

# 1 **Suppressor screen connects HAD protein function to metabolic** 2 **control in *Plasmodium falciparum***

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## 27 28 29 **Abstract**

30 In the malaria parasite *Plasmodium falciparum*, isoprenoid synthesis from glycolytic  
31 intermediates is essential for survival. The antibiotic and antimalarial fosmidomycin (FSM)  
32 inhibits isoprenoid synthesis. In FSM-resistant *P. falciparum*, we identify a loss-of-function  
33 mutation in *HAD2* as causative for resistance. Enzymatic characterization shows that *HAD2*, a  
34 member of the haloacid dehalogenase-like hydrolase (HAD) superfamily, functions as a  
35 nucleotidase. Harnessing a growth defect in *HAD2*-mutant parasites, we select for suppression of  
36 *HAD2*-mediated FSM resistance and uncover hypomorphic suppressor mutations in the locus  
37 encoding the glycolytic enzyme phosphofructokinase. Metabolic profiling demonstrates that

38 FSM resistance is achieved via increased steady-state levels of MEP pathway and glycolytic  
39 intermediates and confirms reduced PFK9 function in the suppressed strains. We identify HAD2  
40 as a novel regulator of malaria glycolytic metabolism and drug sensitivity. Our study informs the  
41 biological functions of an evolutionarily conserved family of metabolic regulators and reveal a  
42 previously undescribed strategy for cellular glycolytic regulation.

43

44 Keywords: Plasmodium, malaria, isoprenoid, resistance, fosmidomycin, suppressor, glycolysis,  
45 metabolism, regulation

46

## 47 **Introduction**

48 As an obligate intracellular parasite of human erythrocytes, the malaria parasite *Plasmodium*  
49 *falciparum* has unique metabolic features that may be exploited to discover new drug targets and  
50 develop new therapies. In the red blood cell niche, *Plasmodium* parasites depend on glucose  
51 metabolism. Infection with *Plasmodium* spp. results in a nearly 100-fold increase in glucose  
52 import in red blood cells (Mehta, Sonawat, & Sharma, 2006; E. Roth, 1990; E. F. Roth, 1987).  
53 Despite these energy requirements, the parasite demonstrates little aerobic respiration via the  
54 TCA cycle. Instead, it relies on anaerobic glycolysis to produce ATP (Bowman, Grant, Kermack,  
55 & Ogston, 1961; Krungkrai, Burat, Kudan, Krungkrai, & Prapunwattana, 1999; MacRae et al.,  
56 2013; Scheibel & Miller, 1969).

57

58 Besides ATP production, glucose also has a number of anabolic fates in *P. falciparum*. One such  
59 fate is the synthesis of isoprenoids. Isoprenoids are a large class of hydrocarbons with extensive  
60 structural and functional diversity (Gershenson & Dudareva, 2007). In the malaria parasite,

61 isoprenoids perform several essential functions, including protein prenylation, dolichylation, and  
62 synthesis of GPI anchors (Guggisberg, Amthor, & Odom, 2014; Imlay & Odom, 2014; Jordão,  
63 Kimura, & Katzin, 2011). Recently, *P. falciparum* isoprenoid intermediates have also been  
64 implicated in modulation of vector feeding behavior (Emami et al., 2017).

65  
66 Despite this diversity, all isoprenoids are synthesized from a common five-carbon building block,  
67 isopentyl pyrophosphate (IPP). Evolution has produced two distinct routes for IPP synthesis: the  
68 mevalonate pathway, found in archaea, fungi, animals, and the cytoplasm of plants; and the  
69 methylerythritol phosphate (MEP) pathway, found in most eubacteria, plant chloroplasts, and  
70 apicomplexan parasites such as *P. falciparum* (Lange, Rujan, Martin, & Croteau, 2000). Because  
71 of its absence in the human host, the MEP pathway is a compelling target for antimalarial  
72 development. The antibiotic and antimalarial fosmidomycin (FSM) is a competitive inhibitor of  
73 the first committed enzymatic step of the MEP pathway, catalyzed by 1-deoxy-D-xylulose-5-  
74 phosphate reductoisomerase (DXR, E.C. 1.1.1.267) (Koppisch, Fox, Blagg, & Poulter, 2002;  
75 Kuzuyama, Shimizu, Takahashi, & Seto, 1998; Steinbacher et al., 2003). FSM has been validated  
76 as a specific inhibitor of the MEP pathway in *P. falciparum* (Zhang et al., 2011) and is a valuable  
77 chemical tool to study MEP pathway biology and essential metabolism in the parasite.

78  
79 Parasites are likely to control the proportion of glucose used for energy production versus  
80 production of secondary metabolites, such as isoprenoids. We previously used a screen for FSM  
81 resistance to identify HAD1, a metabolic regulator whose loss results in increased levels of MEP  
82 pathway intermediates and resistance to MEP pathway inhibition. HAD1 is a cytoplasmic sugar  
83 phosphatase that can dephosphorylate glycolytic intermediates upstream of the MEP pathway

84 (Guggisberg, Park, et al., 2014). HAD1 belongs to the haloacid dehalogenase-like hydrolase  
85 (HAD) enzyme superfamily (Interpro domain IPR023214) and more specifically, the IIB  
86 (IPR006379) and Cof-like hydrolase (IPR000150) subfamilies (Hunter et al., 2012). While  
87 HADs are found in all kingdoms of life, HAD1 is most related to bacterial members of this  
88 superfamily (Guggisberg, Park, et al., 2014; Kuznetsova et al., 2006), and is implicated in  
89 metabolic regulation, stress response, and phosphate homeostasis (M. J. Kang et al., 2005; Y.  
90 Kang, Weber, Qiu, Kiley, & Blattner, 2005; Pratih & Radhakrishnan, 2014; Roberts, Lee,  
91 McCullagh, Silversmith, & Wemmer, 2005; Sun & Vanderpool, 2013). However, many  
92 members of this superfamily remain uncharacterized. The study of HAD proteins in  
93 evolutionarily and metabolically distinct organisms such as *P. falciparum* will greatly facilitate  
94 our understanding of this ubiquitous class of enzymes.

95  
96 In this study, we describe the discovery of HAD2, a second HAD family member in *P.*  
97 *falciparum*. We find that HAD2 is required for metabolic homeostasis and normal asexual  
98 replication in malaria parasites. Loss of HAD2 dysregulates glycolysis and misroutes metabolites  
99 towards the MEP pathway, leading to drug resistance. In vitro, HAD2 dephosphorylates  
100 nucleotides. Selection for suppression of drug resistance identifies mutations in *PFK9*, which  
101 encodes the canonical glycolytic regulatory enzyme, phosphofructokinase. Through our forward  
102 genetics approach, we further define the biological role of the HAD protein superfamily and  
103 uncover a novel mechanism of regulation of cellular central carbon metabolism and drug  
104 sensitivity.

105

106

## 107 **Results**

108

### 109 **A nonsense allele of *HAD2*, homolog of the MEP pathway regulator *HAD1*, in** 110 **FSM<sup>R</sup> strain E2**

111 The MEP pathway is responsible for the synthesis of the essential isoprenoid precursors IPP and  
112 DMAPP. This pathway is specifically inhibited by the antibiotic fosmidomycin (FSM)  
113 (Gisselberg, Dellibovi-Ragheb, Matthews, Bosch, & Prigge, 2013; Yeh & DeRisi, 2011; Zhang  
114 et al., 2011). We previously generated *P. falciparum* strains resistant to FSM. Mutations in  
115 *HAD1* (PF3D7\_1033400) cause the resistance phenotype in a majority of these strains  
116 (Guggisberg, Park, et al., 2014). However, strain E2 remained uncharacterized. We find that E2  
117 is less sensitive to FSM than its parental line (Figure 1A), as previously reported (Guggisberg,  
118 Park, et al., 2014). This resistance phenotype is not due to changes in the *HAD1* locus or changes  
119 in *DXS*, *DXR*, or *HAD1* expression (Figure 1-figure supplement 1). To identify genetic changes  
120 that may result in FSM resistance, we performed whole genome sequencing (and identified the  
121 A469T mutation in PF3D7\_1226300 (PlasmoDB ID), hereafter referred to as *HAD2*  
122 (Aurrecochea et al., 2009). Whole genome sequencing data has been deposited in the NCBI  
123 BioProject and Sequence Read Archive databases (Guggisberg, Kelly, Hodge, & Odom, 2013a,  
124 2013b). Sanger sequencing of the *HAD2* locus in strain E2 confirmed the presence of the A469T  
125 allele. This allele encodes a truncated R157X variant and therefore we expect *HAD2* function is  
126 lost in strain E2.

127

128

129 Interestingly, HAD2 is a close homolog of the known MEP pathway regulator, the sugar  
130 phosphatase HAD1 (Guggisberg, Park, et al., 2014). Sequence homology places both proteins in  
131 the haloacid dehalogenase-like hydrolase (HAD) superfamily and further within the IIB and Cof-  
132 like hydrolase subfamilies (Interpro IPR006379 and IPR000150, respectively) (Hunter et al.,  
133 2012). While no structural information exists for *P. falciparum* HAD2, the structure of the  
134 *Plasmodium vivax* HAD2 (PVX\_123945, PvHAD2) has been solved (PDB ID 2B30). PvHAD2  
135 (93% identical and 98% similar to PfHAD2) possesses the structural motifs found in other HADs,  
136 including a core and cap domain (Figure 1B). HAD2 and HAD1 protein sequences share ~29%  
137 sequence identity and ~53% sequence similarity, and HAD2 possesses the four conserved  
138 sequence motifs found in HAD proteins (Figure 1C). We hypothesized that HAD2, like HAD1,  
139 regulates metabolism in *P. falciparum*.

