199 labelling. The analysis of the labelled fraction for these metabolites showed an immediate
200 conversion of glucose into glycolytic intermediates (Fig. S3), in line with previous analyses
201 (Storm et al., 2014). In agreement with the observation from the spent medium, 3D7 ^{Δ PflipB}
202 mutants displayed a two-fold increase in the relative levels of the glycolytic metabolite pyruvate
203 and of glycerol-3-phosphate, which derive from a glycolytic intermediate (Fig. 4A, B). A similar
204 trend was also displayed by the metabolites 2-oxoglutarate and succinate associated with the
205 TCA cycle, as well as the amino acid aspartate, whereas alanine displayed the reverse
206 tendency (Fig. 4A, B). Conversely, the analysis of 3D7 ^{Δ Pfae3} mutants showed no significant
207 differences in the relative abundances for these metabolites (Fig. 4B).

208 Only a small fraction of [¹³C] labelled triose phosphates was fed into the TCA cycle
209 intermediates in all three parasite lines (Fig. S3), as has been well established in the literature
210 (Ke et al., 2015; MacRae et al., 2013; Storm et al., 2014). Interestingly, whereas the 3D7^{WT}
211 and 3D7 ^{Δ Pfae3} controls had M+4, M+5 and M+6 citrate labelling, indicative of a complete TCA
212 cycle activity (Fig. 4C), this was not the case for 3D7 ^{Δ PflipB}. Rather, in 3D7 ^{Δ PflipB} the signals for
213 M+4 and M+6 citrate were below the detection level, while the M+5 fraction was significantly
214 decreased (Fig. 4C). In both experiments an increment of M+2 citrate was detected for
215 3D7 ^{Δ PflipB} mutants compared to 3D7^{WT}, however, with high variability between the two
216 experiments (Fig. 4C). This labelling pattern, suggested a potential altered flux of glycolytic
217 carbons into the TCA cycle. We further tested this possibility by quantitative western blot
218 analysis of the enzymes operating the first steps of the TCA cycle. Interestingly, the 3D7 ^{Δ PflipB}
219 mutant up-regulate the branched-chain α -keto acid dehydrogenase (BCKDH) component E2,
220 the first enzyme converting pyruvate to acetyl co-A (Oppenheim et al., 2014), while its
221 apicoplast parallel PDC E2 displayed no differences in abundance (Fig. 5A). In contrast,
222 isocitrate dehydrogenase (ICDH), which operates three steps downstream in the TCA cycle,
223 displayed no differences in abundance when compared with 3D7^{WT} (Fig. 5A). Collectively
224 these results point to an altered flux of metabolites through the TCA cycle such that glycolytic
225 derived carbon provide less input while glutamate becomes a major TCA cycle carbon input.

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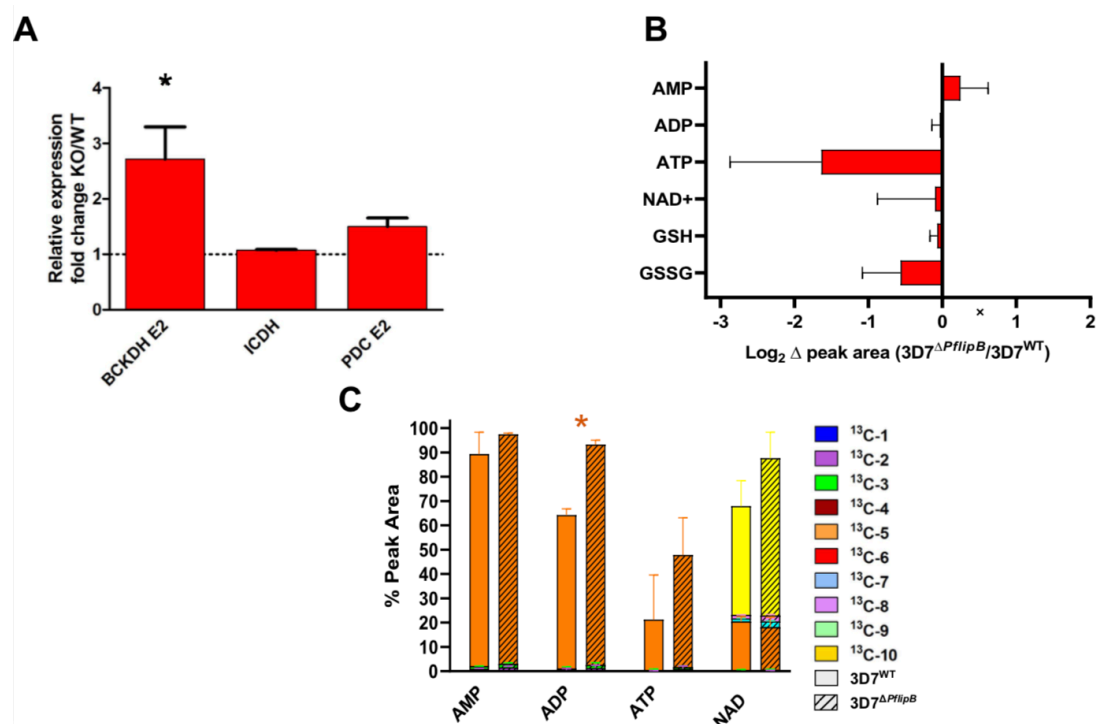


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229 **Figure 4. Metabolomic analyses of 3D7 Δ PflipB mutants and 3D7^{WT} parasites using ¹³C-U-**
 230 **D-glucose labelling.** Results from two independent targeted metabolomics experiments in
 231 biological triplicates comparing 3D7 Δ PflipB mutants and 3D7^{WT} after incubation in culture
 232 medium containing 100% ¹³C-U-D-glucose for 28 hours. (A) Schematics of the *P. falciparum*
 233 central carbon metabolism pathways analysed here, highlighting the metabolic adaptations in
 234 3D7 Δ PflipB mutant compared to wild-type parasites. Arrows shaded in red and blue respectively
 235 correspond to decreased and increased in flux for each specific reaction. Red and black dots
 236 under metabolite names respectively depict the number of labelled and unlabelled carbons,
 237 based on the most abundant labelled form of the metabolite. Abbreviations: Ac-CoA: Acetyl-
 238 CoA; BCKDH: branched-chain ketoacid dehydrogenase; DHAP: Dihydroxyacetone
 239 phosphate; OAA: oxaloacetate; PEP: Phosphoenolpyruvate. (B) Relative intracellular levels
 240 for each metabolite obtained by the sum of all the peak areas for each isotopologue of a
 241 specific metabolite. The differences in abundance for each metabolite are expressed as Log₂
 242 of the 3D7 Δ PflipB/3D7^{WT} (left graph) or Log₂(3D7 Δ Pfae3/3D7^{WT}) (right graph) mean ratio \pm SD
 243 (n=2). (C) Bar graphs summarising the percentage of isotopic incorporation in each identified
 244 metabolite calculated from the chromatographic peak areas. The bars are divided to represent
 245 the mean contribution (n=2) of the different isotopologues to each metabolite labelled fraction
 246 and error bars are SD. Empty bars represent metabolites identified in 3D7^{WT} parasites, while
 247 dashed bars correspond to metabolites from 3D7 Δ PflipB mutants (left graph) or 3D7 Δ Pfae3
 248 mutants (right graph). The variance of citrate M+5 fraction between mutants and 3D7^{WT} is
 249 depicted by an asterisk coloured with the corresponding legend colour and was analysed with
 250 the Student t-test using GraphPad Prism 5; *: P<0.05.
 251

252 Lastly, we tested cofactors involved in carbon and energy metabolism, where we observed a
 253 decrease in relative intracellular levels of ATP in 3D7 Δ PflipB, while all other cofactors tested
 254 were unchanged (Fig. 5B). In addition, 3D7 Δ PflipB mutants had an increase in the ADP M+5
 255 fraction (Fig. 5C). These results may suggest an increased ATP demand in the mutants.
 256 Likewise, an observed increase in incorporation of glucose-derived fully labelled ribose,

257 contributing to the M+5 fraction of this metabolite, may point to an up-regulation of ADP
 258 generation through the addition of the ribose to salvaged hypoxanthine.
 259 In summary, the analysis of 3D7^{ΔPflipB} mutant metabolism reveals an effect on the activity of
 260 both glycolysis and the TCA cycle. The specificity of this phenotype compared to 3D7^{ΔPfae3}
 261 metabolism supports the role of LA biosynthesis in cellular homeostasis.



