Suppression of Drug Resistance Reveals a Genetic Mechanism of Metabolic Plasticity in Malaria Parasites

Ann M. Guggisberg, Philip M. Frasse, Andrew J. Jezewski, Natasha M. Kafai, Aakash Y. Gandhi, Samuel J. Erlinger, Audrey R. Odom John

ABSTRACT In the malaria parasite Plasmodium falciparum, synthesis of isoprenoids from glycolytic intermediates is essential for survival. The antimalarial fosmidomycin (FSM) inhibits isoprenoid synthesis. In P. falciparum, we identified a loss-of-function mutation in HAD2 (P. falciparum 3D7_1226300 [PF3D7_1226300]) as necessary for FSM resistance. Enzymatic characterization revealed that HAD2, a member of the haloacid dehalogenase-like hydrolase (HAD) superfamily, is a phosphatase. Harnessing a growth defect in resistant parasites, we selected for suppression of HAD2-mediated FSM resistance and uncovered hypomorphic suppressor mutations in the locus encoding the glycolytic enzyme phosphofructokinase 9 (PFK9). Metabolic profiling demonstrated that FSM resistance is achieved via increased steady-state levels of methylerythritol phosphate (MEP) pathway and glycolytic intermediates and confirmed reduced PFK9 function in the suppressed strains. We identified HAD2 as a novel regulator of malaria parasite metabolism and drug sensitivity and uncovered PFK9 as a novel site of genetic metabolic plasticity in the parasite. Our report informs the biological functions of an evolutionarily conserved family of metabolic regulators and reveals a previously undescribed strategy by which malaria parasites adapt to cellular metabolic dysregulation.

IMPORTANCE Unique and essential aspects of parasite metabolism are excellent targets for development of new antimalarials. An improved understanding of parasite metabolism and drug resistance mechanisms is urgently needed. The antibiotic fosmidomycin targets the synthesis of essential isoprenoid compounds from glucose and is a candidate for antimalarial development. Our report identifies a novel mechanism of drug resistance and further describes a family of metabolic regulators in the parasite. Using a novel forward genetic approach, we also uncovered mutations that suppress drug resistance in the glycolytic enzyme PFK9. Thus, we identify an unexpected genetic mechanism of adaptation to metabolic insult that influences parasite fitness and tolerance of antimalarials.

KEYWORDS Plasmodium, antimalarial agents, drug resistance mechanisms, fosmidomycin, glycolysis, isoprenoids, malaria, metabolic regulation, metabolism

Malaria remains a global health threat, infecting 216 million people per year and causing nearly half a million deaths, mainly of pregnant women and young children (1). Resistance to current therapies has limited efforts to control malaria (2, 3). New drugs and a deeper understanding of drug resistance mechanisms are urgently needed.

Malaria is caused by infection with unicellular eukaryotic parasites of the genus Plasmodium. The species Plasmodium falciparum is responsible for most life-threatening malarial disease. As an obligate intracellular parasite of human erythrocytes, Plasmo-
*Plasmodium falciparum* has unique metabolic features that may be exploited to discover new drug targets and develop new therapies. In the red blood cell niche, *Plasmodium* parasites are highly dependent on glucose metabolism. Infection with *Plasmodium* spp. results in a nearly 100-fold increase in glucose import in red blood cells (4–6). Despite these energy requirements, the parasite demonstrates little aerobic respiration via the tricarboxylic acid (TCA) cycle. Instead, it relies on anaerobic glycolysis to produce ATP (7–10).

Besides ATP production, glucose also has a number of anabolic fates in *P. falciparum*. One such fate is the synthesis of isoprenoids. Isoprenoids are a large class of hydrocarbons with extensive structural and functional diversity (11). In the malaria parasite, isoprenoids perform several important functions, including protein prenylation, dolichylation, and synthesis of GPI anchors (12–14). Despite this diversity, all isoprenoids are synthesized from a common five-carbon building block, isopentyl pyrophosphate (IPP). Evolution has produced two distinct routes for IPP synthesis: the mevalonate pathway, found in archaea, fungi, animals, and the cytoplasm of plants; and the methylerythritol phosphate (MEP) pathway, found in most eubacteria, plant chloroplasts, and apicomplexan parasites such as *P. falciparum* (15). Because it is both essential for the parasite and absent from the human host, the MEP pathway is a compelling target for antimalarial development. The antibiotic and antimalarial fosmidomycin (FSM) is a competitive inhibitor of the first committed enzymatic step of the MEP pathway, catalyzed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR; EC 1.1.1.267) (16–18). FSM has been validated as a specific inhibitor of the MEP pathway in *P. falciparum* (19) and is a valuable chemical tool to study MEP pathway biology and essential metabolism in the parasite. In this study, we found that FSM is also a useful tool for probing glycolytic metabolism upstream of the essential MEP pathway.

Parasites are likely to control the proportion of glucose used for energy production versus production of secondary metabolites, such as isoprenoids. We previously used a screen for FSM resistance to identify HAD1, a metabolic regulator whose loss results in increased levels of MEP pathway intermediates and resistance to MEP pathway inhibition. HAD1 is a cytoplasmic sugar phosphatase that dephosphorylates a number of sugar phosphate intermediates upstream of the MEP pathway (20, 21). HAD1 belongs to the haloacid dehalogenase-like hydrolase (HAD) enzyme superfamily and, more specifically, to the IIB and Cof-like hydrolase superfamilies (22). While HADs are found in all kingdoms of life, HAD1 is most closely related to bacterial members of this superfamily (20, 23), which have been implicated in metabolic regulation, stress response, and phosphate homeostasis (24–28). However, most members of this superfamily remain uncharacterized.

