Genetic Investigation of Tricarboxylic Acid Metabolism during the *Plasmodium falciparum* Life Cycle

Graphical Abstract

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In Brief

Mitochondria of malaria parasites have features that are divergent from their host's mitochondria. Ke et al. show that six of the TCA cycle enzymes can be disrupted without affecting asexual stages of *Plasmodium falciparum*. The TCA cycle is adaptable and is essential in insect stages of the parasite.

Highlights

- Six of the eight TCA cycle enzymes were knocked out without affecting asexual growth

- Metabolic labeling was analyzed in nine TCA KOs via ^{13}C-labeling and mass spectrometry

- The TCA cycle is adaptable, and the effect of a disrupted TCA cycle is stage specific

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Genetic Investigation of Tricarboxylic Acid Metabolism during the *Plasmodium falciparum* Life Cycle

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SUMMARY

New antimalarial drugs are urgently needed to control drug-resistant forms of the malaria parasite *Plasmodium falciparum*. Mitochondrial electron transport is the target of both existing and new antimalarials. Herein, we describe 11 genetic knockout (KO) lines that delete six of the eight mitochondrial tricarboxylic acid (TCA) cycle enzymes. Although all TCA KOs grew normally in asexual blood stages, these metabolic deficiencies halted life-cycle progression in later stages. Specifically, aconitase KO parasites arrested as late gametocytes, whereas α-ketoglutarate-dehydrogenase-deficient parasites failed to develop oocysts in the mosquitoes. Mass spectrometry analysis of ^{13}C-isotope-labeled TCA mutant parasites showed that *P. falciparum* has significant flexibility in TCA metabolism. This flexibility manifested itself through changes in pathway fluxes and through altered exchange of substrates between cytosolic and mitochondrial pools. Our findings suggest that mitochondrial metabolic plasticity is essential for parasite development.

INTRODUCTION

Malaria is a major global parasitic disease that is responsible for ~300 million infections and ~600,000 deaths per year (WHO, 2013). Although there are a number of effective antimalarial drugs available, the continued emergence of drug-resistant parasites (Ariey et al., 2014) has made finding new treatments a global health priority. Some existing drugs and promising lead compounds target the parasite's mitochondrial functions

(Fry and Pudney, 1992; Nilsen et al., 2013; Phillips et al., 2008). The parasite's mitochondrion is highly divergent from its human counterpart (Vaidya and Mather, 2009), which provides a basis for selective toxicity of antimalarial drugs. However, the tricarboxylic acid (TCA) cycle, a fundamental metabolic pathway within the parasite mitochondrion, has not been fully explored as a potential drug target.

Several lines of evidence support the existence of TCA reactions in the human malaria parasite, *Plasmodium falciparum*. The parasite's genome encodes all of the TCA cycle enzymes (Gardner et al., 2002), which are expressed during the asexual stages (Bozdech et al., 2003). The eight TCA enzymes have been localized to the mitochondrion (Günther et al., 2005; Hodges et al., 2005; Takeo et al., 2000; Tonkin et al., 2004; H.K., J.M.M., M.W.M., and A.B.V., unpublished data), and TCA cycle intermediates are actively synthesized (Olszewski et al., 2009). More recently, isotopic labeling studies have demonstrated an active canonical oxidative TCA cycle. Glutamine and glucose are the main carbon sources for the TCA reactions in *P. falciparum* (Cobbold et al., 2013; MacRae et al., 2013). Glutamine carbon enters the cycle via α-ketoglutarate, whereas glucose appears to provide acetyl-CoA (Cobbold et al., 2013; MacRae et al., 2013), as well as some oxaloacetate (Storm et al., 2014), for entry at the citrate synthase (CS) step. The mitochondrial acetyl-CoA is produced from pyruvate by a branched-chain keto acid dehydrogenase (BCKDH) (Oppenheim et al., 2014).