262

263 **Figure 5. Analysis of the expression of metabolic enzymes and the levels of cofactors**
 264 **involved in glycolysis and in the TCA cycle. (A)** Relative protein levels for the mitochondrial
 265 enzymes branched-chain ketoacid dehydrogenase (BCKDH E2), isocitrate dehydrogenase
 266 (ICDH) and of the apicoplast enzyme dihydrolipoamide transacetylase (PDC E2). Data from
 267 three experiments performed in biological triplicates (n=3) are shown as 3D7^{ΔPflipB}/3D7^{WT} ratio
 268 of the actin-normalised mean fluorescent signals for each protein. Error bars represent SD.
 269 The variance was analysed with the Student t-test using GraphPad Prism 5; *: P<0.05. **(B)**
 270 Relative intracellular levels of metabolic cofactors are represented as Log₂ of the
 271 3D7^{ΔPflipB}/3D7^{WT} ratio of the means from two experiments in biological triplicates. The relative
 272 mean levels are the sum of all peak areas relative to each isotopologue of each metabolite
 273 (n=2). Error bars represent SD. **(C)** Bar graph summarising the percentage of isotope
 274 incorporation in the identified cofactors AMP, ADP, ATP and NAD⁺. Data correspond to two
 275 experiments performed in biological triplicates (n=2). The bars are divided to the mean
 276 contribution of each isotopologue to the total labelled fraction and displays error bars
 277 correspond to SD. Empty bars correspond to metabolites identified in 3D7^{WT} parasites, while
 278 dashed bars refer to metabolites from 3D7^{ΔPflipB} mutants. The variance of each isotopologue
 279 fraction between 3D7^{ΔPflipB} and 3D7^{WT} (n=2) is resented by the asterisk symbol (coloured
 280 according to legend) and was analysed with the Student t-test using GraphPad Prism 5; *:
 281 P<0.05.

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283

284 **Can the *lipB* mutant complete mosquito development?**

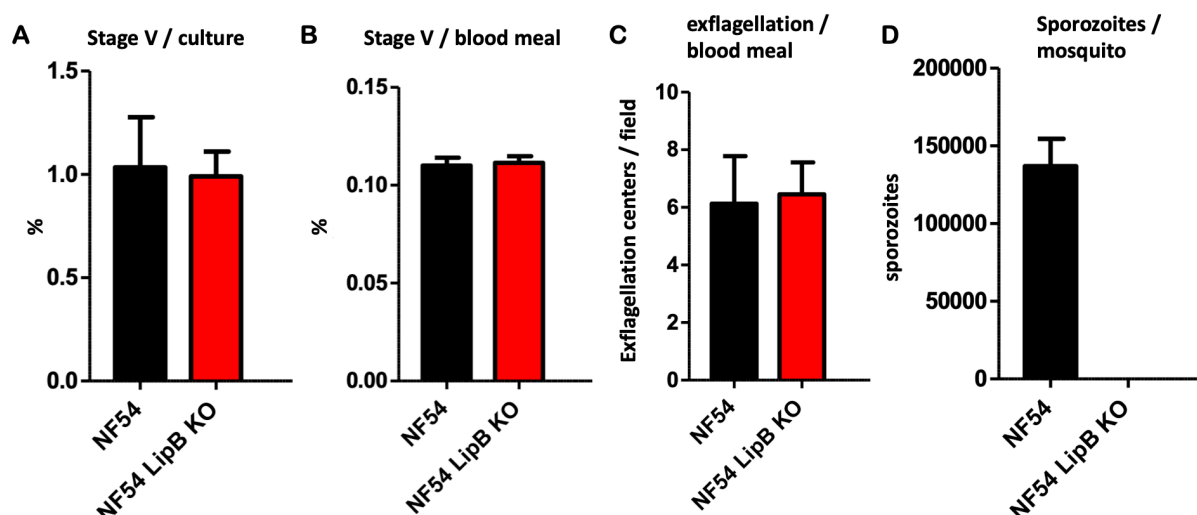
285 Despite the observed changes in redox regulation and metabolic fluxes, 3D7^{Δ*P*lipB} growth in
 286 RBC culture was only mildly affected, showing a slight increase in growth (Günther et al.,
 287 2007). Likewise, we found that at 30 hpi the 3D7^{Δ*P*lipB} mutant showed an increase in
 288 differentiation into schizonts with a 6-hour advance compared to 3D7^{WT} (Fig. S4A). This
 289 accelerated differentiation resulted in faster completion of the trophozoite stage and of the
 290 whole asexual cycle but had no effect on continuous growth in culture and no differences in
 291 the average number of merozoites (Fig. S4B). We thus investigated the development of *lipB*-
 292 deleted parasites in mosquitoes. For this analysis, a second *lipB* deletion line was generated
 293 by double-crossover gene deletion (Fig. S5) in the NF54 background (NF54^{Δ*P*lipB}), which unlike
 294 3D7 parasites, is able to infect mosquitoes. Parasites were maintained in media containing
 295 10% human serum, with the aim of them retaining their capacity to form mature gametocytes.
 296 The deletion of *lipB* did not appear to overtly influence sexual commitment in the parasites
 297 and mature gametocytes developed as in NF54^{WT} (Fig. 6, Table 1).

298

299 **Table 1: Summary of mosquito infection attempts with NF54^{Δ*P*lipB}**

Stage V gametocytemia in cultures		Final concentration of stage V gametocytemia in the infectious blood meals		Wet mount exflagellation of infectious blood meal per field (x40 phase contrast)		Sporozoites/mosquito	
NF54	NF54 LipB KO	NF54	NF54 LipB KO	NF54	NF54 LipB KO	NF54	NF54 LipB KO*
0.40%	1.62%	0.11%	0.11%	5.2	11.7	106,543	0
1.37%	0.63%	0.12%	0.12%	8.1	6.3	150,178	0
1.45%	0.75%	0.11%	0.11%	9.3	4.9	180,991	0
0.92%	0.93%	0.10%	0.10%	1.9	2.7	109,975	0
--	0.87%		0.10%		9.4		0
--	1.10%		0.12%		5		0
--	1.03%		0.12%		5.6		0
1.04% ± 0.24%	0.99% ± 0.12%	0.11% ± 0.004%	0.11% ± 0.003%	6.1 ± 1.7	6.5 ± 1.2	136,922 ± 17,717	0 ± 0

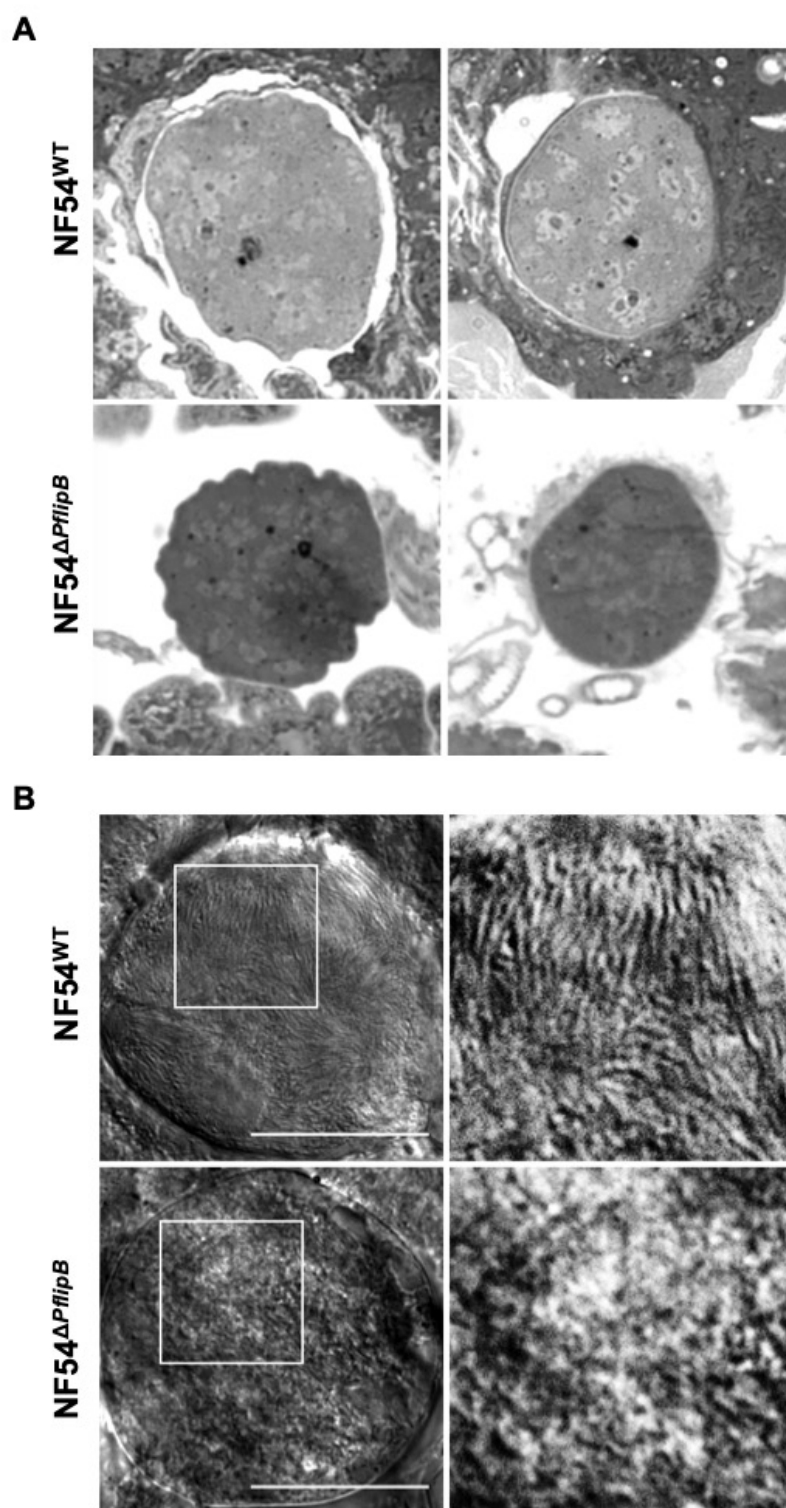
300 Each row represents a separate experiment, with parental NF54 included in the first four.
 301 Despite equivalent numbers of mature gametocytes and rates of exflagellation of male
 302 gametes in comparison with parental NF54 parasites, the LipB KO parasites failed to produce
 303 any salivary gland sporozoites, as determined from dissection and counting of 20 infected
 304 Anopheles mosquitoes per parasite line per experiment. The bottom row shows composite
 305 mean±SEM data.