140

## 141 **HAD2 is a nucleotidase**

142 Based on sequence homology to HAD1 and other HAD proteins, we predicted that HAD2 would  
143 function enzymatically as a phosphatase. The phosphatase activity of purified recombinant  
144 HAD2 was confirmed using *para*-nitrophenyl phosphate (*p*NPP), a chromogenic phospho-  
145 substrate (Kuznetsova et al., 2005, 2006) (specific activity,  $1.05 \pm 0.22$   $\mu\text{mol product}/\text{min}/\text{mg}$   
146 enzyme). Since recent work on the HAD2 homolog from *P. vivax* (PvHAD2) suggested  
147 promiscuous substrate recognition, including sugar phosphates and nucleotides (Srinivasan,  
148 Kempaiah Nagappa, Shukla, & Balaram, 2015), we determined the substrate specificity of  
149 HAD2. Surprisingly, we find markedly weaker activity against sugar phosphate substrates than  
150 by HAD1 and its PvHAD2 homolog (Guggisberg, Park, et al., 2014; Srinivasan et al., 2015). In

151 contrast, we find that HAD2 robustly utilizes several nucleotide substrates (Figure 2). Kinetic  
152 parameters for HAD2 against its top five substrates are found in Table 1.

153

154 We hypothesized that loss of HAD2-mediated nucleotidase function in FSM<sup>R</sup> strain E2 might  
155 result in changes in cellular nucleotide levels. We evaluated nucleotide levels using a recently  
156 described liquid chromatography-mass spectrometry (LC-MS) method (Laourdakis, Merino,  
157 Neilson, & Cassera, 2014). Total steady-state levels of several nucleotides are not altered in  
158 strains lacking functional HAD2 (Figure 2-figure supplement 1).

159

### 160 **Growth defect of *had2*<sup>R157X</sup> parasites drives loss of FSM resistance**

161 FSM<sup>R</sup> *had2*<sup>R157X</sup> parasites are growth-attenuated compared to the parental parasite strain (Figure  
162 3, purple vs. black line), a phenotype that is reversed following prolonged culture without FSM.  
163 Importantly, we find that improved growth rates also correlate with restored FSM sensitivity. We  
164 hypothesized that in FSM<sup>R</sup> *had2*<sup>R157X</sup> parasites, FSM resistance due to loss of HAD2 resulted in a  
165 fitness cost. Over time, the FSM<sup>S</sup> population would thus predominate. To test this possibility, we  
166 selected five clones derived from E2 by limiting dilution in the absence of drug pressure (Figure  
167 4). Three of the five clones were found to be FSM<sup>R</sup> (designated clones E2-R1, -R2, and -R3),  
168 and two were FSM<sup>S</sup> (designated E2-S1 and -S2) (Figure 4). Notably, the FSM<sup>S</sup> clones were no  
169 longer growth impaired (Figure 3, teal vs. purple line). To our surprise, all five E2 clones had  
170 maintained loss of HAD2 via the *had2*<sup>R157X</sup> mutation.

171

### 172 **Long-term culture selects for hypomorphic suppressor mutations in *PFK9***

173 We hypothesized that these FSM<sup>S</sup> E2 clones, driven by a fitness advantage, had acquired new  
174 suppressor mutation(s) at an additional locus, resulting in loss of FSM resistance and an increase  
175 in growth rate. We performed whole genome sequencing on the five E2 clones to identify any  
176 genetic changes that segregated with the FSM<sup>R</sup> and FSM<sup>S</sup> (suppressed) phenotypes. Indeed, we  
177 find that a mutation (*pfk9*<sup>T1206I</sup>) in the locus encoding phosphofructokinase-9 (*PFK9*,  
178 PF3D7\_0915400) is present in all the suppressed (FSM<sup>S</sup>) E2 clones and none of the FSM<sup>R</sup> E2  
179 clones (Figure 4). *PFK9* is the sole mutated genetic locus that segregates with the change in FSM  
180 tolerance.

181  
182 *PFK9* encodes phosphofructokinase (PFK, E.C. 2.7.11), which catalyzes the first committed and  
183 canonically rate-limiting step of glycolysis, the conversion of fructose 6-phosphate to fructose  
184 1,6-bisphosphate. PFK9 is a single polypeptide, comprised of two domains, alpha and beta  
185 (Mony, Mehta, Jarori, & Sharma, 2009) (Figure 5A). While in other eukaryotic systems, the  
186 alpha domain is typically regulatory, previous work on *P. falciparum* PFK9 has demonstrated  
187 catalytic activity for both domains (Bär, Golbik, Hübner, & Kopperschläger, 2000; Kemp &  
188 Gunasekera, 2002; Klinder, Kirchberger, Edelmann, & Kopperschläger, 1998; Mony et al., 2009;  
189 Theodorou, Cornel, Duff, & Plaxton, 1992).

190  
191 We evaluated the effects of the *pfk9*<sup>T1206I</sup> allele on native PFK activity in *P. falciparum* (Beutler,  
192 1984; Mony et al., 2009) (Figure 5B, Figure 5-figure supplement 1). Lysates from both strains  
193 possessing the *pfk9*<sup>T1206I</sup> mutation (E2-S1, S2) have markedly reduced PFK activity compared to  
194 the parental strain (*PFK9*).

195

## 196 **Loss of HAD2 leads to dysregulation of glycolysis at PFK9**

197 Since decreased PFK9 activity restored FSM sensitivity to *had2* mutant strains, we postulated  
198 that HAD2 normally regulates glycolysis, perhaps at the step catalyzed by PFK9. To evaluate  
199 this possibility, we performed targeted metabolic profiling on the parental parasite strain as well  
200 as E2 clones R1-R3 and S1-S2 (Figure 5D). Levels of the MEP pathway intermediate DOXP are  
201 significantly increased in FSM<sup>R</sup> strains lacking HAD2 (Figure 5D,  $p \leq 0.05$ , one-way ANOVA).  
202 As DOXP is the substrate for the FSM target enzyme DXR, our data indicate that FSM<sup>R</sup> strain  
203 E2 has become FSM resistant because increased levels of DOXP counteract competitive  
204 inhibition of DXR by FSM. We also observe increases in the downstream MEP metabolite,  
205 MEcPP, in our FSM<sup>R</sup> strains.

206  
207 To understand the role of PFK9 in suppressing FSM resistance, we also determined the steady-  
208 state levels of glycolytic intermediates (Figure 5D). We find levels of DOXP and MEcPP are  
209 tightly correlated with cellular levels of the PFK9 product, FBP (Pearson  $r \geq 0.9$ ,  $p \leq 0.01$ ).  
210 Clustering indicates that resistant clones are characterized by a metabolic signature of increased  
211 levels of FBP, DOXP, and MEcPP (Figure 5D). Our results suggest that HAD2 controls  
212 metabolic homeostasis at the PFK step. Of note, the *pfk9*<sup>T1206I</sup> suppressor allele restores nearly  
213 parental levels of FBP and downstream MEP pathway intermediates (Figure 5D). Thus, the  
214 hypomorphic allele of *PFK9* suppresses the metabolic dysregulation caused by loss of HAD2.

215

216 **Suppression and complementation support model of HAD2- and PFK9-**  
217 **mediated metabolic regulation**

218 We propose a model of HAD2/PFK9-mediated metabolic regulation (Figure 6). Our data  
219 indicate that HAD2 modulates metabolism through nucleotide dephosphorylation and ultimately  
220 restricts glycolysis at the level of PFK9. Loss of HAD2 misroutes metabolism, leading to  
221 increased substrate availability to the MEP pathway. This results in a substantial fitness cost in  
222 asexual growth, which is rescued by reduced PFK9 activity.

223  
224 In further support of this model, we independently repeated our genetic selection with three  
225 FSM<sup>R</sup> E2 clones (*had2*<sup>R157X</sup>, *PFK9*<sup>WT</sup>), cultured without FSM for >1 month (Figure 4). As before,  
226 these strains also lost their FSM resistance phenotype (Figure 4) and restored normal growth  
227 (Figure 3). We sequenced *HAD2* and *PFK9* in these three suppressed strains (E2-S3, -S4, and -  
228 S5). We find that all strains again maintain the *had2*<sup>R157X</sup> mutation and acquire new, independent  
229 *PFK9* mutations, correlating with increased growth rate and FSM sensitivity (Figures 3 and 4).  
230 Of the four *PFK9* variants identified in this study, three variants map to the alpha domain, while  
231 one variant (S335L) maps to the beta domain (Figure 5A). As with the original T1206I variant  
232 found in strains S1 and S2, these additional variants show a significant reduction in PFK activity  
233 from lysate (Figure 5B).

234  
235 Our model also predicted that restoration of functional HAD2 in FSM<sup>R</sup> strain E2 should restore  
236 FSM sensitivity. Using a piggyback transposon system to express GFP-tagged HAD2 (Balu,  
237 Shoue, Fraser, & Adams, 2005; Guggisberg, Park, et al., 2014; Muralidharan, Oksman, Pal,  
238 Lindquist, & Goldberg, 2012), we were repeatedly unable to obtain successful transfectants from  
239 the FSM<sup>R</sup> clones (*had2*<sup>R157X</sup>, *PFK9*), perhaps due to reduced overall fitness. However, we were  
240 able to rescue loss of HAD2 in a suppressed FSM<sup>S</sup> E2 clone (*had2*<sup>R157X</sup>, *pfk9*<sup>T1206I</sup>) (Figure 5-

241 figure supplement 2A). Consistent with our model, expression of HAD2-GFP in *had2*<sup>R157X</sup>,  
242 *pfk9*<sup>T1206I</sup> parasites led to FSM hyper-sensitivity (Figure 5E, Figure 5-figure supplement 2B).  
243 The existence of the *pfk9*<sup>T1206I</sup> allele in the presence of functional HAD2 seems to reduce parasite  
244 fitness, as evidenced by reduced growth (Figure 3, grey vs. teal lines).