In this report, we describe the discovery of HAD2, a second HAD family member in *P. falciparum*. We found that HAD2 is a cytosolic phosphatase required for metabolic homeostasis. Loss of HAD2 dysregulates glycolysis and misroutes metabolites toward the MEP pathway, conferring drug resistance. In our study, we harnessed a fitness defect in had2 parasite strains to employ an innovative screen for suppression of drug resistance in the parasite. Selection for suppression of drug resistance identified mutations in *PFK9*, which encodes the canonical glycolytic regulatory enzyme phosphofructokinase (PFK). Reduction in PFK9 activity rescued the metabolic dysregulation in our resistant mutants and restored FSM sensitivity. Our unique approach thus reveals PFK9 as a site of exceptional metabolic plasticity in the parasite and uncovers a novel genetic mechanism by which *P. falciparum* malaria parasites may adapt to metabolic stress and drug selective pressure.

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**RESULTS**

An FSM-resistant (FSMr) strain possesses a nonsense allele of HAD2, homolog of the MEP pathway regulator HAD1. The MEP pathway is responsible for the synthesis of the essential isoprenoid precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). This pathway is specifically inhibited by the
antibiotic FSM (19, 30, 31). We previously generated *P. falciparum* strains resistant to FSM. Mutations in *HAD1* (*P. falciparum* 3D7_1033400 [PF3D7_1033400]) cause the resistance phenotype in a majority of these strains (20). However, the genetic and biochemical basis of FSM resistance in strain E2 remained unknown. As previously reported, we found that E2 is less sensitive to FSM than its wild-type (WT) 3D7 parental line (Fig. 1A) (20). Strain E2 showed an FSM half-maximal inhibitory concentration (IC50) of 4.8 ± 1.2 μM, significantly greater than that shown by its parent strain (0.9 ± 0.06 μM) (*P* ≤ 0.01 [unpaired Student’s *t* test]).

We found that this resistance phenotype was not due to changes in expression of the genes encoding the first two (rate-limiting) steps of the MEP pathway, *DXS* and *DXR* (32–35) (see Fig. S1A in the supplemental material). In addition, strain E2 does not have genetic changes in the known FSM resistance locus and MEP pathway regulator, *HAD1*, and *HAD1* appears to be expressed in strain E2 (Fig. S1B).

To identify new genetic changes that may result in FSM resistance, we performed whole-genome sequencing on strain E2 and identified an A469T mutation in PF3D7_1226300 (PlasmoDB identifier [ID]), here referred to as *HAD2* (36). Variant data for strains sequenced in this study can be found in Data Set S1 in the supplemental material. Sanger sequencing of the *HAD2* locus in strain E2 confirmed the presence of the A469T single nucleotide polymorphism (SNP). The A469T SNP yielded a truncated (R157X) protein variant, and we therefore expected that HAD2 function would be lost in strain E2. Interestingly, HAD2 is a close homolog of a known MEP pathway regulator, the sugar phosphatase HAD1 (20). Sequence homology places both proteins in the haloacid dehalogenase-like hydrolase (HAD) superfamily and, further, within the IIB and Cof-like hydrolase subfamilies (Interpro IPR006379 and IPR000150, respectively) (22).

While no structural information exists for *P. falciparum* HAD2, the structure of the *Plasmodium vivax* HAD2 (PVX_123945; *P. vivax* HAD2 [PvHAD2]) has been solved (PDB ID 2B30). PvHAD2 (93% identical and 98% similar to *P. falciparum* HAD2 [PfHAD2]) contains the common structural motifs found in other HADs, including a core and cap domain (Fig. 1B). HAD2 possesses the four conserved sequence motifs found in HAD proteins (Fig. 1C), which are involved in binding of the substrate, coordination of the phosphoryl group and Mg2+ ion, and hydrolysis of the substrate phosphate (37–39). Overall, HAD2 and HAD1 protein sequences share ~29% sequence identity and ~53% sequence similarity (Fig. 1C). We hypothesized that HAD2, like HAD1, regulates metabolism in *P. falciparum* and that loss of HAD2-mediated metabolic control was responsible for FSM resistance in malaria parasite strain E2.

**HAD2 is a functional phosphometabolite phosphatase.** We have previously established that *P. falciparum* HAD1 is a promiscuous sugar phosphatase, with activity against a wide range of phosphometabolites. Similarly, *P. vivax* HAD2 has been enzy-
matically characterized and found to possess phosphatase activity against various monophosphorylated substrates, including glycerol 2-phosphate (glc2P) and pyridoxal phosphate (PLP) (40). Recombinant PvHAD2 also utilizes additional monophosphorylated substrates, such as AMP and glycerol 1-phosphate (glc1P), with moderate activity.

On the basis of the previous characterization of a close Plasmodium homolog, as well as sequence homology to HAD1 and other HAD proteins, we predicted that PfHAD2 would also function enzymatically as a phosphatase. We successfully purified recombinant PfHAD2 in Escherichia coli and confirmed the phosphatase activity of recombinant PfHAD2 using para-nitrophenyl phosphate (pNPP), a promiscuous, chromogenic phosphosubstrate (Fig. 2A) (23, 41). Because E. coli expresses a number of HAD-like phosphatases (23), we confirmed that the phosphatase activity was specific to purified PfHAD2 by expression and purification of a catalytically inactive mutant (HAD2D26A). The Asp26 residue was chosen for mutagenesis because the corresponding residue in PfHAD1 (Asp27) has been previously shown to be required for catalysis (21).

We also established the activity of PfHAD2 against a panel of phosphorylated substrates and determined that its substrate profile closely mirrors that of PvHAD2 (Fig. 2B). Overall, we found that PfHAD2 is a phosphatase with activity against small phosphosubstrates, such as glc2P. These data suggest that, like HAD1 and related HADs in microbes and plants (23, 42–44), HAD2 is a phosphatase with the potential to utilize a variety of monophosphorylated phosphometabolites.