306
307
308

Figure 6. Bar graphs representing the data from Table 1.

309 This allowed us to proceed to evaluate parasite development in the mosquito. Seven mosquito
310 infection experiments were performed with NF54^{ΔPflipB} gametocytes (Table 1). While midgut
311 oocysts were detectable in all experiments, no sporozoites were detected in any of the infected
312 mosquitoes in any of the seven experiments, while an average of 136,922 ± 17,717
313 sporozoites was detected in four experiments performed with the parental NF54 (Table 1, Fig.
314 6). These data indicated a major defect in development in the mosquito in the NF54^{ΔPflipB}
315 parasites. To explore this further, we examined the morphology of the midgut oocysts (Fig. 7).
316 NF54^{ΔPflipB} parasites were unable to form midgut oocyst sporozoites, suggesting an attenuated
317 sexual development for this line that could not complete its transmission cycle in the
318 *Anopheles* vector.



319

320 **Figure 7. Oocyst morphology on day 7 and 13 post-feeding of gametocytes to**
321 ***Anopheles* mosquitoes shows a defect in sporozoite development for LipB knockout**
322 **parasites. (A) Two representative light microscopy images of NF54^{ΔlipB} and of NF54^{WT} oocysts**
323 **on day 7 post feeding. (B) A representative DIC microscopy image of NF54^{ΔlipB} and of NF54^{WT}**
324 **oocysts on day 13. The insets are shown in white squares. Scale bar 25 μm. The NF54^{ΔPflipB}**
325 **oocysts show malformation in both methods and time points.**

326 DISCUSSION

327 The cellular redox balance of *Plasmodium* parasites is constantly under threat of oxidative
328 stress generated by the metabolic functions of the parasite and by the metabolic activities and
329 defence mechanisms of the host (Becker et al., 2004; Müller, 2015; Nepveu and Turrini, 2013;
330 Patzewitz et al., 2013). Apicoplast-specific redox balance is an integral part of the overall
331 cellular redox steady-state (Biddau et al., 2018; Kehr et al., 2010; Mohring et al., 2017). While
332 fragmented information is available about the different apicoplast redox control pathways, their
333 importance is evident in the series of specific antioxidant systems it hosts (Kehr et al., 2010)
334 and in the redox regulators controlling its biogenesis (Biddau et al., 2018). Likewise,
335 apicoplast-hosted pathways are coupled to redox reactions, including the biosynthesis of
336 isoprenoid precursors, which is coupled to the reduction of NADP⁺ to NADPH plus H⁺ (Seeber
337 et al., 2005; Seeber and Soldati-Favre, 2010), and the activity of PDC as discussed here (Fig.
338 1). LA is proposed to contribute to redox regulation in other systems (Tibullo et al., 2017), and
339 here we provide evidence in support of this role within the apicoplast of *Plasmodium*.

340

341 Evidence supporting the proposed role of LA as an apicoplast redox regulator

342 LA is a powerful antioxidant with low redox potential (Bilska and Włodek, 2005; Packer et al.,
343 1995), which prompted us to test how interfering with LA biosynthesis affected apicoplast
344 redox regulation. Our results revealed that the LipB deletion mutant (3D7^{ΔPflipB}) shows
345 transcriptional changes of the apicoplast redox enzymes peroxidase-like enzyme TPx_{GI},
346 thioredoxin ATrx2, and the peroxiredoxin AOP (Fig. 2A). We propose that these changes
347 promote apicoplast redox homeostasis in response to the oxidative stress caused by the
348 depletion of the LA antioxidant function. In support of this hypothesis, up-regulation of TPx_{GI}
349 also occurs in response to other oxidative stresses in *P. falciparum* (Akide-Ndunge et al.,
350 2009). Likewise, we recently reported that the ortholog of PfATrx2 in the related parasite
351 *Toxoplasma gondii* (TgATrx2) controls apicoplast gene expression, likely via a redox state-
352 controlled interaction with proteins in transit to the apicoplast lumen (Biddau et al., 2018). If
353 PfATrx2 performs a similar function, then the changes in its expression in 3D7^{ΔPflipB} may serve

354 to control protein transit to the apicoplast lumen in response to organelle redox imbalance.
355 The different roles of TP_{X_{GI}} and ATrx2 may account for the different pattern in their
356 transcriptional changes.
357 Our observations provide evidence linking the depletion of apicoplast LA to changes in the
358 apicoplast antioxidant response (Fig. 2A). We thus suggest that the apicoplast P_fPDC E2
359 enzyme may operate as an apicoplast antioxidant through its prosthetic LA. Examples of
360 DHLA acting as an electron donor to both GSH and thioredoxin systems have been previously
361 described in other organisms (Packer et al., 1995). DHLA bound to PDC-E2 and to α -
362 ketoglutarate dehydrogenase (KGDH) acts as an electron donor to glutaredoxins in an *E. coli*
363 mutant with both the thioredoxin and the GSH systems were disrupted (Feeney et al., 2011).
364 Likewise, *Mycobacterium tuberculosis* KGDH E2 uses DHLA to transfer electrons received
365 from E3 to peroxiredoxins and contributes to antioxidant defence (Bryk et al., 2002).
366 Additionally, KADH E2-mediated reduction of thioredoxins was observed in mammals (Bunik
367 and Follmann, 1993). A similar redox regulatory role of the apicoplast PDC E2 may explain
368 why it is expressed during the intra-erythrocytic stages (Foth et al., 2005; McMillan et al.,
369 2005), despite the dispensability of fatty acid biosynthesis in this stage (Vaughan et al., 2009).
370 Our attempts to delete PDC E2 in *P. falciparum* using gene replacement were unsuccessful
371 (data not shown), raising the possibility that its role during intra-erythrocytic development is
372 essential. In agreement with this observation, *Pfpdce2* was proposed to be essential during
373 intra-erythrocytic development in a recent whole-genome random mutagenesis screen (Zhang
374 et al., 2018). This hypothetical role for LA in redox regulation raises questions about the
375 sources of electrons for LA/DHLA recycling. This might not be attributed exclusively to the flux
376 of glycolytic pyruvate to PDC as this was suggested to contribute to a build-up of acetyl-CoA
377 in the organelle (Lim and McFadden, 2010). A possible alternative candidate for LA/DHLA
378 recycling might be the apicoplast-targeted GSH reductase (GR) (Müller, 2015). The GR-
379 mediated reduction of LA was demonstrated in rats, and, in particular, the use of NADPH +H⁺
380 as an electron donor was described in mitochondrial fractions (Haramaki et al., 1997; Pick et
381 al., 1995).