245  
246  
247  
248 Using fluorescence microscopy, we observe that HAD2-GFP is localized throughout the  
249 cytoplasm in asexual *P. falciparum* trophozoites and schizonts (Figure 5-figure supplement 2C),  
250 but excluded from the digestive food vacuole. This is consistent with the lack of a predicted  
251 signal sequence for HAD2 using SignalP, PlasmoAP, and PlasMit algorithms (Foth et al., 2003;  
252 Petersen, Brunak, von Heijne, & Nielsen, 2011; Zuegge, Ralph, Schmuker, McFadden, &  
253 Schneider, 2001). Overall, our localization suggests that HAD2 primarily accesses  
254 monophosphorylated nucleotides in the cytosol.

255

## 256 **Discussion**

257

258 Cells must control levels of critical metabolites in order to efficiently utilize carbon sources for  
259 energy and biosynthesis of essential molecules. Cells may regulate their metabolism via  
260 transcriptional, post-transcriptional, post-translational, allosteric, or enzymatic mechanisms that  
261 are necessary for growth (Blume et al., 2015; Brown, Brown, Netea, & Gow, 2014; Jurica et al.,  
262 1998; Kronstad et al., 2012). In the glucose-rich red blood cell niche, *Plasmodium* spp. malaria  
263 parasites display a unique dependence on glycolysis for energy and biosynthesis.

264

265 Using resistance to a metabolic inhibitor, we identify a nucleotidase, HAD2, as a novel regulator  
266 of metabolism. Cells lacking HAD2 exhibit marked dysregulation of central carbon metabolism,  
267 including altered steady-state levels of glycolytic intermediates and isoprenoid precursors.  
268 HAD2 is necessary for optimum parasite fitness, and strains lacking HAD2 acquire additional  
269 genetic changes that suppress HAD2-mediated drug resistance and growth defect. We find that  
270 mutations in phosphofructokinase (*PFK9*) restore wild-type growth rates and FSM sensitivity.  
271 Our study thus directly genetically connect the function of HAD2, a HAD superfamily member,  
272 to control of essential central carbon metabolism, as outlined in our model (Figure 6).

273

274 In our study, we find that inhibitor resistance is defined by a distinct metabolic signature,  
275 characterized by increased levels of the MEP pathway metabolites DOXP and MEcPP and the  
276 key glycolytic metabolite FBP. This finding suggests that MEP pathway metabolism is precisely  
277 linked to FBP production. In other microbial systems, FBP levels reflect metabolic flux and are  
278 cued to environmental perturbations (Kochanowski et al., 2012). FBP-centered metabolic  
279 regulation is also important to the related apicomplexan *Toxoplasma gondii*, which constitutively  
280 expresses the fructose 1,6-bisphosphatase (FBPase) to fine-tune glucose metabolism (Blume et  
281 al., 2015). While *P. falciparum* does not appear to possess an FBPase, necessary for  
282 gluconeogenesis, the parasite may possess alternative FBP-sensing mechanisms to tune  
283 metabolism, perhaps via regulators such as HAD1 and HAD2.

284

285 HAD2 is a member of the HAD superfamily and a homolog of the previously described  
286 metabolic regulator HAD1. With our previous studies on HAD1 (Guggisberg, Park, et al., 2014;

287 Park, Guggisberg, Odom, & Tolia, 2015), we define the role of these proteins in *P. falciparum*  
288 and contribute to the greater understanding of the HADs, an evolutionarily conserved and  
289 widespread protein family. Both enzymes belong to the IIB (IPR006379) and Cof-like hydrolase  
290 (IPR000150) subfamilies (Hunter et al., 2012). HAD enzymes display diverse substrate  
291 preferences (Cabello-Díaz et al., 2015; José A Caparrós-Martín, McCarthy-Suárez, & Culiáñez-  
292 Macià, 2013; José Antonio Caparrós-Martín, Reiland, Köchert, Cutanda, & Culiáñez-Macià,  
293 2007; Huang et al., 2015; Kuznetsova et al., 2006; Liu et al., 2015; Roberts et al., 2005; Titz et  
294 al., 2007), and their biological functions are largely unknown. These subfamilies are comprised  
295 of proteins from bacteria and plastid-containing organisms (plants and apicomplexans), most of  
296 which notably also employ the MEP pathway, suggesting a common function of HADs. As *P.*  
297 *falciparum* HADs influence easily quantified phenotypes (drug tolerance, growth, metabolite  
298 levels), the malaria parasite remains an attractive system to study general HAD biology and  
299 function.

300  
301 Our study uncovers two critical, parasite-specific features of metabolism that are required for *P.*  
302 *falciparum* fitness. First, our data indicate that HAD2 is necessary for efficient asexual  
303 replication in *P. falciparum*. As HAD2 lacks close mammalian homologs, targeted therapeutics  
304 directed against HAD2 are of great interest. Second, our work highlights the central role of the  
305 glycolytic enzyme PFK9. While the parasite tolerates substantial reductions in PFK activity in  
306 the *had2*<sup>R157X</sup> background, such defects reduce the fitness of parasites rescued with HAD2-GFP.  
307 A recent kinetic model of parasite glycolysis confirms that PFK has a high flux-control  
308 coefficient, is sensitive to competitive inhibition, and can effectively reduce glycolytic flux  
309 (Penkler et al., 2015; van Niekerk, Penkler, du Toit, & Snoep, 2016). We thus propose that PFK9

310 is a leading candidate for strategies to target essential glucose metabolism in malaria. Like  
311 HAD2, PFK9 is plant-like and evolutionarily divergent from its mammalian homologs (Mony et  
312 al., 2009). These differences may be exploited for PFK inhibitor design.

313

314 Finally, our approach demonstrates the power of forward genetics to uncover novel biology in a  
315 clinically relevant, non-model organism. We employ a previously described screen for FSM  
316 resistance (Guggisberg, Park, et al., 2014) to uncover a novel resistance locus and employ a  
317 second selection for parasite fitness to identify changes that suppress our resistance phenotype.

318 While fitness costs associated with antimalarial resistance are well known [summarized in  
319 (Gabryszewski et al., 2016; Hastings & Donnelly, 2005; Rosenthal, 2013)], this study represents,  
320 to our knowledge, the first to harness this evolutionary trade-off to identify suppressor mutations  
321 in a non-target locus. Fitness assessment of resistance mutations may allow for suppressor  
322 screening for other antimalarials or other target pathways to reveal new aspects of biology and  
323 drug resistance.

324

325

## 326 **Materials & Methods**

327

### 328 **Parasite culture maintenance**

329 Unless otherwise indicated, parasites were maintained in a 2% suspension of human erythrocytes  
330 in RPMI medium (Sigma Aldrich, St. Louis, MO) modified with 27 mM NaHCO<sub>3</sub>, 11 mM  
331 glucose, 5 mM HEPES, 0.01 mM thymidine, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 10  
332 µg/mL gentamycin, and 5 g/L Albumax (Thermo Fisher Scientific, Waltham, MA). Parasites  
333 were maintained at 37 °C in 5% O<sub>2</sub>/5% CO<sub>2</sub>/90% N<sub>2</sub>.

334

### 335 **Generation of FSM<sup>R</sup> strain E2 and clones**

336 FSM<sup>R</sup> strain E2 was generated as previously described (Guggisberg, Park, et al., 2014). Briefly, a  
337 clone of genome reference strain 3D7 (MRA-102, MR4, ATCC, Manassas, VA) was selected  
338 with 3 µM FSM. Clones of strain E2 were isolated by limiting dilution.

339

### 340 **Quantification of FSM resistance**

341 Assays were performed in opaque 96-well plates with FSM concentrations ranging from 0.63 nM  
342 to 1 mM. Asynchronous cultures were seeded at 0.5-1.0% parasitemia. After 3 days, media was  
343 removed and parasitemia was measured by DNA content using Picogreen fluorescence as  
344 previously described (Corbett et al., 2004). Cells were stained for 10 min in a solution containing  
345 10 mM Tris HCl, 1 mM EDTA, 2% Triton-X 100, and 0.5% Picogreen reagent (Thermo Fisher  
346 Scientific). Fluorescence was measured on a POLARStar Omega spectrophotometer (BMG  
347 Labtech, Ortenberg, Germany). Half maximal inhibitory concentration (IC<sub>50</sub>) values were

348 calculated using Graphpad Prism software (Graphpad Software, La Jolla, CA). At minimum, all  
349 assays were performed in triplicate.

350

### 351 **Quantitative PCR**

352 Total RNA was extracted from saponin-lysed parasites using the Ambion Purelink RNA Mini kit  
353 (Thermo Fisher Scientific) according to kit instructions. An on-column DNase (Qiagen, Venlo,  
354 Netherlands) treatment was performed after the first wash step. To synthesize cDNA, one  
355 microgram of total RNA was used in reverse transcriptase reactions using the Quantitect Reverse  
356 Transcription kit (Qiagen).

357

358 PCR reactions were run using Fast SYBR Green PCR Master Mix (Applied Biosystems,  
359 Waltham, MA) and 300 nM each primer. Primers used are listed in Supplementary File 1.

360

361 Thermocycling was performed on an Applied Biosystems 7500 Fast RT PCR System with the  
362 following parameters: 95 °C for 30 seconds and 40 cycles of 95 °C for 3 seconds, 60 °C for 30  
363 seconds. Controls lacking reverse transcriptase and template both produced no significant signal.  
364 Melt curve analysis was used to verify that all primer sets produce single products.

365

366 Relative expression levels were calculated using the  $\Delta\Delta C_t$  method.  $\Delta C_t$  represents the  $C_t$  value of  
367 the reference genes (average of *beta tubulin* and *18s rRNA*) subtracted from the target.  $\Delta\Delta C_t$   
368 represents the  $\Delta C_t$  value of the parental sample subtracted from the sample of interest. Fold  
369 changes are calculated as  $2^{-\Delta\Delta C_t}$ . All assays were performed using technical and biological  
370 triplicates.