In vitro evolution of mutations suppressing FSM resistance. During routine culturing of E2 FSMr parasites, we observed that the E2 strain appeared to be growth attenuated compared to its parental parasite strain. Surprisingly, during prolonged culture in the absence of FSM, this growth phenotype resolved, and improved growth rates correlated with a return to FSM sensitivity (Fig. 3A). From these observations, we hypothesized that had2R157X-mediated FSM resistance led to a fitness cost in cultured parasites. We sought to harness this fitness cost to drive in vitro evolution of an FSM-sensitive (FSMs) population possessing additional, novel mutations that might suppress FSM resistance in had2R157X parasite strains.

FSM-resistant strain E2 was cultured through multiple passages in the absence of FSM selection. Through limiting dilution, we derived five E2-based clones in the absence of drug pressure (Fig. 3B). Of the five clones, three (designated clones E2-R1, E2-R2, and E2-R3) remained FSM+, but two of these (designated E2-S1 and E2-S2) were found to be FSM− (Fig. 3B and 4A). To validate our novel suppressor screen approach, we independently repeated this genetic selection with the three FSM+ E2 clones by again culturing in the absence of FSM for >1 month (Fig. 3B). As before, these strains (E2-S3, E2-S4, and E2-S5) also lost their FSM resistance phenotype (Fig. 4A).

Consistent with our initial observation that our had2R157X FSM-resistant strain grew poorly, we found that the FSM+ clones (E2-RX) grew at a significantly reduced rate.

FIG 2 PfHAD2 is a phosphatase. (A) HAD2 is an active phosphatase, and HAD2D26A is a catalytic mutant (cat. mut.) that can be used as a negative control for HAD2-specific activity. “No enz.” represents a no-enzyme control. Data shown represent the enzyme activities seen using the synthetic phosphatase substrate pNPP. Error bars represent standard errors of the means (SEM) (****, P ≤ 0.0001 [unpaired t test]; n.s., not significant). (B) Activity of HAD2, normalized to the activity of the catalytic mutant (HAD2D26A), for a variety of substrates (2-GlcP, 2-glycerol-phosphate; M6P, mannose-6-phosphate; FBP, fructose-2,6-bisphosphate; dAMP, deoxy-AMP). Error bars represent SEM.
compared to the parental strain, while the FSMs clones (E2-SX) had restored growth rates similar to that of the wild-type parental strain (Fig. 4B).

Loss of FSM resistance might have occurred by reversion of the had2R157X mutation in E2-derived strains. Instead, we found that all E2-SX clones maintained loss of HAD2 via the had2R157X mutation. We hypothesized that the FSMs E2 clones, driven by a fitness advantage, had acquired a new suppressor mutation(s) at an additional locus. We performed whole-genome sequencing on the original five E2 clones to identify any genetic changes that segregated with FSM sensitivity. Sequencing revealed that

**FIG 3** Leveraging resistance-associated growth attenuation to identify genetic changes that modulate FSM sensitivity. (A) Prolonged culture resulted in loss of FSM resistance in strain E2. Shown are FSM dose responses of the strain E2 before (day 9) and after (day 79) prolonged culture without FSM. Nine days after thawing resistant strain E2, we observed an FSM IC50 of 4.9 μM, while after 79 days of culture without FSM, E2 had an FSM IC50 of 1.3 μM. The dose responses were part of routine evaluation of individual strain phenotypes at discrete points in time. Each data point is representative of the mean from two technical replicates. Error bars represent SEM. (B) Parasites are colored according to FSM phenotype (teal, FSMs; purple, FSMr). Cloned strains are named according to FSM phenotype (E2-SX, sensitive; E2-RX, resistant). An FSMs parental strain was selected under conditions of FSM pressure to enrich for FSMr strain E2 (had2R157X). After relief of FSM pressure, a fitness advantage selected for spontaneous suppressor mutations in PFK9 (pfk9mut, yellow star) that resulted in FSM sensitivity. FSMr clones were grown without FSM pressure, and a fitness advantage again selected for suppressor mutations in PFK9 that resulted in an increased growth rate and loss of FSM resistance.

**FIG 4** Suppressor strains with PFK9 mutations display changes in FSM tolerance and growth. (A) Suppressed clones have significantly lower FSM IC50s (****, \( P < 0.0001 \)). Error bars represent SEM. HAD2 and PFK9 genotypes for each strain are indicated. For reference, the parental (par) strain data are shown in the black column. All data are representative of results from ≥3 independent experiments. (B) FSM resistance results in a fitness cost. FSMr clones with the had2R157X allele (R1 to R3, purple lines) had reduced growth rates compared to the wild-type parental (par) strain (black) (*, \( P < 0.05 \)). The growth defect was rescued in clones with mutations in PFK9 (S1 to S5, teal lines). Growth was normalized to parasitemia on day 0. Error bars represent SEM of results from ≥3 independent growth experiments.
a new mutation (C3617T) in the locus encoding phosphofructokinase-9 (PFK9; PF3D7_0915400) was present in both of the suppressed (FSM) E2 clones but in none of the three FSMr E2 clones (Fig. 3B; see also Data Set S1). The C3617T mutation results in a PFK9T1206I protein variant. PFK9 contained the only SNP that segregated with the change in FSM tolerance. Two other loci had indels that also segregated with our FSM phenotype. These loci encode a tyrosine recombinase (MAL13P1.42) (45) and an erythrocyte surface protein (PIESP1; PFC0435w). Given their predicted functions and the presence of A/T indels in poly(A) and poly(T) tracts, we concluded that mutations in these loci were unlikely to result in our suppressed phenotype and prioritized PFK9 as the likely locus of our suppressor mutation.