382 **Changes in the expression of cytosolic redox regulators and in the asexual cycle**
383 **progression upon *lipB* deletion suggest putative apicoplast-to-cytosol signalling**

384 Our data identified changes in cytosolic antioxidant expression in 3D7^{Δ*Pf*lipB} parasites. Both
385 enzymes for which we observed upregulation, GST and 1-CysPx (Fig. 2B), are highly
386 abundant in the *P. falciparum* cytosol (Liebau et al., 2002), and both have demonstrated
387 antioxidant activity in this compartment (Deponete and Becker, 2005; Harwaldt et al., 2002;
388 Krnajski et al., 2001; Liebau et al., 2002). One potential explanation for this phenotype is
389 plastid-to-cytosol redox signalling, as described in plant chloroplasts (reviewed in Dietz et al.,
390 2016). In *Arabidopsis thaliana*, chloroplast-originated H₂O₂ signal induces upregulation of the
391 expression of genes encoding different GSTs as well as enzymes involved in glycolysis and
392 the pentose phosphate pathway (PPP) (Sewelam et al., 2014). Moreover, chloroplast-
393 originated ROS signalling induce changes in the cell cycle progression (Bode et al., 2016). It
394 is thus possible that the accelerated cell cycle progressions described before for 3D7^{Δ*Pf*lipB}
395 (Günther et al., 2007) may be triggered in response to a redox signalling originating from the
396 apicoplast. Furthermore, trophozoites appear to be the asexual stage most affected by redox
397 imbalance in culture (Akide-Ndunge et al., 2009), which is in line with our observations of
398 accelerated differentiation at that stage in the mutant (Fig. S4).

399

400 **Deletion of *lipB* affects energy metabolism in *P. falciparum* blood stages.**

401 Metabolic analysis of 3D7^{Δ*Pf*lipB} mutants highlighted an increased glycolytic activity, which is
402 the main energy releasing pathway in blood stage *P. falciparum* (Salcedo-Sora et al., 2014).
403 Increased glycolytic activity in 3D7^{Δ*Pf*lipB} mutants was mostly evident through increased
404 glucose demand and increased lactate production via spent medium analysis (Fig. 3).

405 A potential model tying these phenotypes together goes as follows: changes to apicoplast
406 redox balance due to depletion of LA also induce changes in cytosolic redox through plastid-
407 to-cytosol signalling. This signal leads to temporary accelerated differentiation, which results
408 in a higher demand on glycolysis. However, we cannot rule out that the changes in cytosolic
409 redox regulator expression and asexual cycle progression are the result of the metabolic

410 changes taking place in this mutant and not the cause. The uncertainty stems from the
411 potential dual roles of LA in i/ regulating redox and ii/ supporting PDC dependent fatty acid
412 synthesis. However, accumulating evidence suggests that the latter is not essential during
413 intra-erythrocytic development (Cobbold et al., 2013) unless fatty acid starvation occurs (Botté
414 et al., 2013). We thus propose that the changes in carbon metabolism observed upon *lipB*
415 deletion are not the consequence of disruption of fatty acid metabolism. This hypothesis is
416 further supported by the absence of similar carbon metabolic variations in the 3D7^{ΔP_{fae3}} mutant
417 (Fig. S2), which lacks a fully-functional PDC due to the deletion of aE3, and which has only
418 modest changes in cytosolic redox (Laine et al., 2015).

419

420 **How is *lipB* deletion influencing the TCA cycle?**

421 The main change we detected in the TCA cycle using targeted metabolomics analysis was in
422 the labelling of citrate, the first product of the cycle. In 3D7^{ΔP_{lipB}} mutants, citrate presented
423 mainly as M+2 labelling and showed a significant decrease in M+5 while all other
424 isotopologues for this metabolite were below detection levels (Fig. 4C). These results point to
425 a change in the cycle flow, whereby unlabelled glutamate and M+2 pyruvate are likely
426 generating unlabelled and M+2 labelled citrate (Fig 4A). Furthermore, the apparent absence
427 of M+4 and M+6 citrate fractions may suggest a non-cyclic pathway that starts with 2-
428 oxoglutarate from glutamine and stops at citrate. The increase in relative intracellular levels
429 for 2-oxoglutarate and succinate further support that the cycle is fuelled mainly by the
430 glutamate branch.

431 The link between *lipB* deletion and the changes seen in the TCA cycle is unclear, and the lack
432 of change in ICDH protein levels (Fig. 5A) is puzzling. One possible explanation is that the
433 enzymatic activity of ICDH, or indeed citrate synthase and aconitase, may be inhibited in
434 response to the observed cellular redox changes. Studies on plant mitochondria highlight the
435 role of thiol redox switches in adjusting mitochondrial function in light of external stresses
436 (reviewed in Nietzel et al., 2017), and citrate synthase, aconitase and ICDH are all substrate

437 for thiol based redox regulation (Yoshida et al., 2013; Schmidtman et al., 2014; Yoshida and
438 Hisabori, 2014).

439 Interestingly, alongside the altered levels of citrate labelling, we observed an increase in
440 relative levels of BCKDH E2 (Fig. 5A) and no change in the total relative intracellular levels of
441 citrate (Fig. 4B). Previous studies suggested that citrate could take part in a malate shuttle,
442 whereby cytosolic citrate is used to generate oxaloacetate and acetyl-CoA, which in turn take
443 part in carbon fixation and protein acetylation (Cobbold et al., 2013; Storm et al., 2014). The
444 apparent absence of M+4 and M+6 citrate in 3D7^{ΔPflipB} mutants is in line with this scenario,
445 and could suggest that the citrate fraction that is not cycling in the TCA cycle but may instead
446 be channelled towards this malate shuttle. If true, this would result in increased availability of
447 cytosolic acetyl-CoA, which in turn may affect histone acetylation and thus gene expression
448 patterns (Cobbold et al., 2016). This hypothetical model could explain the altered progression
449 through the cell cycle, which typically depends on very tight regulation of gene expression
450 (Bozdech et al., 2003).

451

452 **LA biosynthesis is essential to complete sporogony**

453 We identified a severe defect in sporozoite production in the NF54^{ΔPflipb} mutant (Table 1, Fig.
454 6,7), despite the fact that normal numbers of oocysts were produced. This observation raises
455 the possibility that LA synthesis in the apicoplast is essential for sporoblast development,
456 through a yet undefined mechanism. This finding is surprising considering the evidence from
457 the rodent parasite *P. berghei*, where deletion of *lipB* caused no defect to the production of
458 salivary gland sporozoites but showed a moderate defect in liver-stage development (Falkard
459 et al., 2013). This is not the first example for such a discrepancy, PDC E1α is dispensable for
460 mosquito development in the rodent malaria parasite *P. yoelii* but was necessary for
461 sporozoite maturation in *P. falciparum* (Cobbold et al., 2013). Taken together these findings
462 point to different dependency of human and rodent malaria on PDC enzymes for development
463 in the mosquito. A possible reason for this difference may be the increased number of

464 sporozoites produced per oocyst, which is four-fold higher in the human malaria parasites *P.*
465 *falciparum* (Rungsiwongse and Rosenberg, 1991) than in rodent malaria parasites (Lindner et
466 al., 2013; Shimizu et al., 2010). High sporozoite numbers in rodent malaria oocysts may
467 require enhanced metabolism, which would depend on both PDC activity and a functional
468 redox regulation network.

469

470 **Material and Methods**

471 **Parasite culture and assessment of growth**

472 - ***P. falciparum* culturing and synchronization**

473 *P. falciparum* 3D7 parasites (isolated in the Netherlands) were cultured in RPMI 1640
474 (Invitrogen) supplemented with 11 mM D-Glucose (Sigma Aldrich), 0.5% w/v AlbuMAX II
475 (Invitrogen), 200 mM hypoxanthine (Sigma Aldrich), and 20 mg/ml gentamycin (PAA) in
476 human erythrocytes at 5% haematocrit (Trager and Jensen, 1976). Parasites were cultured
477 maintaining a reduced oxygen atmosphere (1% O₂, 3% CO₂ and 96% N₂) and a constant
478 temperature of 37°C (referred to as standard procedures). Parasitemias were determined by
479 microscopy analysis of Giemsa-stained thin smears and synchronisations were performed
480 following the sorbitol procedure (Lambros and Vanderberg, 1979). Tighter synchronisation
481 was obtained by a combination of sorbitol treatment with magnetic-activated cell sorting
482 (MACS) using LD columns (Miltenyi Biotech). Briefly, sorbitol-synchronised parasites were
483 maintained in culture until they reached the early ring stage. Cultures were synchronised twice
484 with sorbitol 6 hours apart and then cultured until they reached the late schizont stage.
485 Schizonts were purified over a MACS column once cultures had reached a schizont:ring ratio
486 of 1:2. Schizonts were then placed in culture for 1 hour with gentle shaking and cultures
487 synchronised again using the sorbitol method to obtain highly synchronous ring stages with 1-
488 hour synchrony. Highly-synchronous parasites were used for all time point experiments and
489 RNA extractions, with triplicate cultures for each condition.