371

372

### 373 **Antisera generation**

374 Anti-HAD1 antisera has been previously described (MRA-1256, MR4, ATCC, Manassas, VA)

375 (Guggisberg, Park, et al., 2014). Polyclonal anti-HAD2 antisera was raised against 6xHis-HAD2

376 in rabbits, with Titermax as an adjuvant (Cocalico Biologicals, Reamstown, PA). Antisera

377 specificity was confirmed by immunoblot of *P. falciparum* lysate lacking HAD2.

378

### 379 **Immunoblotting**

380 Lysates were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride

381 membrane. Membranes were blocked in 5% non-fat dry milk, 0.1% Tween-20 in PBS. Rabbit

382 polyclonal antisera were used at the following dilutions: anti-HAD2, 1:2,000-5,000; anti-HAD1

383 (Guggisberg, Park, et al., 2014), 1:20,000; anti-heat shock protein 70 (Hsp70), 1:5,000 (AS08

384 371, Agrisera Antibodies, Vännäs, Sweden). All blots used an HRP-conjugated goat anti-rabbit

385 IgG secondary antibody at 1:20,000 (ThermoFisher Scientific 65-6120). When necessary, blots

386 were stripped by washing with 200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2 before re-

387 probing.

388

### 389 **Whole genome sequencing and SNP analysis**

390 Library preparation, Illumina sequencing, read alignment, and variant analysis were performed

391 by the Washington University Genome Technology Access Center (GTAC, St. Louis, MO).

392 Parasite genomic DNA was prepared for sequencing as previously described (Guggisberg, Park,

393 et al., 2014). One microgram of genomic DNA was sheared, end repaired, and adapter ligated.

394 Libraries were sequenced on an Illumina HiSeq 2500 in Rapid Run mode to generate 101 bp  
395 paired end reads. Reads were aligned to the *P. falciparum* 3D7 reference genome (PlasmoDB  
396 v7.2) using Novoalign (V2.08.02). Duplicate reads were removed. SNPs were called using  
397 samtools, using a quality score cutoff of 20 and a read depth cutoff of 5. SNPs were annotated  
398 using snpEff. Parental SNPs were removed using previously sequenced parental genomes  
399 (Guggisberg, Park, et al., 2014). Data has been deposited in the NCBI BioProject database  
400 (PRJNA222697) and Sequence Read Archive (SRP038937).

401

### 402 **Sanger sequencing**

403 The E2 A469T (R157X) *HAD2* SNP was verified by amplifying *P. falciparum* genomic DNA  
404 using primers HAD2\_R157X\_F and HAD2\_R157X\_R. The *PFK9* locus was amplified using the  
405 PFK9\_F and PFK9\_R primers. Amplicons were sequenced using the PFK9\_seq\_(1-8) primers.  
406 Primer sequences can be found in Supplementary File 1.

407

### 408 **Generation of recombinant protein**

409 The predicted coding sequence of *HAD2* (PlasmoDB ID PF3D7\_1226300) was amplified from *P.*  
410 *falciparum* genomic DNA by PCR using the HAD2\_LIC\_F and HAD2\_LIC\_R primers  
411 (Supplementary File 1). Ligation-independent cloning was used to clone the PCR product into  
412 vector BG1861 (Alexandrov et al., 2004), which introduces an N-terminal 6xHis fusion to the  
413 expressed protein. BG1861:6xHis-HAD2 construct was transformed into One Shot  
414 BL21(DE3)pLysS *Escherichia coli* cells (Thermo Fisher Scientific) according to supplier  
415 instructions. Protein expression was induced for 3 hours with isopropyl- $\beta$ -D-thiogalactoside at  
416 mid-log phase (OD<sub>600</sub> 0.4 – 0.5). Cells were collected by centrifugation and stored at -20°C.

417  
418 Induced cell pellets were lysed in buffer containing 1 mg/mL lysozyme, 20 mM imidazole, 1 mM  
419 dithiothreitol, 10 mM Tris HCl (pH 7.5), 30 U benzonase (EMD Millipore, Darmstadt, Germany),  
420 and Complete Mini EDTA-free protease inhibitor tablets (Roche Applied Science, Penzberg,  
421 Germany). 6xHis-HAD2 was bound to nickel agarose beads (Gold Biotechnology, Olivette, MO)  
422 and eluted in 300 mM imidazole, 20mM Tris HCl (pH 7.5), and 150 mM NaCl. This eluate was  
423 further purified by size-exclusion gel chromatography using a HiLoad 16/600 Superdex 200 pg  
424 column (GE Healthcare, Chicago, IL) equilibrated in 50 mM Tris HCl (pH 7.5), 1 mM  
425 dithiothreitol, and 1 mM MgCl<sub>2</sub>. The elution fractions containing HAD2 were pooled into a  
426 centrifugal filter and concentrated to approximately 3 mg/ml. Glycerol was added to a final  
427 concentration of 10% (w/v), and the solution was immediately flash frozen in liquid nitrogen and  
428 stored at -80°C. The purification of recombinant HAD1 has been previously described  
429 (Guggisberg, Park, et al., 2014).

430

### 431 **HAD2 activity assays**

432 Recombinant 6xHis-HAD2 was generated in *E. coli*. HAD2 phosphatase activity was measured  
433 against the substrate *p*NPP (New England Biolabs, Ipswich, MA). Phosphatase activity of  
434 recombinant enzyme was initially evaluated against the substrate *para*-nitrophenyl phosphate  
435 (New England Biolabs). Reactions contained 10 mM *p*NPP, 50 mM Tris HCl (pH 7.5), 5 mM  
436 MgCl<sub>2</sub>, 0.5 mM MnCl<sub>2</sub>, and 0.25-4 µg enzyme. *Para*-nitrophenyl production was quantified by  
437 absorbance at 405 nm. All reactions were performed at 37°C.

438

439 Specific enzyme activity against phosphorylated substrates was measured using the EnzChek  
440 Phosphate Assay Kit (Thermo Fisher Scientific) according to supplier instructions. Reactions  
441 were performed in 50  $\mu$ L volumes containing 1 mM substrate and 150–750 ng enzyme. Kinetic  
442 parameters were determined by continuous measurement of product formation in response to  
443 0.06 – 10 mM substrate. All reactions were performed at 37°C.

444

### 445 **Metabolic profiling**

446 All metabolic profiling was performed on  $\sim 1 \times 10^9$  early trophozoites synchronized by treatment  
447 with 5% sorbitol. Parasite-infected erythrocytes were lysed with saponin, washed with cold 2 g/L  
448 glucose in PBS, and frozen at -80 °C. For measurement of nucleotide levels, samples were  
449 extracted and prepared for ultra-high performance liquid chromatography tandem mass  
450 spectrometry (IP-RP-UPLC-MS/MS) as previously described (Laourdakis et al., 2014).

451

452 For measurement of MEP pathway and glycolytic intermediates, samples were extracted in 600  
453  $\mu$ L of ice-cold extraction solvent [chloroform, methanol, and acetonitrile (2:1:1, v/v/v)] using  
454 two liquid-nitrogen cooled 3.2 mm stainless steel beads and homogenization in a Tissue-Lyser II  
455 instrument (Qiagen) at 20 Hz for 5 minutes in a cold sample rack. Ice-cold water was added and  
456 samples were homogenized for 5 more minutes at 20 Hz. Samples were centrifuged at 14,000 rcf  
457 at 4°C for 5 min. The polar phase was lyophilized and re-dissolved in 100  $\mu$ L water and analyzed  
458 by LC-MS/MS. LC-MS/MS was performed on a 4000QTRAP system (AB Sciex, Framingham,  
459 MA) in multiple-reaction monitoring mode using negative ionization and 10 mM  
460 tributylammonium acetate (pH 5.1-5.5) as the ion pair reagent. The specific parameters used for  
461 analysis of MEP pathway metabolites have been previously described (Zhang et al., 2011).

462 Liquid chromatography separation was performed using ion pair reverse-phase chromatography  
463 (Luo, Groenke, Takors, Wandrey, & Oldiges, 2007). The referenced method (Luo et al., 2007)  
464 was modified: (1) RP-hydro 100 mm × 2.0 mm, 2.5 µm high performance liquid chromatography  
465 column (Phenomenex, Torrance, CA), (2) flow rate of 0.14 mL/min, (3) solvent A of 10 mM  
466 tributylammonium acetate in 5% methanol, (4) binary LC gradient (20% solvent B (100%  
467 methanol) from 0 to 2.5 min, 30% B for 12.5 min, 80% B for 5 min, and column equilibration at  
468 for 5 minutes), and (5) 20 µL autosampler injection volume.

469

#### 470 ***P. falciparum* growth assays**

471 Asynchronous cultures were seeded at 1% parasitemia (percent of infected red blood cells).  
472 Media was exchanged daily. All assays were performed in the absence of drug pressure. Samples  
473 were fixed in 4% paraformaldehyde, 0.05% glutaraldehyde in PBS. Fixed samples were stained  
474 with 0.01 mg/ml acridine orange and parasitemia was determined on a BD Biosciences LSRII  
475 flow cytometer (Thermo Fisher Scientific). All assays were performed using biological  
476 triplicates, at minimum.

477

#### 478 **Assay of PFK9 activity from lysate**

479 Sorbitol-synchronized trophozoites were isolated using 0.1% saponin. Cell pellets were washed  
480 three times in buffer [100 mM Tris-HCl (pH 7.5), 1 mM MgCl<sub>2</sub>, 1 mM DTT, 10% glycerol, and  
481 EDTA-free protease inhibitor tablets (Roche)]. Lysates were prepared by sonication at 4°C  
482 (Fisher Scientific Model 550 Sonic Dismembrator, amplitude 3.5), followed by centrifugation at  
483 4°C (10,000 x g, 10 min).