To verify whether mutations in PFK9 were responsible for suppressing FSM resistance in all of our suppressed strains, we investigated HAD2 and PFK9 in the E2-S3, -S4, and -S5 strains, which were derived through independent evolution of the E2-R1, -R2, and -R3 populations in the absence of FSM. By Sanger sequencing, we found that, as before, all strains maintained the had2R157X mutation and acquired new, independent PFK9 mutations (Fig. 4A). The independent acquisition of four different PFK9 alleles during selection, each of which was associated with both improved growth and loss of FSM resistance, strongly indicates that loss of PFK9 function is responsible for these phenotypes in strains lacking HAD2.

Loss of HAD2 is necessary for FSM resistance in had2R157X parasites. HAD2 was not the sole genetic change in FSMr strain E2. In addition, because intraerythrocytic P. falciparum parasites are haploid, we cannot distinguish recessive from dominant or gain-of-function mutations. Therefore, we sought to establish whether restoring HAD2 expression in trans in a had2R157X strain would restore FSM sensitivity. Using a previously described expression system enabled by the piggyBac transposase (20, 46), we expressed HAD2-green fluorescent protein (HAD2-GFP) driven by the heat shock protein 110 (Hsp110) promoter (47). We confirmed that the transfected had2R157X E2-R2 clone maintained the had2R157X allele at the endogenous locus and successfully expressed HAD2-GFP (Fig. 5A). Additionally, the had2R157X allele does not appear to result in a truncated protein product, as evidenced by immunoblotting of the E2-R2 clone and the corresponding rescued strain (Fig. 5A). This suggests that complete loss of HAD2, as opposed to a truncated protein isoform, is responsible for the observed phenotypes in the E2-RX mutants. Expression of HAD2-GFP in had2R157X parasites results in restoration of FSM sensitivity (Fig. 5B; see also Fig. S2), confirming that loss of HAD2 is necessary for FSM resistance in this strain. The resistant clone (E2-R2) has an FSM IC50 of 3.9 ± 0.2 μM, significantly higher than that of the wild-type parent strain (0.9 ± 0.06 μM, P ≤ 0.001 [one-way analysis of variance (ANOVA) and Sidak’s posttest]). Expression of HAD2-GFP in E2-R2 results in an IC50 of 0.6 ± 0.02 μM for FSM, signifi-
significantly lower than that seen with the E2-R2 strain \( (P < 0.001) \) but not significantly different from that of the parental strain \( (P > 0.5) \).

Using fluorescence microscopy, we also investigated the localization of HAD2-GFP in our E2-R2 Hsp110:HAD2-GFP strain. We observed that HAD2-GFP was diffusely present throughout the cytoplasm in asexual *P. falciparum* trophozoites and schizonts but excluded from the digestive food vacuole (Fig. S3). This finding is consistent with the lack of a predicted signal sequence for HAD2 as determined using SignalP, PlasmoAP, and PlasMit algorithms \( (48–50) \).

**PFK9 mutations in suppressed strains are hypomorphic.** The PFK9 locus encodes the enzyme phosphofructokinase (PFK; EC 2.7.11), which catalyzes the first committed and canonically rate-limiting step of glycolysis, which is the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. PFK9 is comprised of two domains, alpha (\( \alpha \)) and beta (\( \beta \)), which are typically encoded by independent genes in nonapicomplexans \( (51) \) (Fig. 6A). While in other systems the \( \alpha \) domain is regulatory, previous work on *P. falciparum* PFK9 has demonstrated catalytic activity for both domains \( (51–55) \).

Of the four PFK9 variants identified in this study, three variants map to the \( \alpha \) domain, while one variant (S335L) maps to the \( \beta \) domain (Fig. 6A). We projected our mutations onto a three-dimensional model of PfPFK9 to reveal a possible structural basis for altered PFK function. Three variants (S335L, T1206I, and S1267L) align to and model currently available crystal structures of PFK, while a fourth allele (N1359Y) does not. While three (T1206L, S1267L, and N1359Y) of the four mutations map to the \( \alpha \) domain of PIPFK9, these mutations do not appear to cluster in any particular region. All mutations affect amino acid residues that are physically distant from the substrate-binding pocket of either domain and are not predicted to impact binding or specific catalytic residues.
Consistent with a previous study on PfPFK9 (51), attempts to purify recombinant full-length PFK9 were unsuccessful. Thus, to understand the enzymatic impacts of our PFK9 variants, we quantified the native PFK-specific activity in P. falciparum (51, 56) (Fig. 6B; see also Fig. S4). Lysates from strains possessing PFK9 mutations (E2-SX strains) have markedly reduced specific activity of PFK compared to the parental strain (Fig. 6B). Combined with the diverse mutation locations (Fig. 6A), the reduced lysate PFK activity in E2-SX strains suggests that a variety of genetic strategies to alter PFK function can lead to resistance suppression.

Metabolic profiling reveals mechanisms of resistance and suppression in HAD2 and PFK9 mutant parasites. Reduced activity of PFK9, which catalyzes the canonical rate-limiting step in glycolysis, is associated with restored FSM sensitivity of had2 mutant strains. Therefore, we anticipated that metabolic changes might underlie both resistance and suppression in our E2 clones. We performed targeted metabolic profiling on the parental parasite strain as well as E2 clones R1 to R3 and S1 and S2 (Fig. 7A; see also Table S1 and Fig. S5 in the supplemental material). We found that levels of the MEP pathway intermediate DOXP (1-deoxy-D-xylulose 5-phosphate) were significantly increased in FSMr (had2R157X PFK9) strains (Fig. 7A) (P/H11349 0.05 [one-way ANOVA and Sidak’s posttest]). FSM is competitive with DOXP for inhibition of its target enzyme, DXR. Therefore, our data are consistent with the hypothesis that FSMr strains achieve resistance via increased levels of DOXP, which outcompetes FSM. We also observed a significant increase in the downstream MEP metabolite, MEcPP (2-C-methyl-D-erythritol-2,4-cyclopyrophosphate), in our FSMr strains (P/H11349 0.05).