490

491

492 - **Spent medium metabolite quantification**

493 Analyses of D-glucose and L-lactate concentrations from spent culture medium samples were
494 performed using the D-Glucose-HK and L-Lactic acid kits from Megazyme following
495 manufacturer's protocol. Briefly, parasite cultures for each condition were synchronised using
496 a double sorbitol treatment (Lambros and Vanderberg, 1979) with a 6 hour window, and split
497 in triplicate cultures at the same parasitemia. At each time point, an aliquot of the culture was
498 collected from each condition and erythrocytes were pelleted by centrifuging at 1000g for 5
499 min. The resulting supernatant was then stored at -20°C. For the enzymatic assay, parasite
500 spent medium samples were diluted by 1:6 with double distilled water.

501

502 **Evaluation of antioxidant gene expression**

503 - **RT-qPCR**

504 Highly synchronous parasites (see above) were cultured in triplicate for each condition until
505 they reached 26, 30 and 34 hpi. Pellets of infected RBCs at 6-8% parasitemias were then
506 washed three times in PBS and kept at -80°C. Nucleic acids were extracted using the RNeasy
507 kit (QIAGEN). Contaminating DNA was removed using the Turbo DNA-free kit (Thermo Fisher
508 Scientific). RNA samples were then reverse transcribed using the RETRO-script kit (Thermo
509 Fisher Scientific). qPCR was performed using the Power SYBR Green Master Mix (Thermo
510 Fisher Scientific) adding 20 ng of cDNA for each reaction and 300 nM of each primer (see
511 Table S1). All reactions were run in a 7500 Real-Time PCR System (Applied Biosystems).
512 The calculation of relative gene expression was performed using the $\Delta\Delta(Ct)$ method (Livak
513 and Schmittgen, 2001).

514 - **Protein extraction and quantitative fluorescent western blot**

515 For protein extraction, saponin-lysed parasite pellets were resuspended in 2D lysis buffer (100
516 mM Hepes pH 7.4, 5 mM MgCl₂, 10 mM EDTA, 0.5% (v/v) Triton X-100, 5 µg/ml RNase A, 1x

517 complete protease inhibitor cocktail (Roche) in ddH₂O water). Samples were subjected to
518 three rounds of freeze-thaw in dry ice and incubated at 4°C for 5 min in a sonicated water
519 bath. Samples were then centrifuged at 13000 g for 20 min at 4°C. Supernatants containing
520 protein fractions were quantified using the Protein Assay kit (Bio-Rad), with bovine serum
521 albumin used to generate a reference quantification curve.

522 Western blot analysis was performed by separating 20 µg of protein sample by SDS-PAGE
523 with NuPage Novex 4–12% and 15% (w/v) Bis-Tris gels (Invitrogen). Separated proteins were
524 transferred to Protran nitrocellulose membranes (Schleicher & Schuell) using a Transblot
525 semi-dry transfer system (BioRad). Membranes were blocked with 5% (w/v) dried skimmed
526 milk in PBS for 1-18 hours and incubated for 1 hour with two or more primary antibodies.
527 Primary antibodies used for relative quantification included *P. falciparum* rabbit anti-actin
528 antibody (1:12,000, loading control), *P. falciparum* rabbit anti-1-CysPx (1-Cys peroxiredoxin)
529 antibody 1:50,000; *P. falciparum* rabbit anti-2-CysPx (2-Cys peroxiredoxin) antibody at
530 1:70,000; *P. falciparum* rabbit anti-BCKDH E2 antibody at 1:5,000; *P. falciparum* rabbit anti-
531 isocitrate dehydrogenase antibody at 1:10,000 and *P. falciparum* rabbit anti-PDC E2 lipoyl
532 domain antibody at 1:250. Membranes were washed three times in PBS containing 0.2% (v/v)
533 Tween 20 and 2.5% (w/v) dried skimmed milk. Blots were then probed with IR dye-conjugated
534 antibody (1:10,000, IRDye800CW goat anti-rabbit antibody; LI-COR Biosciences) for 1 hour
535 and washed again twice. Membranes were loaded in an Odyssey SA scanner (LI-COR
536 Biosciences) and fluorescent signal intensities quantified with the Image Studio software (LI-
537 COR Biosciences). All antibodies were custom made by Eurogentec.

538 **Metabolomics experiment and analysis**

539 - **¹³C-U-D-glucose labelling experiment setup**

540 Parasites were cultured until parasitemias attained 6-8%. Two sorbitol treatments were
541 performed at approximately 8 and 14 hpi. After synchronisation, triplicate parasites cultures
542 were set using a medium where D-glucose was replaced with ¹³C-U-D-glucose (99%, CK Gas
543 Products Ltd) and the haematocrit was set to 1%. A control culture of uninfected RBCs was

544 prepared with the same conditions. All cultures were incubated for 20 hours following standard
545 procedures until late trophozoites. At this point, parasite culture metabolism was rapidly
546 quenched at 4°C in a bath of dry ice and 70% ethanol (Vincent and Barrett, 2015). Erythrocyte
547 pellets were then obtained and washed in ice-cold PBS by centrifugation at 800g for 5 min at
548 4°C. Infected RBCs in the pellets were enriched using MACS LD column purification (Miltenyi
549 Biotech) and a QuadroMACS magnet (Miltenyi Biotech) with all steps performed at 4°C.
550 Enriched samples were quantified using a Neubauer cell counting chamber and a Scepter 2.0
551 Handheld Automated Cell Counter (Millipore) to have 2.0×10^8 infected RBC per sample. The
552 same number of uninfected RBCs was collected as a control. All the samples were then added
553 to a solution of HPLC-grade chloroform:methanol:water (1:3:1; v/v/v), at concentration of
554 2×10^8 parasites per 0.5 mL solution, incubated in ice in a sonicating water bath for 2 min and
555 extracted for 1 hour at 4°C and 1500 rpm on an orbital shaker. After extraction, samples were
556 centrifuged at 13000g for 20 min at 4°C, and supernatants transferred into glass mass
557 spectrometry vials (Thermo) and stored at -80°C until LC-MS analysis.

558 - **Liquid chromatography-mass spectrometry analyses**

559 Metabolomics analyses were performed by liquid chromatography-mass spectrometry using
560 an Ultimate 3000 LC system (Dionex, UK) connected to a Q Exactive HF Hybrid Quadrupole-
561 Orbitrap mass spectrometer, (Thermo Fisher Scientific). The system was controlled by the
562 software Chromeleon (Dionex, UK) and Xcalibur (Thermo Scientific), acquiring both positive
563 and negative ionisation mode. Chromatographic separation was performed with a ZIC-pHILIC
564 chromatography column (150 mm \times 4.6 mm \times 65 μ m; Sequant, Umeå, Sweden) using a two
565 solvent system consisting of solvent A: 20 mM ammonium carbonate and solvent B:
566 acetonitrile. The table shows chromatographic conditions:

Solvent A (%)	Solvent B (%)	Time (min)
20	80	0
80	20	30
95	5	31
95	5	35
20	80	36
20	80	46

567

568 - **Metabolomic data analysis**

569 Vendor-specific raw data were initially centroided and converted into the open format mzXML
570 for subsequent processing. PeakML files (Scheltema et al., 2011) were then generated by
571 extracting the chromatographic peaks contained in the mzXML files using the detection
572 algorithm from XCMS (Tautenhahn et al., 2008). The data processing pipeline mzMatch.R
573 (Jankevics et al., 2012) was used to sort and combine all PeakML files corresponding to
574 replicates and to exclude all non-reproducible data. Further steps of noise-filtering, gap-filling,
575 and metabolite identification were performed on PeakML files utilising data obtained from
576 metabolic standards run in parallel. For each metabolite of interest, the proportions of each
577 isotopologue and its relative abundance in the sample were determined. The
578 PeakML.Isotope.TargetedIsotopes function of mzMatch-ISO (Chokkathukalam et al., 2013)
579 was used to scan the PeakML files for labelled metabolite quality and quantity. All metabolites
580 of interest in this study were reliably identified by comparison of the chromatographic retention
581 times and the m/z values with an authentic metabolic standard processed in parallel. These
582 should be then considered as “identified compounds” or level 1 according to the Metabolomic
583 Standard Initiative (Sumner et al., 2007). All metabolomics data was corrected for natural
584 carbon isotope abundance and reagent impurity using the software IsoCor (Millard et al.,
585 2019).