484

485 PFK9 activity in lysate was monitored by linking it to the oxidation of NADH, as described  
486 previously (Beutler, 1984; Mony et al., 2009). Reactions contained 100 mM Tris-HCl (pH 7.5), 1  
487 mM MgCl<sub>2</sub>, 1 mM DTT, 0.25 mM NADH, 1 mM ATP, 3 mM fructose 6-phosphate, as well as  
488 excess of linking enzymes aldolase (7.5 U), triose-phosphate isomerase (3.8 U), and glycerol 3-  
489 phosphate dehydrogenase (3.8 U). After adding fresh cell lysate (15 µg total protein), the  
490 absorbance at 340 nm was measured at 37°C for 40 min. Activity (µmol/min/mg total protein)  
491 was determined by linear regression using Graphpad Prism software. All assays were performed  
492 using technical and biological triplicates.

493

#### 494 **pTEOE110:HAD2 plasmid construction**

495 The pTEOE110:HAD2-GFP construct contains the *heat shock protein 110* (PF3D7\_0708800) 5'  
496 UTR and a C-terminal GFP tag. Human dihydrofolate reductase (hDHFR) is present as a  
497 selectable marker. Inverted terminal repeats are included for integration into the genome by a co-  
498 transfected piggyBac transposase (pHTH, MRA912, MR4, ATCC, Manassas, VA).

499

500 *HAD2* was amplified with the HAD2\_XhoI\_F and HAD2\_AvrII\_R primers (Supplementary File  
501 1) and cloned into AvrII and XhoI sites in the pTEOE110 plasmid.

502

#### 503 **Parasite transfections**

504 Transfections were performed as previously described (Guggisberg, Park, et al., 2014). Briefly,  
505 50-100 µg of plasmid DNA was precipitated and resuspended in Cytomix (25 mM HEPES pH  
506 7.6, 120 mM KCl, 0.15 mM CaCl<sub>2</sub>, 2mM EGTA, 5 mM MgCl<sub>2</sub>, 10 mM K<sub>2</sub>HPO<sub>4</sub>).

507

508 A ring-stage *P. falciparum* culture was washed with Cytomix and resuspended in the  
509 DNA/Cytomix solution. The culture was electroporated at 950  $\mu$ F and 0.31 kV, washed with  
510 media, and returned to normal culture conditions. Parasites expressing the construct were  
511 selected by continuous treatment with 5 nM WR92210 (Jacobus Pharmaceuticals, Princeton, NJ).  
512 Transfectants were cloned by limiting dilution and presence of the HAD2-GFP construct was  
513 verified by PCR using gene-specific and GFP-specific primers (HAD2\_R157X\_F and GFP\_R,  
514 Supplementary File 1). The presence of the endogenous *had2*<sup>R157X</sup> and *pfk9*<sup>T1206I</sup> mutations were  
515 verified by Sanger sequencing.

516

### 517 **Live fluorescent microscopy**

518 Erythrocytes infected with E2 Hsp110:HAD2-GFP parasites were stained with 10 ng/ $\mu$ L Hoescht  
519 33258 and mounted under coverslips on polysine adhesion slides. Cells were viewed on an  
520 Olympus BH8 microscope (Tokyo, Japan). Minimal adjustments to contrast and brightness were  
521 applied equally to all microscopy images using Inkscape.

522

523 **Author contributions**

524 Conceptualization, A.M.G., A.Y.G., and A.R.O.J.; Investigation, A.M.G., A.Y.G., E.F.M., and  
525 M.B.C.; Resources, A.M.G. and S.J.E.; Writing – Original Draft, A.M.G. and A.R.O.J.; Writing  
526 – Review and Editing, A.M.G., A.Y.G., S.J.E., E.F.M, M.B.C, and A.R.O.J; Visualization,  
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528

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543

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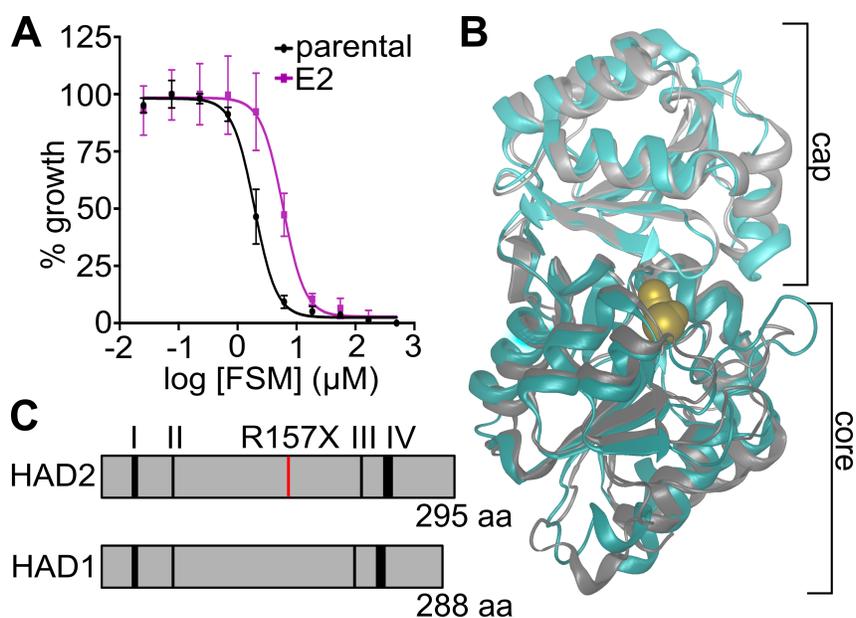
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## 743 Figures and Tables

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745

746 **Figure 1. FSM<sup>R</sup> strain E2 possesses a mutation in HAD2, a homolog of the MEP pathway**

747 **regulator HAD1. (A)** Representative FSM dose-response of the parental strain and strain E2.

748 Strain E2 has a mean IC<sub>50</sub> of 4.8 ± 1.2 μM (n=7), greater than the parent strain (0.8 ± 0.1 μM),

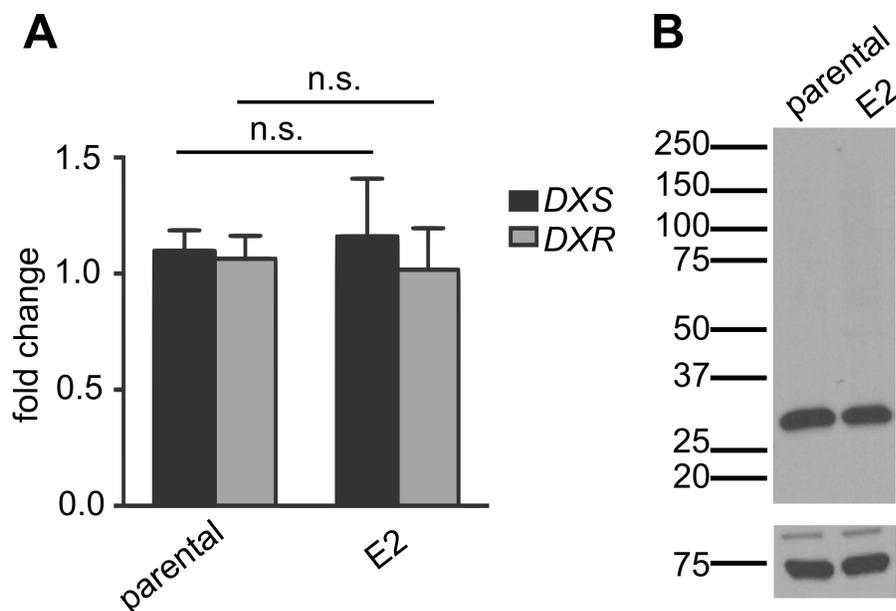
749 p≤0.01, unpaired Student's t-test. **(B)** *P. vivax* HAD2 (teal, PDB 2B30) is structurally similar to

750 PfHAD1 (grey, PDB 4QJB). Ions (Mg<sup>2+</sup>, Ca<sup>2+</sup>, Cl<sup>-</sup>) are shown in yellow. **(C)** HAD2 is a homolog

751 of HAD1 (~29% identity and ~53% similarity) and possesses all HAD conserved sequence

752 motifs required for catalysis (Burroughs, Allen, Dunaway-Mariano, & Aravind, 2006).

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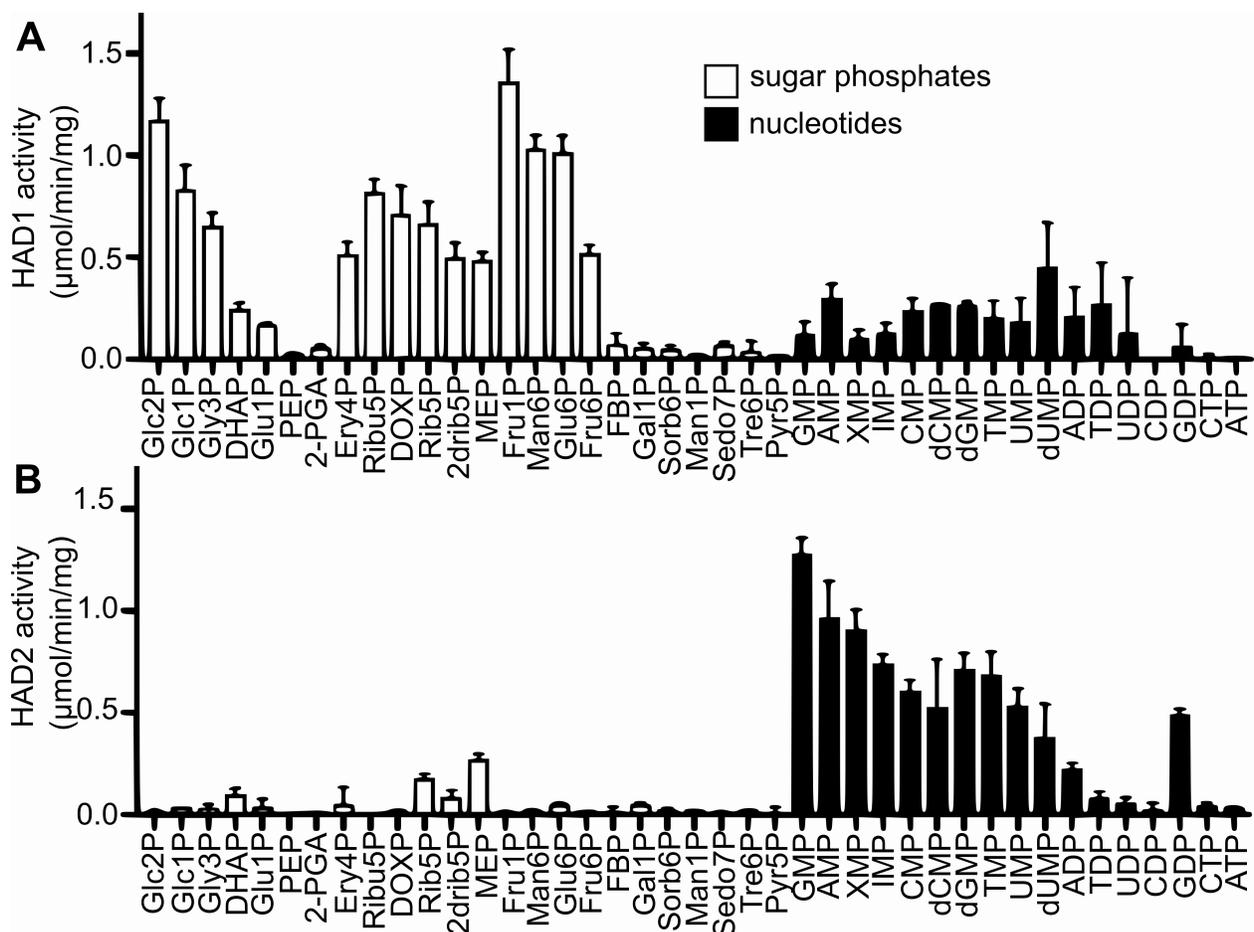
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755 **Figure 1-figure supplement 1. FSM<sup>R</sup> strain E2 does not have increased expression of *DXS*,**  
756 ***DXR*, or *HAD1*.**