To understand the role of PFK9 in conferring and suppressing FSM resistance, we determined the steady-state levels of intermediates from glycolysis, metabolites of which feed into the MEP pathway (Fig. 7A; see also Table S1 and Fig. S5). Hierarchical clustering indicates that resistant clones are characterized by a metabolic signature of increased levels of FBP (fructose 1,6-bisphosphatase), DOXP (1-deoxy-D-xylulose 5-phosphate) were significantly increased in FSMr (had2R157X PFK9) strains (Fig. 7A) (P/H11349 0.05 [one-way ANOVA and Sidak’s posttest]). FSM is competitive with DOXP for inhibition of its target enzyme, DXR. Therefore, our data are consistent with the hypothesis that FSMr strains achieve resistance via increased levels of DOXP, which outcompetes FSM. We also observed a significant increase in the downstream MEP metabolite, MEcPP (2-C-methyl-D-erythritol-2,4-cyclopyrophosphate), in our FSMr strains (P/H11349 0.05).

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Of note, the pfk9T1206I suppressor allele in strains S1 and S2 restored nearly parental levels of FBP and downstream MEP pathway intermediates (Fig. 7A), consistent with our finding that PFK activity was reduced in lysate from these strains (Fig. 6B).
DISCUSSION

Cells must control levels of critical metabolites in order to efficiently utilize carbon sources for energy and biosynthesis of essential molecules. Cells may regulate their metabolism via transcriptional, posttranscriptional, posttranslational, allosteric, or enzymatic mechanisms that are necessary for growth (57–60). In the glucose-rich red blood cell niche, Plasmodium malaria parasites display a unique dependence on glycolysis for energy and biosynthesis.

Using resistance to a metabolic inhibitor, we identified a phosphatase member of the HAD superfamily, HAD2, as a novel regulator of metabolism in P. falciparum. Importantly, HAD2 controls substrate availability to the parasite-specific MEP pathway for synthesis of isoprenoids, which are promising drug targets for much needed new antimalarials. We found that tolerance of inhibitors such as FSM is a robust and sensitive readout of metabolic perturbation. HAD2 is necessary for metabolic homeostasis in malaria parasites. Cells lacking HAD2 exhibit marked dysregulation of central carbon metabolism, including altered steady-state levels of glycolytic intermediates and isoprenoid precursors. We found that mutations in phosphofructokinase (PFK9) restored wild-type growth rates and FSM sensitivity to our had2 mutant strains. Our report thus genetically connects the function of HAD2, a HAD superfamily member, to control of essential central carbon metabolism. In addition, our work revealed a previously undescribed strategy by which malaria parasites may respond to cellular metabolic dysregulation through mutation in the gene encoding the rate-limiting glycolytic enzyme PFK9.

HAD2 is a member of the HAD superfamily and a homolog of the previously described metabolic regulator HAD1. Together with our previous studies on HAD1 (20, 21), we define the cellular role of these proteins in P. falciparum and contribute to the greater understanding of the HADs, an evolutionarily conserved and widespread protein family. Both enzymes belong to the IIB (IPR006379) and Cof-like hydrolase (IPR000150) subfamilies (22). HAD enzymes display diverse substrate preferences (23, 27, 43, 44, 61–64), and their biological functions are largely unknown. Like other HAD homologs, including PfHAD1 (20), HAD2 appears to be a cytoplasmic phosphatase with a preference for small, monophosphorylated substrates. While the HAD superfamily is thought to consist of a highly evolvable pool of enzymes with broad substrate specificity (37, 42), our work strongly suggests that, like HAD1 and HAD2, other members of this superfamily are likely to perform specific and biologically important cellular functions.

We found that HAD1 and HAD2 influence central carbon metabolism. In our studies of fosmidomycin resistance, we were uniquely positioned to detect these related but distinct mechanisms of metabolic regulation through the study of the MEP pathway, whose substrate availability is closely linked to glycolysis. had2 mutations were found at a lower rate than had1 mutations (20) and appeared to have a fitness cost in FSM-resistant parasites, suggesting that, despite their homology, HAD1 and HAD2 have distinct metabolic roles in vivo. As has been suggested by others in the HAD field (42, 65, 66), HADs are amenable to evolution of their substrate specificity and may quickly adopt divergent cellular functions, which may result in different fitness phenotypes upon mutation.

The exact mechanism by which HAD2 enacts its regulation on parasite glycolysis remains unclear. Possible mechanisms include direct or indirect regulation of PFK9 by HAD2, as well as HAD2-mediated regulation of glycolysis downstream of PFK9, such that mutation of PFK9 is a “bypass” mechanism in had2 mutants (Fig. 8). HAD2 itself may also be subject to regulation, and understanding this regulation may be key in uncovering HAD2’s in vivo function and mechanism of glycolytic regulation. HAD2 from P. vivax is sensitive to inhibition by free phosphate (40), which may influence its in vivo substrate specificity in a cellular context. PfHAD2 has also appears to be phosphorylated in vivo (67, 68), and changes in phosphorylation often greatly influence enzymatic activity in vivo. As P. falciparum has a smaller repertoire of HADs than bacterial species...
and *Plasmodium* HADs influence easily quantified phenotypes (drug tolerance, growth, metabolite levels), the malaria parasite may be an attractive system for study of the molecular mechanisms by which HAD proteins control metabolic homeostasis and growth.