586 **Double cross-over deletion of *PfLipB* in NF54 strain by *Cre-loxP* system**

587 *P. falciparum* strain NF54 was cultured in complete medium containing RPMI 1640 salts and
588 10% heat-inactivated human serum (Graves et al., 1984). The strategy for double cross over
589 deletion of *pflipB* using *Cre-loxP* is depicted in **Fig. S5**. Briefly, we employed a double
590 crossover recombination strategy to generate parasite lines lacking a functional *pflipB* locus
591 (PlasmoDB ID: PF3D7_0823600). A 0.5 kb fragment of *pflipB*, PCR-amplified from NF54
592 gDNA using primers p1/p2 (Table S1), was cloned into PCC1-*cdup-hdhfr*-DXO between the
593 *SacII* and *AflII* sites and served as the homology region for the first cross-over (O'Neill et al.,
594 2011). The second homologous fragment (0.5 kb) was PCR amplified using primers p3/p4 and

595 cloned between *EcoRI* and *AvrII* sites to give rise to 8.5 kb PCC1-*cdup*-*hdhfr*- Δ *lipB* plasmid.
596 50 μ g of this plasmid was used to transfect NF54 strain by electroporation and transformed
597 parasites were selected with 1.5 nM WR99210. Drug pressure was maintained until 7 days
598 and parasites were then cultured in drug-free medium up to day 27 when the parasites
599 reappeared. WR99210-resistant parasites were then treated with 1.0 μ M 5-Fluorocytosine
600 (in DMSO) to remove single crossover-integrated plasmid and episomal forms, both of which
601 contain the suicidal *cdup* marker (Maier et al., 2006). To unmark the parasites, pTET-BSD-
602 *Cre* (kind gift from Allan Cowman) was mobilized into recombinant NF54 and cultured in 2.5
603 μ M blasticidin S-hydrochloride for 7 days, after which single-cell cloning was performed
604 (Goodyer and Taraschi, 1997).

605 **Generation of *Pf* Δ *lipB* gametocytes and production of salivary gland sporozoites**

606 Gametocytes were induced from *Pf* Δ *lipB* and WT NF54 parasite lines by multiple rounds of
607 sub-culturing for 14-18 days with nutrient deprivation, as described (Ponnudurai et al., 1986).
608 Gametocyte induction and maturation was monitored microscopically by Giemsa-stained thin
609 blood smears from culture samples. Mature gametocyte pellets were mixed with fresh O-type
610 Rh⁺ blood and human serum to produce an artificial blood meal at approximately 50%
611 hematocrit for mosquito feeds. The final concentration of stage V gametocytes in artificial
612 blood meals was 1.0% \pm 0.1% (mean \pm SEM across 7 experiments), which was equivalent to the
613 gametocyte numbers observed with NF54 (**Table S1**). *Anopheles stephensi* mosquitoes were
614 fed 3-5 days after their emergence from pupae. Fed mosquitoes were maintained for 13–16
615 days before recovery of salivary gland sporozoites (SPZ) by hand dissection.

616 **Ultra-structure analysis of midgut oocysts**

617 The infected midgut oocysts were fixed in 4%/0.1% formaldehyde/glutaraldehyde and
618 immersed in a 50:50 glycerol/water solution for Differential Interference Contrast (DIC)
619 imaging. Using a Nikon Ti Eclipse inverted microscope, cut midgut sections were pre-screened
620 for the presence of oocysts at 10 \times magnification using an automated tiling feature of the Nikon

621 NIS Elements 3.2 software. Individual oocysts were imaged at high magnification using a 60×
622 NA 1.4 oil immersion objective with a matching DIC slider. Images were compiled using the
623 NIH's ImageJ program.

624 Morphology of developing oocysts (Fig 6A) was done by fixing dissected mosquito midguts
625 in 2.5% glutaraldehyde in PBS, and then in 0.5% OsO₄, dehydration in an ethanol series,
626 embedding in London Resin White, and semi-thin sections (400nm) were mounted on glass
627 slides and stained with 0.5% toluidine blue (w/v): 0.1% Na₂CO₃ (w/v) for 10 seconds, and
628 then imaged on an Olympus BH-2 light microscope.

629

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639

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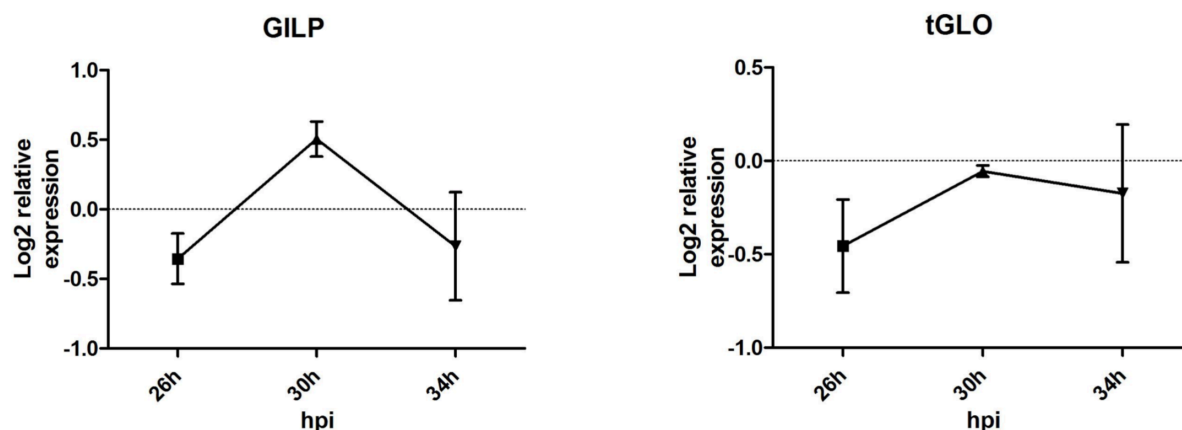
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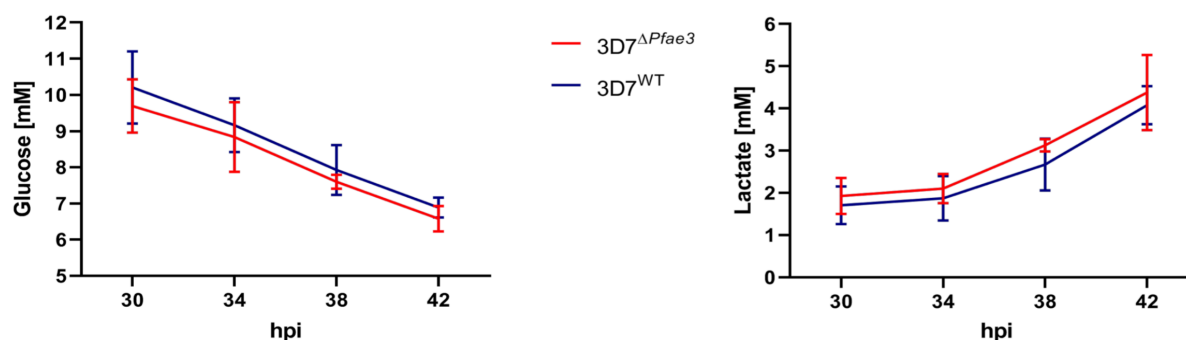
997 **Supplemental figures**