757 (A) *DXS* and *DXR* transcript levels are unchanged in FSM<sup>R</sup> strain E2. Shown are mean relative  
758 mRNA expression levels determined by qPCR. Error bars represent S.E.M. from duplicate  
759 biological replicates, measured using three technical replicates (N.s. = not significant,  $p > 0.05$ ,  
760 unpaired Student's t-test). (B) *HAD1* expression is unchanged in FSM<sup>R</sup> strain E2. Shown are  
761 immunoblots of the parent strain and FSM<sup>R</sup> strain E2. Marker units are kilodaltons (kDa). Top  
762 panel was probed with anti-*HAD1* antisera. Bottom panel was probed with anti-heat shock  
763 protein 70 antisera as a loading control. Expected protein masses: *HAD1*, 33 kDa; Hsp70, 74  
764 kDa. Blot is representative of three independent experiments. Minimal brightness adjustments  
765 were applied equally to all blot images.

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769 **Figure 2. HAD2 is a nucleotidase.**

770 Shown are mean specific enzyme activities for (A) HAD1 and (B) HAD2. Error bars represent

771 S.E.M. (n≥3). HAD1 activity against sugar phosphates was previously described (Guggisberg,

772 Park, et al., 2014). Sugar phosphates (white bars) are ordered from left to right by increasing

773 number of carbon atoms (3-15). Nucleotides (black bars) are ordered from left to right by

774 increasing degree of phosphorylation. Abbreviations: Glc2P, glycerol 2-phosphate; Glc1P,

775 glycerol 1-phosphate; Gly3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate;

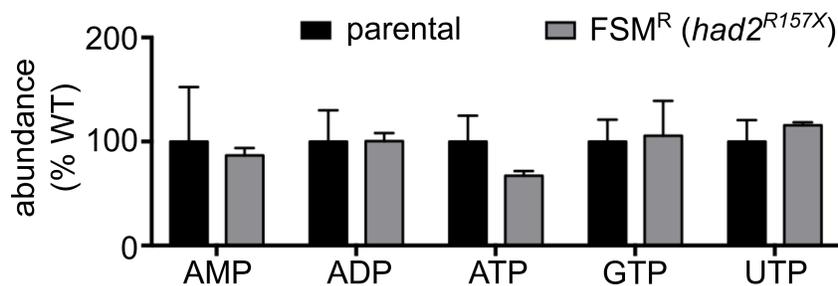
776 Glu1P, glucose 1-phosphate; PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglyceric acid; Ery4P,

777 erythrose 4-phosphate; Ribu5P, ribulose 5-phosphate; DOXP, deoxyxylulose 5-phosphate; Rib5P,

778 ribose 5-phosphate; 2drib5P, 2-deoxyribose 5-phosphate; MEP, methylerythritol phosphate;

779 Fru1P, fructose 1-phosphate; Man6P, mannose 6-phosphate; Glu6P, glucose 6-phosphate; Fru6P,  
780 fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; Gal1P, galactose 1-phosphate; Sorb6P,  
781 sorbitol 6-phosphate; Man1P, mannose 1-phosphate; Sedo7P, sedoheptulose 7-phosphate; Tre6P,  
782 trehalose 6-phosphate; Pyr5P, pyridoxal 5'-monophosphate; GMP, guanosine 5'-  
783 monophosphate; AMP, adenosine 5'-monophosphate; XMP, xanthosine 5'-monophosphate; IMP,  
784 inosine 5'-monophosphate; CMP, cytidine 5'-monophosphate; dCMP, 2'-deoxycytidine 5'-  
785 monophosphate; dGMP, 2'-deoxyguanosine 5'-monophosphate; TMP, thymidine 5'-  
786 monophosphate; UMP, uridine 5'-monophosphate; dUMP 2'-deoxyuridine 5'-monophosphate;  
787 ADP, adenosine 5'-diphosphate; TDP, thymidine 5'-diphosphate; UDP, uridine 5'-diphosphate;  
788 CDP, cytidine 5'-diphosphate; GDP, guanosine 5'-diphosphate; CTP, cytidine 5'-triphosphate;  
789 ATP, adenosine 5'-triphosphate.

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793 **Figure 2-figure supplement 1. Nucleotide levels are unchanged in E2 FSM<sup>R</sup> parasites.**

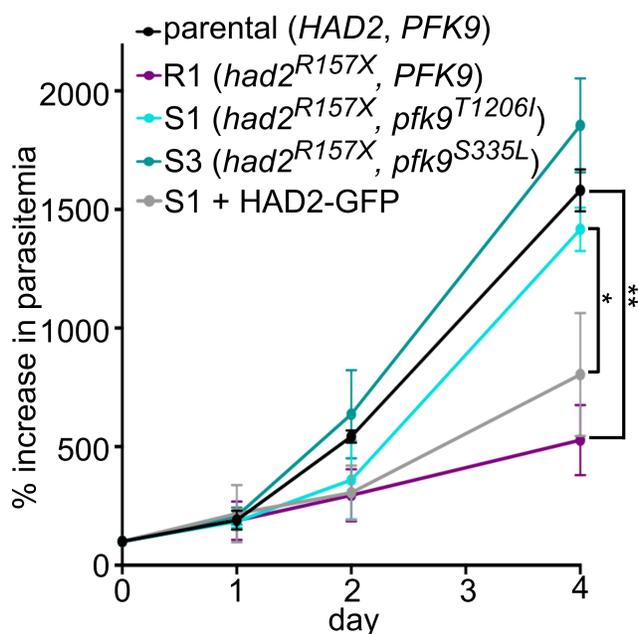
794 Error bars represent S.E.M. (n=3). A full panel of nucleotides was assessed. Of those detected,

795 no metabolite is significantly changed in the FSM<sup>R</sup> (*had2*<sup>R157X</sup>) strain (p>0.05, unpaired

796 Student's t-test). Other nucleotides were below the limit of detection.

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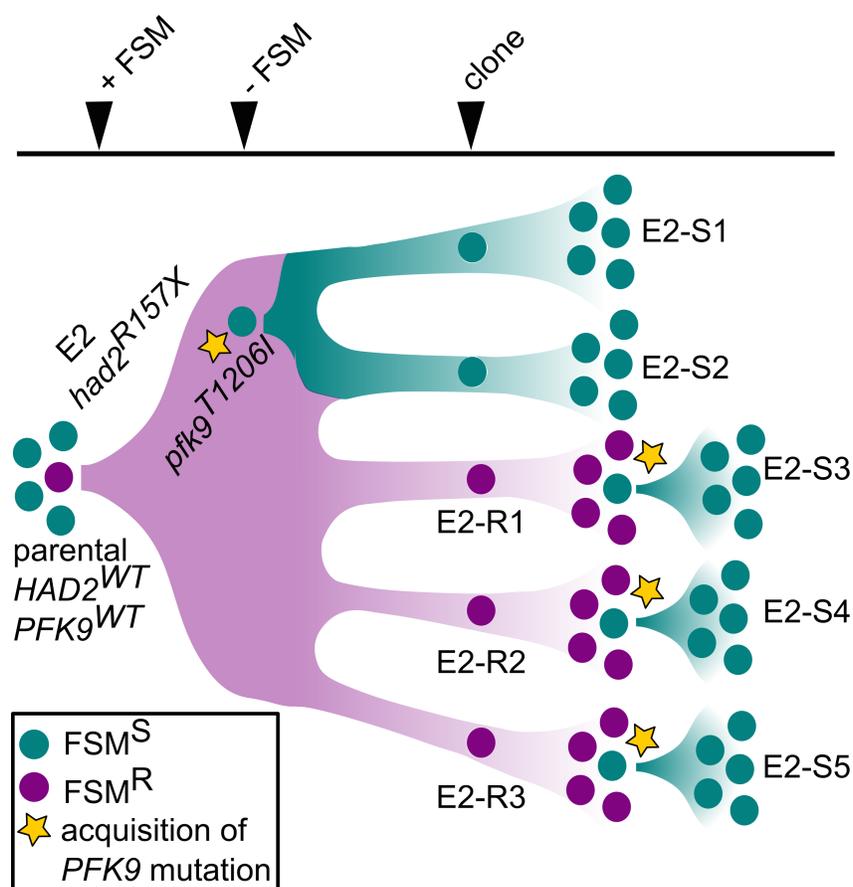
799

800 **Figure 3. FSM resistance results in a fitness cost.**

801 A representative FSM<sup>R</sup> clone with the *had2*<sup>R157X</sup> allele (R1, purple line) has a reduced growth  
802 rate compared to the wild-type parental strain (black). The growth defect is rescued in two  
803 representative clones with mutations in *PFK9* (S1 and S3, teal lines). Growth is normalized to  
804 percent infected red blood cells on day 0. Error bars represent S.E.M. from independent growth  
805 experiments ( $n \geq 4$ , \*\* =  $p \leq 0.01$ , \* =  $p \leq 0.05$ , one-way ANOVA).