Metabolic profiling reveals that loss of HAD2 function leads to metabolic dysregulation, which is centered on the canonical rate-limiting step of glycolysis, catalyzed by PFK9. While the cellular abundance of the PFK9 product FBP is increased in *had2* parasites, HAD2 does not directly utilize FBP as an enzymatic substrate, suggesting an indirect mechanism of HAD2-mediated metabolic regulation. However, the distinct metabolic signature of *had2* parasites, characterized by increased levels of the MEP pathway metabolites DOXP and MEcPP and the key glycolytic metabolite FBP, suggests that MEP pathway metabolism is precisely linked to FBP production. In other microbial systems, FBP levels reflect metabolic flux and are cued to environmental perturbations (69). FBP-centered metabolic regulation is also important for the related apicomplexan *Toxoplasma gondii*, which constitutively expresses fructose 1,6-bisphosphatase (FBPase) to fine-tune glucose metabolism (57). While *P. falciparum* does not appear to possess an FBPase (necessary for gluconeogenesis), the parasite may possess alternative FBP-sensing mechanisms to tune metabolism, perhaps via regulators such as HAD1 and HAD2.

The metabolic dysregulation that we have observed in the *had2* mutant strains appears to be associated with a fitness disadvantage. Under conditions of FSM selective pressure, the benefits of dysregulated metabolism outweigh the costs. However, in the absence of FSM, *had2* parasites achieve metabolic relief through secondary mutation in PFK9. The improved growth of *had2 pfk9* double mutant parasites, compared to parasites with a *had2* single mutation, argues that the growth and metabolic phenotypes are linked. However, our complementation studies cannot strictly discern whether restoring HAD2 directly increases the growth rate of the *had2* strain, as transfection and complementation of HAD2 inherently constitute additional selection for increased fitness. Of note, two recent essentiality screens performed in *Plasmodium* spp. found that HAD2 is dispensable for growth and that loss of HAD2 was not
associated with any significant fitness defect (70, 71). However, it is unknown whether the mutant strains generated in these screens have also acquired additional suppressor mutations, such as polymorphisms in PFK9, that have facilitated their growth.

Likewise, PFK9 provides an additional case study of the context dependence of gene essentiality in Plasmodium spp. Given its involvement in the canonical rate-limiting step in glycolysis, PFK9 is strongly predicted to be essential for asexual growth of malaria parasites (70, 71). In the context of a had2 mutation, our strains readily develop mutations in PFK9 that reduce function but are nonetheless associated with increased fitness. Indeed, it is surprising that parasites that are entirely dependent on glycolysis for ATP production tolerate such a significant loss of activity in this enzyme. Because we identified mutations across the length of PFK9 in our suppressed strains, our studies do not appear to point to a specific disrupted function, such as alterations in an allosteric binding pocket or a dimer interface. The observed mutability of PFK9 points to a remarkable and unexpected metabolic plasticity in the parasite. That is, even though the parasite inhabits a highly controlled intraerythrocytic niche, a wide range of metabolic states of P. falciparum growth are still permissive for parasite growth. This previously undescribed metabolic plasticity centered on PFK9 should be considered in future efforts to target essential metabolism in Plasmodium.

Combined with the study described above, our work highlights the central role of the glycolytic enzyme PFK9. A recent kinetic model of parasite glycolysis confirms that PFK has a high flux-control coefficient, is sensitive to competitive inhibition, and can effectively reduce glycolytic flux (72, 73). Like HAD2, PFK9 is plant-like and evolutionarily divergent from its mammalian homologs (51). These differences may be exploited for PFK inhibitor design and may indicate that PFK9 can be specifically targeted for antimalarial development. However, our work cautions that the parasite has a surprising capacity to adapt to perturbations in central carbon metabolism, which may present challenges in targeting these pathways.

Finally, our approach demonstrates the power of forward genetics to uncover novel biology in a clinically relevant, non-model organism. We employ a previously described screen (20) to uncover a novel resistance locus and employ a second selection for parasite fitness to identify changes that suppress our resistance phenotype. Of the 19 strains in our original FSM resistance screen (20), we identified only one had2 mutant, likely due to the reduced fitness associated with resistance in this strain. While fitness costs associated with antimalarial resistance are well known (74–77), this study represents, to our knowledge, the first to harness this evolutionary trade-off to identify suppressor mutations in a nontarget locus. Additional methods to identify low-fitness resistant mutants have recently been recently described (77), and fitness assessment of resistance mutations may allow suppressor screening for other antimalarials or other target pathways to reveal new aspects of biology and drug resistance in malaria parasites.

**MATERIALS AND METHODS**

**Parasite strains and culture.** Unless otherwise indicated, parasites were maintained at 37°C in 5% O2–5% CO2–90% N2 in a 2% suspension of human erythrocytes in RPMI medium (Sigma-Aldrich) modified with 27 mM NaHCO3, 11 mM glucose, 5 mM HEPES, 0.01 mM thymidine, 1 mM sodium pyruvate, 0.37 mM hypoxanthine, 10 μg/ml gentamicin, and 5 g/liter Albumax (Thermo Fisher Scientific).

FSM strain E2 was generated by selecting a clone of genome reference strain 3D7 (MRA-102 from MR4; ATCC, Manassas, VA) under conditions of continuous treatment with 3 μM FSM, as previously described (20). Clones of strain E2 were isolated by limiting dilution.

**Quantification of FSM resistance.** Opaque 96-well plates were seeded with asynchronous cultures at 0.5% to 1.0% parasitemia (percentage of infected red blood cells). After 3 days, media were removed and parasitemia was measured via Picogreen fluorescence on a POLARStar Omega spectrophotometer (BMG Labtech), as previously described (78). Half-maximal inhibitory concentration (IC50) values were calculated using GraphPad Prism. Unless otherwise indicated, all IC50 data are representative of means of results from ≥3 independent experiments performed with technical replicates.