998

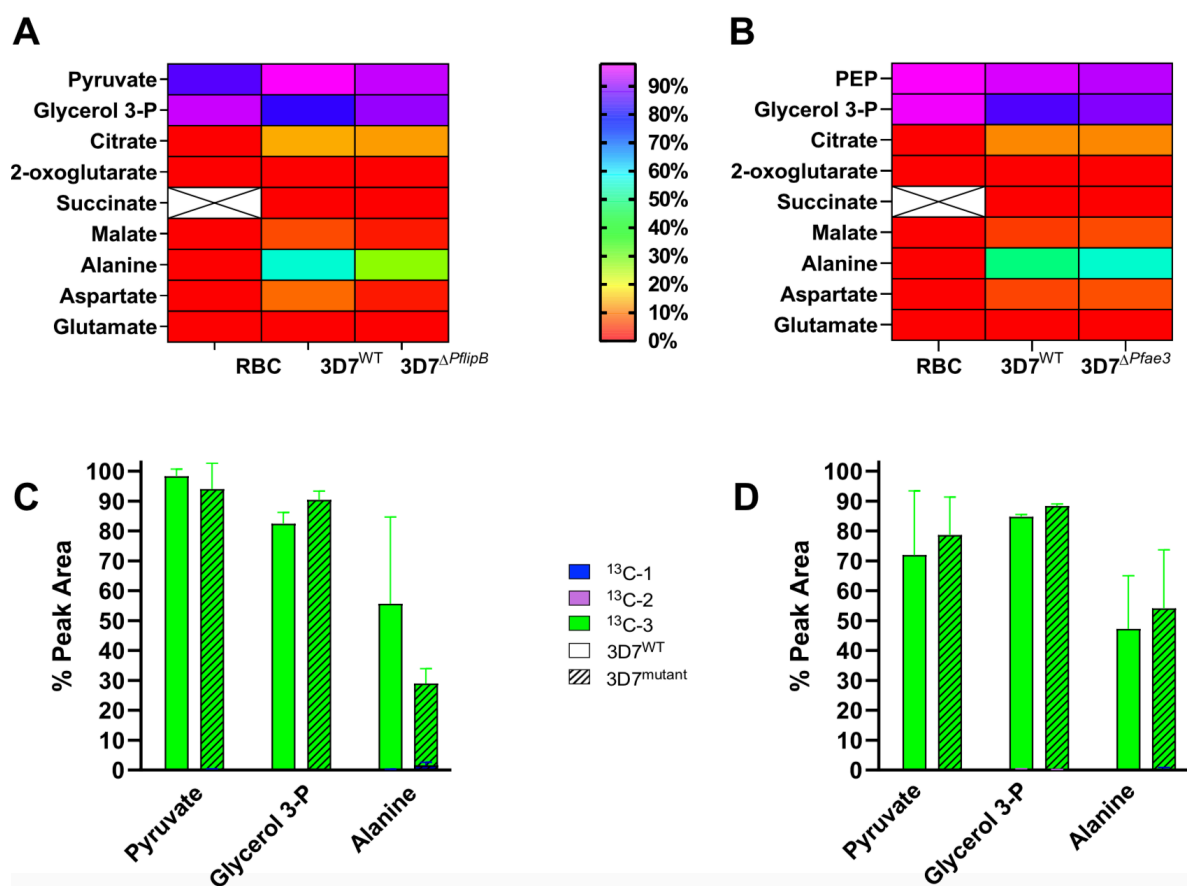
999 **Figure S1. Analysis of the apicoplast glyoxalase system enzymes relative expression**
1000 **levels.** Relative expression levels for the enzymes glyoxalase-1-like protein (GILP) and
1001 glyoxalase 2 (tGLO) were determined in three independent experiments from highly
1002 synchronised parasites following the sorbitol and MACS protocol (see Materials and Methods).
1003 Samples were harvested at 26, 30 and 34 hpi. Differences are expressed as Log_2 of the
1004 $3D7^{\Delta PflipB}/3D7^{WT}$ ratio of the mean signals from three experiments \pm SD.

1005



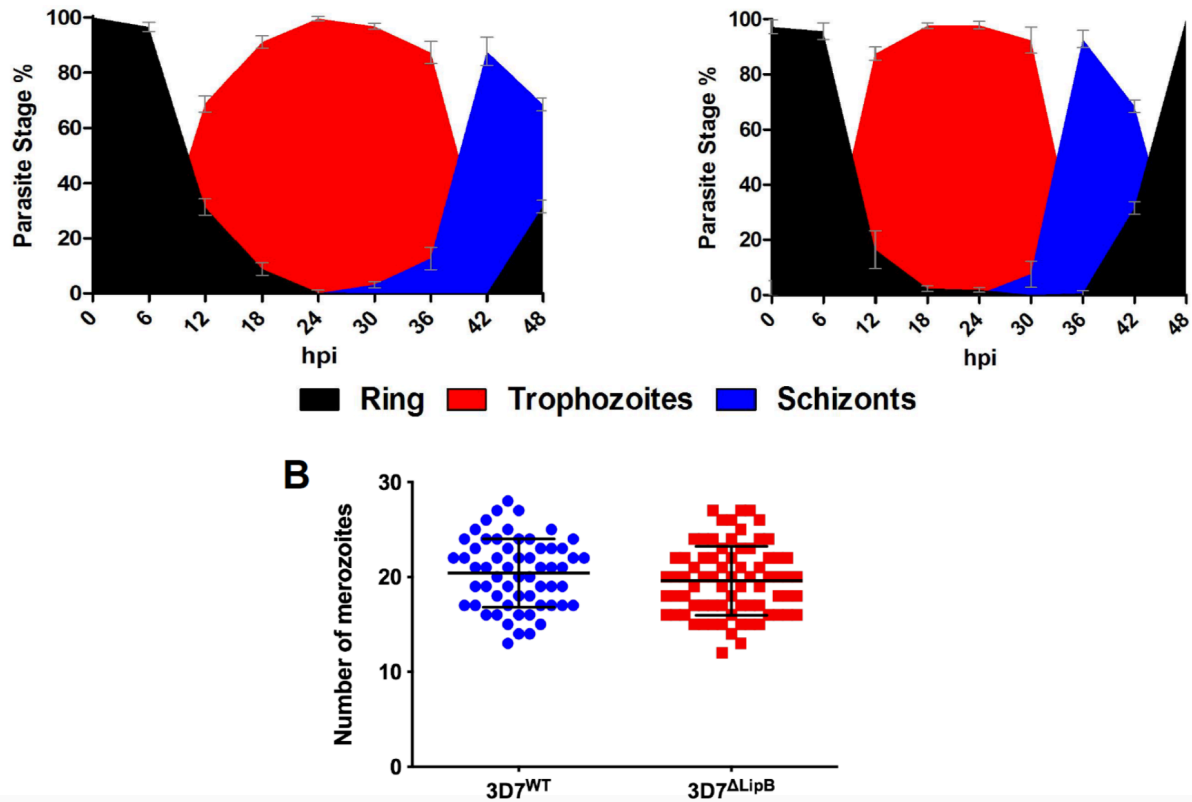
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1007 **Figure S2. Analysis of D-glucose (left) and L-lactate (right) in spent medium samples**
1008 **from $3D7^{\Delta Pfae3}$ mutants and $3D7^{WT}$ parasites cultures.** Spent medium samples were
1009 analysed using a commercial enzymatic assay for D-glucose and L-lactate. Three
1010 independent experiments in triplicate cultures at 4% parasitemia were used for each parasite
1011 line tested in this experiment. Results are reported as mean \pm SD (n=3). The variance between
1012 the lines at each time point was analysed with the Student t-test using GraphPad Prism 5.
1013