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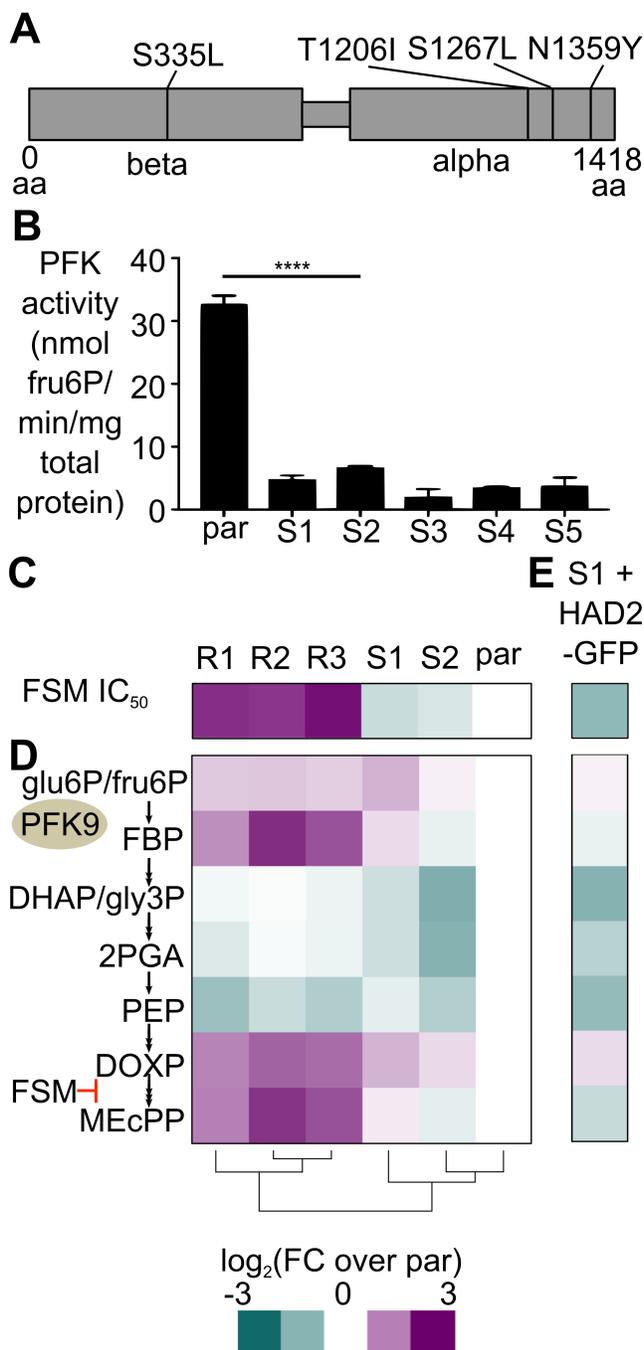


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809 **Figure 4. Parasite selection scheme and genetic changes that modulate FSM sensitivity.**

810 Parasites are colored by their FSM phenotype (teal, FSM<sup>S</sup>; purple, FSM<sup>R</sup>). Cloned strains are  
811 named by FSM phenotype (E2-SX, sensitive; E2-RX, resistant). A FSM<sup>S</sup> parental strain was  
812 selected under FSM pressure to enrich for FSM<sup>R</sup> strain E2 (*had2*<sup>R157X</sup>). After relief of FSM  
813 pressure, a fitness advantage selects for spontaneous suppressor mutations in *PFK9* (*pfk9*<sup>mut</sup>,  
814 yellow star) that result in FSM sensitivity. FSM<sup>R</sup> clones are grown without FSM pressure and a  
815 fitness advantage again selects for suppressor mutations in *PFK9* that result in increased growth  
816 rate and loss of FSM resistance.

817



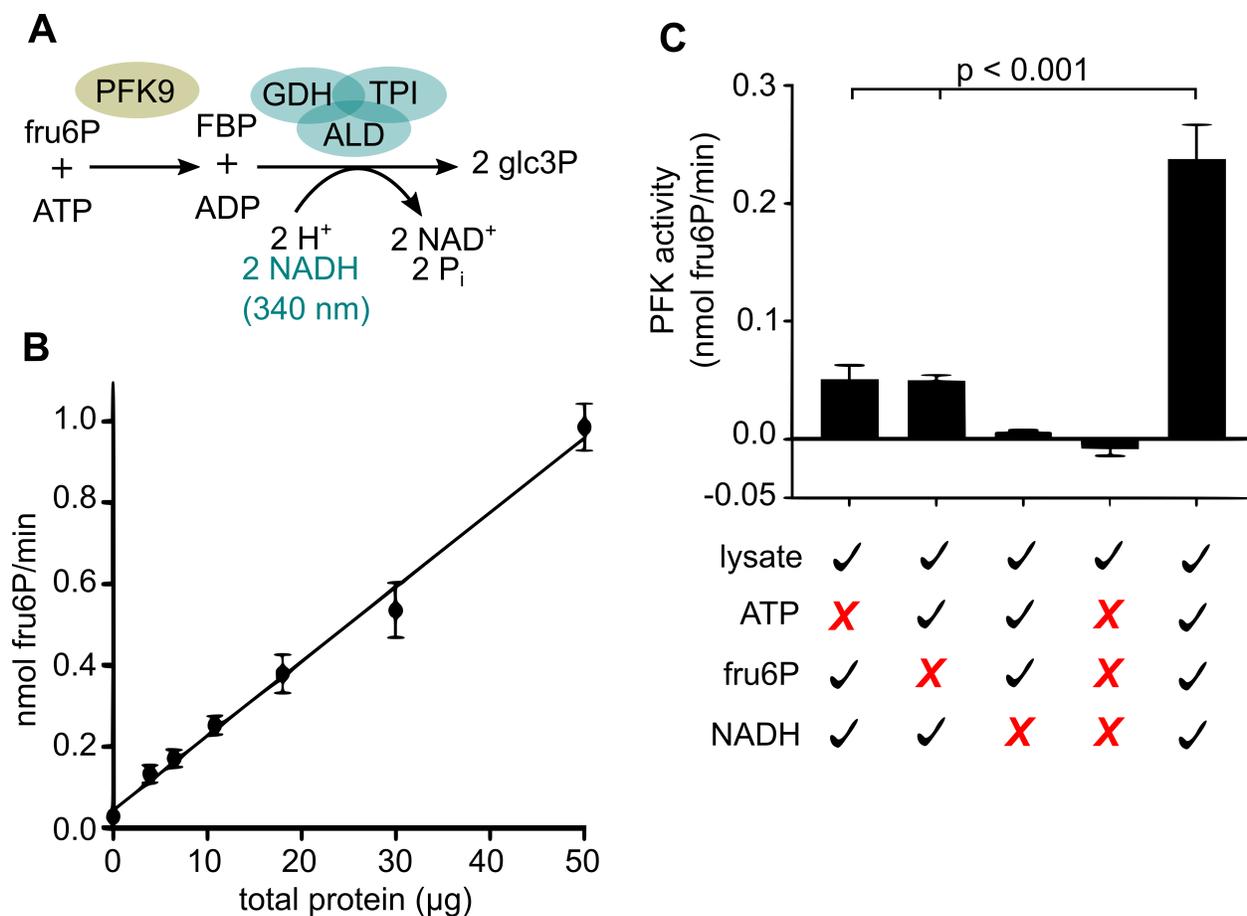
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819 **Figure 5. *HAD2* and *PFK9* alleles alter FSM resistance, PFK activity, and metabolite levels**  
 820 **in *P. falciparum*.**

821 For heatmaps, colors represent the log<sub>2</sub> of the fold change (FC) over the parental strain, as

822 indicated in the legend. Cloned strains are named by FSM phenotype (SX, sensitive; RX,