**HAD2 structural alignment.** Structures were aligned using the TM-align algorithm in Lasergene Protein 3D software (root mean square deviation (RMSD) of 1.9 Å).

**Whole-genome sequencing and variant analysis.** Library preparation, sequencing, read alignment, and variant analyses were performed by the Washington University Genome Technology Access Center. One microgram of parasite genomic DNA was sheared, end repaired, and adapter ligated. Libraries were
sequenced on an Illumina HiSeq 2500 system in Rapid Run mode to generate 101-bp paired-end reads. Reads were aligned to the *P. falciparum* 3D7 reference genome (PlasmoDB v7.2) using Novaalign (V2.08.02). Duplicate reads were removed. SNPs were called using samtools (quality score of ≥20, read depth of ≥5) and annotated using snpEff. Background variants were removed using previously sequenced genomes from parental and control strains (20). Mixed-variant calls and variants in highly variable surface antigen loci (79, 80) were removed.

**Sanger sequencing.** The HAD2 (Plasmodb PF3D7_1226300) A469T (R157X) SNP was amplified and sequenced using the HAD2_R157X_F and HAD2_R157X_R primers. The PFK9 locus was amplified using the PFK9_F and PFK9_R primers. PFK9 amplicons were sequenced using the PFK9_seq (1–8) primers. Primer sequences can be found in Table S2 in the supplemental material.

**Generation of recombinant HAD2.** The predicted coding sequence of HAD2 was amplified using the HAD2_LIC_F and HAD2_LIC_R primers (Table S2). A catalytic mutant (D26A) was also generated to use as a negative control in activity assays. The had2D26A allele was created using the HAD2_D26A_F and HAD2_D26A_R site-directed mutagenesis primers (Table S2).

Ligation-independent cloning was used to clone HAD2 and had2D26A into vector BG1861 (81), which introduces an N-terminal 6×His fusion into the expressed protein. BG1861 6×His-HAD2 was transformed into One Shot BL21(DE3)pLyS5 *Escherichia coli* cells (Thermo Fisher Scientific). Protein expression was induced for 3 h with 1 mM isopropyl-β-D-thiogalactoside at mid-log phase (optical density at 600 nm [OD600] of 0.4 to 0.5). Cells were collected by centrifugation and stored at –20°C.

Cells were lysed in buffer containing 1 mg/ml lysozyme, 20 mM imidazole, 1 mM diithiothreitol, 1 mM MgCl2, 10 mM Tris HCl (pH 7.5), 30 U benzonase (EMD Millipore), and EDTA-free protease inhibitor tablets (Roche). 6×His-HAD2 was bound to nickel agarose beads (Gold Biotechnology), washed with a mixture containing 20 mM imidazole, 20 mM Tris HCl (pH 7.5), and 150 mM NaCl, and eluted in a mixture containing 300 mM imidazole, 20 mM Tris HCl (pH 7.5), and 150 mM NaCl. This eluate was further purified by size exclusion gel chromatography using a HiLoad 16/600 Superdex 200-pg column (GE Healthcare) equilibrated in a mixture containing 25 mM Tris HCl (pH 7.5), 250 mM NaCl, and 1 mM MgCl2. The elution fractions containing HAD2 were pooled and concentrated, and glycerol was added to reach a concentration of 10% (wt/vol). Protein solutions were immediately flash frozen and stored at –80°C.

**HAD2 activity assays.** The rate of para-nitrophenyl phosphate (pNPP; Sigma-Aldrich 50942) hydrolysis by HAD2 was determined by continuous measurement of absorbance at 405 nm. Assays were performed at 37°C in a 50-μl volume consisting of 50 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 10 mM pNPP, and 1.2 μM enzyme.

Hydrolysis of other phosphorylated substrates by HAD2 was measured using an EnzChek phosphate assay (Thermo Fisher Scientific). The reaction buffer was modified to contain 50 mM Tris HCl (pH 8.0), 20 mM MgCl2, 0.2 mM 2-amino-6-mercaptop-7-methylpurine riboside (MESG), and 1 U/ml purine nucleoside phosphorylase (PNP). Reaction mixtures contained 5 mM substrate and 730 nM enzyme. The activity of catalytically inactive 6×His-had2D26A was measured for all substrates, and the data were used to normalize the activity found for the WT HAD2 enzyme. Activity was normalized to that obtained from catalytically inactive 6×His-had2D26A. All data represent means of results from ≥3 independent experiments performed with technical replicates.

**P. falciparum growth assays.** Asynchronous cultures were seeded at 1% parasitemia. Media (no drug) were exchanged daily. Samples were taken at indicated time points and fixed in phosphate-buffered saline (PBS)—4% paraformaldehyde—0.05% glutaraldehyde. Cells were stained with 0.01 mg/ml acridine orange, and parasitemia was determined on a BD Biosciences LSRII flow cytometer (Thermo Fisher Scientific). Parasite growth was measured by continuous measurement of absorbance at 600 nm [OD600].

**Parasite transfections.** Transfections were performed as previously described (20). Briefly, 50 to 100 μg of plasmid DNA was precipitated and resuspended in Cytomix (25 mM HEPES [pH 7.6], 120 mM KCl, 0.15 mM CaCl2), 2 mM EGTA, 5 mM MgCl2, 10 mM KH2PO4).