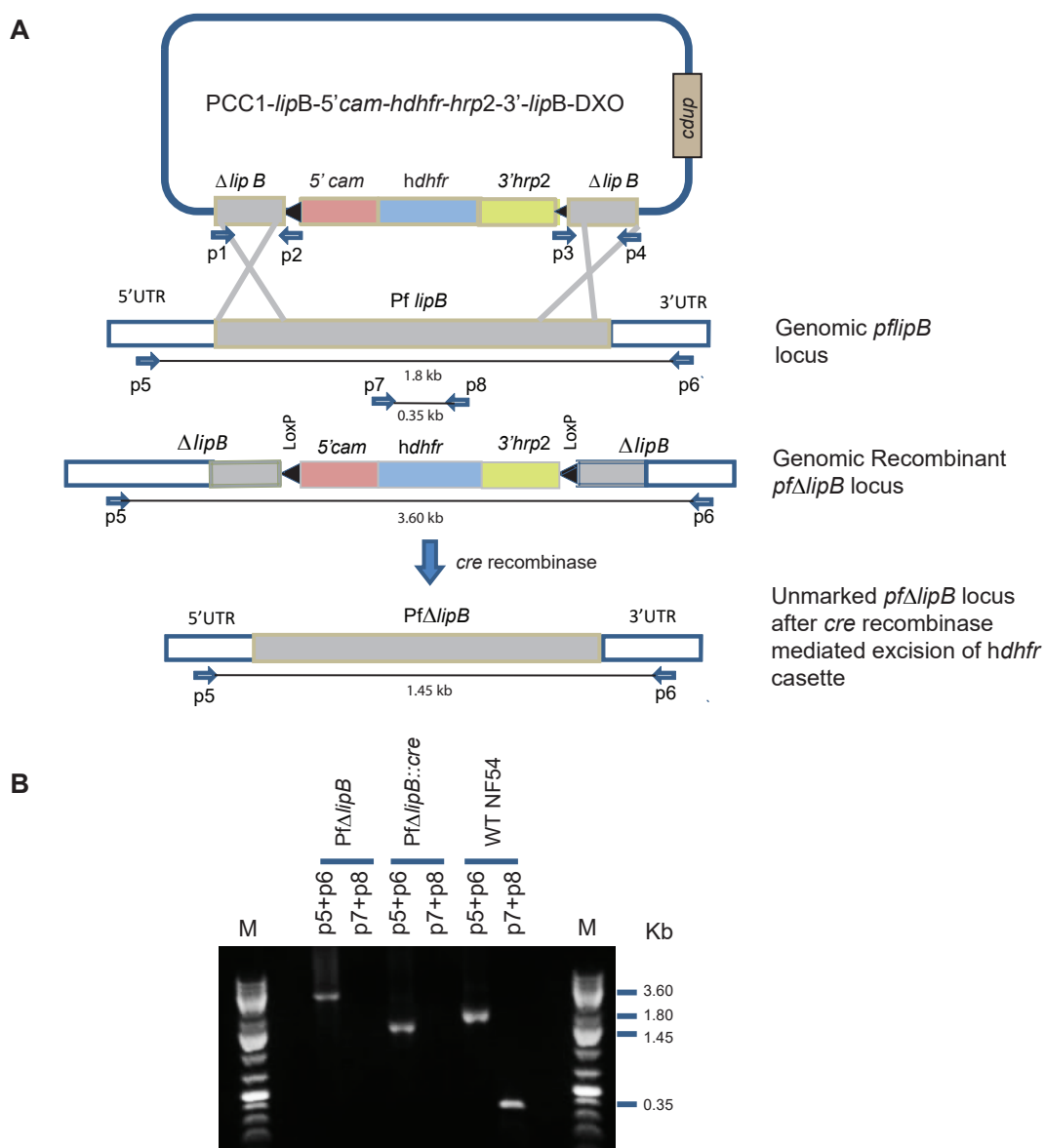
1014

1015 **Figure S3. Metabolomic analyses of 3D7^{ΔPflipB} (A,C) 3D7^{ΔPfae3} (B,D) mutants and 3D7^{WT}**
 1016 **parasites using ¹³C-U-D-glucose labelling.** Results from two independent targeted
 1017 metabolomics experiments in biological triplicates comparing 3D7^{ΔPfae3} or 3D7^{ΔPflipB} mutant to
 1018 3D7^{WT} line after incubation in culture medium containing 100% ¹³C-U-D-glucose for 28 hours.
 1019 (A-B) Heatmap representing the total labelling incorporation from ¹³C-U-D-glucose in RBC,
 1020 3D7^{WT} and 3D7^{ΔPflipB} (A) or 3D7^{ΔPfae3} (B) mutants. Crossed squares shows that succinate could
 1021 not be detected in that analysis. (C-D) Bar graphs summarising the percentage of isotopic
 1022 incorporation in each identified metabolite relative to the peak area presented as mean ± SD
 1023 (n=2). Empty bars represent metabolites identified in 3D7^{WT} parasites, while dashed bars
 1024 correspond to metabolites from 3D7^{ΔPflipB} (C) or 3D7^{ΔPfae3} (D) mutants.



1025

1026 **Figure S4. Growth pattern of 3D7^{ΔPflipB} mutants and 3D7^{WT} parasites.** (A) The variation for
1027 each parasite stage during asexual development was estimated by counting 200 random
1028 infected RBCs for each time point for 3D7^{WT} (left) and 3D7^{ΔPflipB} mutants (right). Fractions are
1029 presented as the mean percentage from three cultures for each condition, error bars
1030 correspond to SD. (B) The graph represents the number of merozoite per segmenter (n=100)
1031 in 3D7^{ΔPflipB} mutants and 3D7^{WT}. Bars represent mean ± SD.



1032

1033 **Figure S5. Generation and genotypic analysis of *PfΔlipB* in *P. falciparum* NF54.** (A) To
 1034 generate parasite lines lacking a functional *pflipB* locus (PF3D7_0823600) we employed a
 1035 double crossover recombination strategy using *Cre-loxP*. 0.5 kb fragments on both ends of
 1036 *pflipB* served as homology regions for double cross-over recombination and the *LoxP* site was
 1037 incorporated into the genomic locus that flanks the *hdhfr* cassette. Primers used to amplify the
 1038 double-recombination plasmid and to test for integration are shown. (B) PCR results showing
 1039 *pflipB* deletion in the knockout strain. Lane 1: Marker (M) NEB 1kb plus DNA ladder, 3-4:
 1040 *PfΔlipB*; Lanes 5-6: *PfΔlipB::Cre*, Lanes 7-8: wild-type NF54. Primers p5/p6 amplified a 3.6 kb
 1041 fragment only from *PfΔlipB* strain due to the incorporation of the *hdhfr* cassette. A much
 1042 smaller 1.45 kb fragment in *PfΔlipB::Cre* strain due to unmarking action of *Cre* recombinase.
 1043 In both these strains primers p7/p8 did not give a product. Alternatively, primers p5/p6
 1044 amplified a 1.8 kb fragment in the wild-type NF54 strain. Primers p7/p8 amplified a product of
 1045 0.35 kb as the deleted fragment was retained in the non-recombinant WT NF54 strain.
 1046

1047 **Table S1** - List of primers used in this work including names, sequences and purpose.
 1048 Restriction enzyme sites are italicized. LoxP site are indicated in lower case letters. The
 1049 underlined bases constitute STOP codon.

Primer Number	Primer Name	Sequence	Restriction site	Purpose
p1	LipB-F1	ACACCGCGGT <u>TAA</u> ATAAAATGTAAACGTACACTTAAC	<i>SacII</i>	Amplification of <i>PflipB</i> gene flanking regions with loxP inserts
p2	LipB-LoxP-R1	ATGCTTAAGataactcgtatagcatacattatacgaagtatt <u>TAA</u> ATATC ACTACAATTTGCTACACTAC	<i>AflII</i>	
p3	LipB-LoxP-F2	ACACTAGAA <u>TT</u> Cataactcgtataatgtatgctatacgaagttat <u>TAA</u> CG CACTTTTGATTTGCGCACAAC	<i>EcoRI</i>	
p4	LipB-R2	ACAACACCTAGGT <u>TA</u> GAAAAAGATAAGTATCCCTTAGATG	<i>AvrII</i>	
p5	LipB-5'UTR-F	GTAGCATATAGATTTACACAACACTAAC	/	Amplification of <i>PflipB</i> gene locus
p6	LipB-3'UTR-R	CTGTACCCGTTATGGTGACATAAA	/	
p7	del-LipB-F	TAATTTGTATTCGAATGAAATTATTC	/	Control primers for <i>PflipB</i> excision
p8	del-LipB-R	CTTACTGTTTGAATTTGTATATATG	/	
p9	GILPF	TTGGGGCTTGGAGTACACTT	/	Amplification of <i>PfGILP</i> by qPCR
p10	GILPR	AAATCTCCGTACCTGAGCCT	/	
p11	tGLOF	TGTGGTCATGAATATACACTCGA	/	Amplification of <i>PfGLO</i> by qPCR
p12	tGLOR	TCGTTCTGATCACATCTTAGGAA	/	
p13	AOPF	TTTACGCCTACTTGCAAGTACA	/	Amplification of <i>PfAOP</i> by qPCR
p14	AOPR	TCTCCAAGGTCTCATTCCCA	/	
p15	TP _{X(GI)} F	GCAAGAGGATTGGAGGTATACA	/	Amplification of <i>PfTP_{X(GI)}</i> by qPCR
p16	TP _{X(GI)} R	TGTGGGGAAAGCTAAGATCTAAA	/	
p17	ATrx2F	TCATAATCTAAGAACTGGGCGA	/	Amplification of <i>PfATrx2</i> by qPCR
p18	ATrx2R	TCGTTTGGTGGTTCGTTTGG	/	
p19	PfACTF	GAAGCAGCAGGAATCCACAC	/	Amplification of <i>PfActin</i> by qPCR
p20	PfACTR1	GTTGATGGTGCAAGGGTTGT	/	

1050