823 resistant). **(A)** Schematic of suppressor mutations identified in PFK9. Amino acid changes are  
824 shown. **(B)** Measurement of PFK activity of *P. falciparum* lysate indicates that E2-SX clones  
825 with *PFK9* suppressor mutations have significantly reduced PFK activity ( $n \geq 3$ , \*\*\*\* =  $p \leq 0.0001$ ,  
826 one-way ANOVA). Error bars represent S.E.M. Assay is linear and specific for PFK activity  
827 (Figure 5-figure supplement 1). **(C)** FSM  $IC_{50}$ s for parental (par) strain and E2 clones. **(D)**  
828 Metabolic profiling and clustering of parental and E2 clone strains demonstrates a metabolic  
829 signature of resistance, which includes increased levels of MEP pathway intermediates DOXP  
830 and MEcPP and the glycolytic metabolite FBP. Data are representative of at least three  
831 independent experiments. Glu6P/fru6P and DHAP/gly3P represent isomer pairs that cannot be  
832 confidently distinguished. Clustering performed using the heatmap function in R. **(E)** When loss  
833 of HAD2 is rescued in *had2<sup>R157X</sup>*, *pfk9<sup>T1206I</sup>* parasites, the resulting strain is hyper-sensitive to  
834 FSM, due to functional HAD2 and a hypomorphic *PFK9* allele.  
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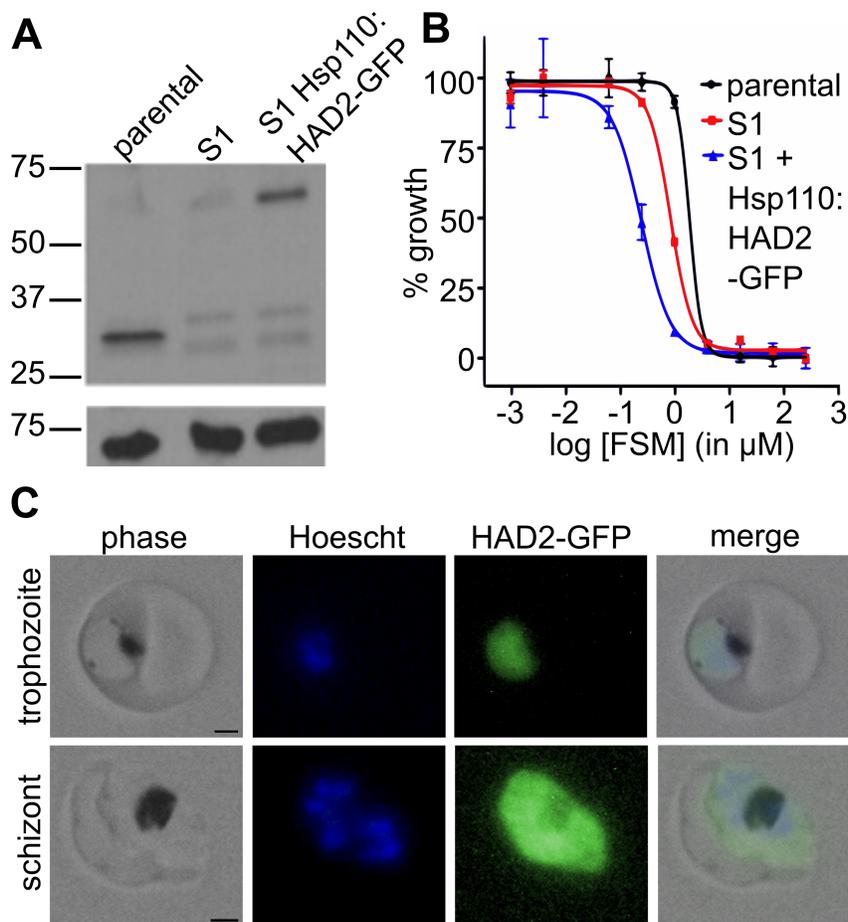


837

838 **Figure 5-figure supplement 1. Assay of PFK activity from *P. falciparum* lysate is linear and**  
 839 **specific.**

840 (A) Schematic of linked enzyme assay of PFK activity. PFK catalyzes the phosphorylation of  
 841 fructose 6-phosphate (fru6P) to fructose 1,6-bisphosphate (FBP). Excess linking enzymes  
 842 aldolase (ALD), triose-phosphate isomerase (TPI), and glycerol 3-phosphate dehydrogenase  
 843 (GDH) produce glycerol 3-phosphate. NADH utilization is monitored spectrophotometrically at  
 844 340 nm. (B) The assay is linear with respect to total lysate protein content. Error bars represent  
 845 S.E.M. (n=2). (C) The assay is specific for *P. falciparum* lysate-dependent PFK activity and  
 846 detects PFK activity above background (n=2, p<0.001). Error bars represent S.E.M.

847



848

849 **Figure 5-figure supplement 2. Rescue of HAD2 confirms its role as a negative regulator.**

850 (A) Successful expression of pTEOE110:HAD2-GFP in strain S1 (*had2*<sup>R157X</sup>, *pfk9*<sup>T1206I</sup>) was  
851 confirmed by immunoblot. Marker units are kilodaltons (kDa). Expected sizes: HAD2, 33 kDa;  
852 HAD2-GFP, 60 kDa. The top blot was probed with anti-HAD2 antisera. The bottom blot was  
853 probed with anti-heat shock protein 70 antisera as a loading control. Blot is representative of  
854 three independent experiments. Minimal brightness adjustments were applied equally to all blot  
855 images. (B) Representative FSM dose-response demonstrating expression of HAD2-GFP in  
856 strain S1 (*had2*<sup>R157X</sup>, *pfk9*<sup>T1206I</sup>) results in oversensitivity to FSM. The parental strain has a FSM  
857 IC<sub>50</sub> of  $0.8 \pm 0.1 \mu\text{M}$ , and S1 has an IC<sub>50</sub> of  $0.5 \pm 0.08 \mu\text{M}$ . When loss of HAD2 is rescued in  
858 strain S1, the resulting strain has an IC<sub>50</sub> of  $0.3 \pm 0.1 \mu\text{M}$ . Data shown are means and S.E.M. (n $\geq$   
859 3). (C) Live microscopy of E2 HAD2-GFP parasites indicates that HAD2-GFP localizes to the

860 parasite cytoplasm. Hoescht 33258 was used as a nuclear stain. Scale bars, 2  $\mu\text{m}$ . Image

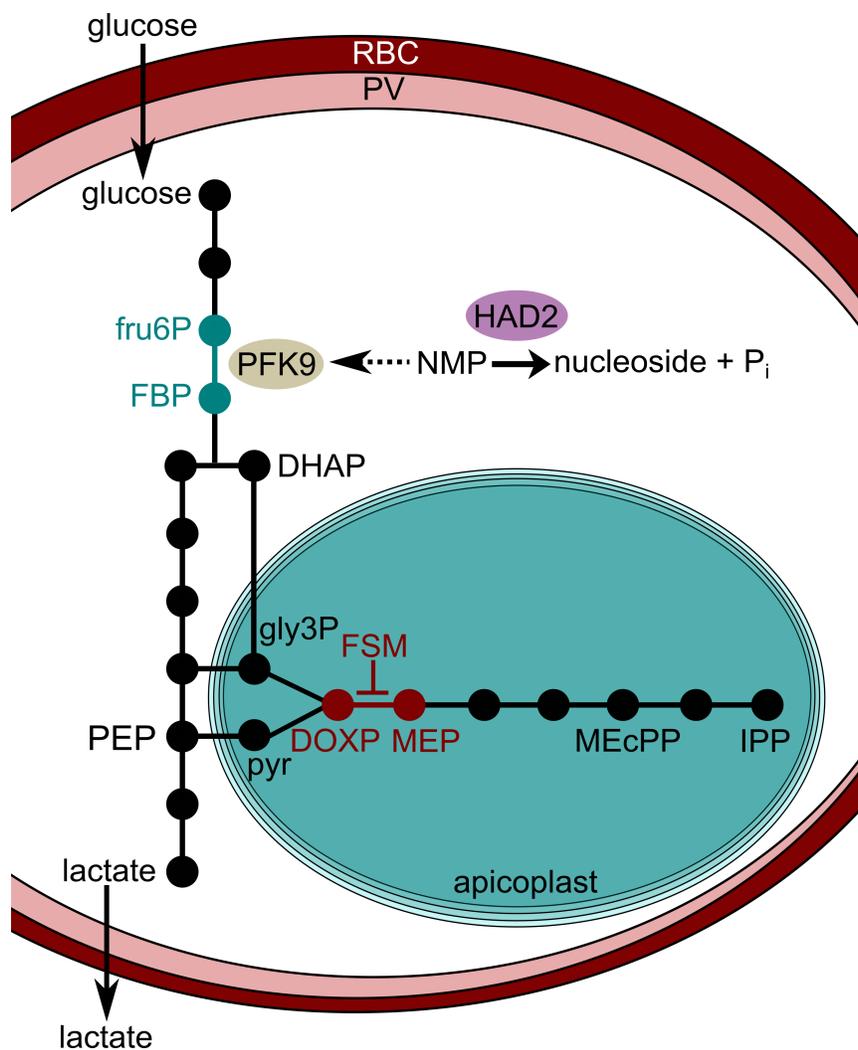
861 adjustments were applied equally to all images.

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867 **Figure 6. Model of HAD2-PFK9 metabolic regulation.**

868 HAD2 may function as a negative glycolytic regulator at PFK9 by dephosphorylating  
869 nucleotides that function as positive allosteric regulators of PFK9. Loss of HAD2 results in  
870 increased flux through glycolysis and the MEP pathway, resulting in FSM resistance. Reduced  
871 PFK activity relieves this increased flux and restores FSM sensitivity. Abbreviations: RBC, red  
872 blood cell; PV, parasitophorous vacuole; NMP, nucleotide monophosphate.

873

874 **Table 1. Kinetic parameters for HAD2.**

875 Shown are the mean kinetic parameters for HAD2 against various substrates. Error bars represent  
876 S.E.M. ( $n \geq 3$ ). Abbreviations used: AMP, adenosine 5'-monophosphate; GMP, guanosine 5'-  
877 monophosphate; 2dGMP, 2'-deoxyguanosine 5'-monophosphate; XMP, xanthosine 5'-  
878 monophosphate; IMP, inosine 5'-monophosphate.

879

<b>Substrate</b>	<b><math>K_m</math> (mM)</b>	<b><math>K_{cat}</math> (sec<sup>-1</sup>)</b>	<b><math>k_{cat}/K_m</math> (M<sup>-1</sup> sec<sup>-1</sup>)</b>
AMP	1.0 ± 0.2	1.7 ± 0.3	1.7 × 10 <sup>3</sup>
GMP	0.30 ± 0.07	2.0 ± 0.1	6.9 × 10 <sup>3</sup>
2dGMP	0.30 ± 0.01	3.2 ± 0.5	11.0 × 10 <sup>3</sup>
XMP	0.73 ± 0.09	3.6 ± 0.1	5.0 × 10 <sup>3</sup>
IMP	8.4 ± 0.6	2.5 ± 0.1	0.30 × 10 <sup>3</sup>

880