A ring-stage *P. falciparum* culture was washed with Cytomix and resuspended in the DNA/Cytomix solution. Cells were electroporated using a Bio-Rad Gene Pulser II electroporator at 950 μF and 0.31 kV. Electroporated cells were washed with media and returned to normal culture conditions. Parasites expressing the construct were selected by continuous treatment with 5 nM WR92210 (Jacobus Pharmaceuticals). Transfected cells were cloned by limiting dilution, and the presence of the HAD2-GFP construct expressing the construct were selected by continuous treatment with 5 nM WR92210 (Jacobus Pharmaceuticals).

**Antiserum generation.** Polyclonal anti-HAD2 antiserum was raised against 6×His-HAD2 in rabbits, with TiterMax as an adjuvant (Cocalico Biologicals). Antiserum specificity was confirmed by immunoblotting of lysate lacking HAD2. Polyclonal anti-HAD1 antiserum was previously described (MRA-1256 from MRA; ATCC) (20).

**Immunoblotting.** Lysates were separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in 5% nonfat dry milk—0.1% Tween 20—PBS. Rabbit polyclonal antiserum was used at the following dilutions: 1:2,000 to 5,000 anti-HAD2 and 1:20,000 anti-HAD1 (20). For all blots, 1,200,000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG
antibody was used as a secondary antibody (ThermoFisher 65-6120). Blots were stripped with 200 mM glycine-0.1% SDS-1% Tween 20 (pH 2.2) and reprobed with 1:5,000 rabbit anti-heat shock protein 70 (Hsp70) (AS08 371; Agrisera Antibodies) as a loading control. All blots shown are representative of results from ≥3 independent experiments. Minimal adjustments were applied equally to all blot images.

**PFK model construction.** PFKK subunits were searched against the HHpred server for protein remote homology detection and three-dimensional (3D) structure prediction using statistics as previously described (82–85). The *Borella burgdorferi* PFK structure (PDB 1KZH) (86) returned the highest similarity for both PFKK domains and was used to predict the 3D structure for each domain using the program MODELLER. PFK product orientation in the active site of the model was predicted via the alignment tool, using PyMOL software against the *E. coli* PFK crystal structure (PDB 1PFK) (87). The α domain model encompasses amino acids 779 to 1347, and the β domain model encompasses amino acids 110 to 638.

**Assay of native PFK9 activity.** Sorbitol-synchronized trophozoites were isolated using 0.1% saponin. Cells were washed in buffer containing 100 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM dithiothreitol (DTT), 10% glycerol, and EDTA-free protease inhibitor tablets (Roche) and lysed by sonication at 4°C (Fisher Scientific model 550 Sonic Dismembrator; amplitude of 3.5), followed by centrifugation at 4°C (10,000 × g, 10 min). An “RBC carryover” control was comprised of the trace cellular material remaining after saponin lysis, centrifugation, and washing of uninfected erythrocytes.

Lysate PFK9 activity was monitored by linking it to the oxidation of NADH, as previously described (51, 56). Reaction mixtures contained 100 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM DTT, 0.25 mM NADH, 1 mM ATP, 3 mM fructose 6-phosphate, and excess volumes of linking enzymes aldolase (7.5 U), triose-phosphate isomerase (3.8 U), and glyceral 3-phosphate dehydrogenase (3.8 U). After fresh cell lysate (10 to 15 μg total protein) was added, absorbance at 340 nm was measured at 37°C for 40 min. Activity was determined by linear regression using GraphPad Prism software. Unless otherwise indicated, data represent means of results from ≥3 independent experiments.

**Metabolite profiling.** Approximately ~1 × 10⁶ sorbitol-synchronized early trophozoites were isolated using 0.1% saponin, washed with ice-cold PBS–2 g/liter glucose, and frozen at −80°C. Samples were extracted in 600 μl of ice-cold extraction solvent (chloroform, methanol, and acetonitrile [2:1:1 (vol/vol/ vol)]) using two liquid-nitrogen-cooled 3.2-mm-diameter stainless steel beads and homogenization in a Tissue-Lyser II instrument (Qiagen) at 20 Hz for 5 min in a cold sample rack. Ice-cold water was added, and samples were homogenized for 5 min at 20 Hz. Samples were centrifuged at 14,000 relative centrifugal force (rcf) at 4°C for 5 min. The polar phase was lyophilized and redissolved in 100 μl water and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS was performed on a 4000QTRAP system (AB Sciex) in multiple-reaction monitoring mode using negative ionization and 10 mM tributylammonium acetate (pH 5.1 to 5.5) as the ion pair reagent. The specific parameters used for analysis of MEP pathway metabolites have been previously described (19). Liquid chromatography separation was performed using ion pair reverse-phase chromatography (88) with the following modifications: (i) RP-hydro high-performance liquid chromatography column (Phenomenex) (100 mm by 2.0 mm, 2.5-μm pore size); (ii) flow rate of 0.14 ml/min; (iii) solvent A, consisting of 10 mM tributylammonium acetate–5% methanol; (iv) binary LC gradient (20% solvent B [100% methanol] from 0 to 2.5 min, 30% solvent B for 12.5 min, 80% solvent B for 5 min, and column equilibration at for 5 min); and (v) a 20-μl autosampler injection volume.

**Additional methods.** Additional methods are provided in Text S1 in the supplemental material.

**Accession number(s).** All genome data have been deposited in the NCBI BioProject database (PRJNA222697) and Sequence Read Archive (SRP038937).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.01193-18.

**TEXT S1.** DOCX file, 0.02 MB.

**FIG S1.** TIF file, 0.4 MB.

**FIG S2.** TIF file, 0.2 MB.

**FIG S3.** TIF file, 2.4 MB.

**FIG S4.** TIF file, 0.8 MB.

**FIG S5.** TIF file, 0.5 MB.

**TABLE S1.** DOX file, 0.02 MB.

**TABLE S2.** DOX file, 0.02 MB.

**DATA SET S1.** PDF file, 0.2 MB.

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