Although recent studies have investigated metabolic flow through the TCA cycle in *Plasmodium* parasites (Cobbold et al., 2013; MacRae et al., 2013; Oppenheim et al., 2014; Storm et al., 2014), a broad analysis of TCA metabolism using genetic disruptions in *P. falciparum* has not been conducted until now. Previously, succinate dehydrogenase (*SDH*) was knocked out in the rodent parasite *P. berghei* (Hino et al., 2012), and knocked down in the human parasite *P. falciparum* (Tanaka et al., 2012),

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Figure 1. TCA Architecture in the Asexual Blood Stages of WT *P. falciparum*

Bar graphs show the percent isotopic enrichment (y axes) for ^{13}C isotopomers (x axes) of TCA metabolites extracted from D10 WT parasites incubated for 4 hr with either U-^{13}C glucose (blue bars) or U-^{13}C glutamine (orange bars). Please note different scales for distinct metabolites. These data are the average of three biological replicates, each carried out in triplicate. The molecular structures corresponding to the most-abundant glucose and glutamine-derived isotopomers are shown (*). Abbreviations: ACO, aconitase; CS, citrate synthase; FH, fumarate hydratase; IDH, isocitrate dehydrogenase; KDH, α-ketoglutarate dehydrogenase; MQO, malate quinone oxidoreductase; SCS, succinyl-CoA synthase; SDH, succinate dehydrogenase. Cofactors: NAD$^+$, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NADP$^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; Q, ubiquinone; QH$_2$, ubiquinol. Error bars indicate SEMs of three biological replicates.

without associated metabolomic analyses. MacRae et al. (2013) conducted a metabolomic study of TCA and associated intermediates in *P. falciparum* combined with chemical inhibition of the single TCA enzyme aconitase. Disruption of *BCKDH* in *P. berghei* forced the parasite to grow in reticulocytes (Oppenheim et al., 2014); consequently, reticulocyte metabolites might influence metabolomic analysis of this KO line. Storm et al. (2014) investigated the role of phosphoenolpyruvate carboxylase (PEPC) in *P. falciparum* but did not directly follow the TCA cycle enzymes. Therefore, we undertook a study to look at the essentiality, redundancy, and functions of the TCA cycle enzymes in *P. falciparum*. Here, we generated 11 KO lines, disrupting six of the eight TCA cycle enzymes in *P. falciparum*, and analyzed phenotypic and metabolomic features of these KO lines in different life cycle stages. The availability of these KO lines also provides a resource for further detailed metabolic studies.

RESULTS

TCA Architecture in Wild-Type *P. falciparum*

To establish the baseline metabolic architecture of wild-type (WT) parasites, we incubated infected red blood cells (RBCs;

D10 strain; ~90% parasitemia at the late trophozoite/schizont stages) in a culture medium containing either uniformly ^{13}C-labeled (U-^{13}C) glutamine or U-^{13}C glucose for 4 hr and monitored the appearance of ^{13}C in TCA intermediates by high-performance liquid chromatography-mass spectrometry (HPLC-MS). As controls, uninfected RBCs were labeled with U-^{13}C glutamine or U-^{13}C glucose for 4 hr. In agreement with a previous report (Ellinger et al., 2011), RBCs converted U-^{13}C glutamine into glutamate and α-ketoglutarate but no other TCA cycle intermediates (Table S2). Similarly, RBCs did not convert U-^{13}C glucose into TCA cycle intermediates during 4 hr incubations (Table S2). In contrast, WT parasites readily converted U-^{13}C glutamine into malate (Figure 1). The abundant +4 isotopomers (normal mass plus four atomic mass units) of succinate, fumarate, and malate observed indicated that TCA metabolism progressed through canonical oxidative reactions with the majority of carbon entering the cycle as α-ketoglutarate and leaving the cycle as malate (Figure 1). The presence of +4 citrate in these samples indicated that a small fraction of +4 malate was oxidatively converted into citrate. Oxidative turning of the TCA cycle was further confirmed by analyzing metabolites extracted from parasites incubated in U-^{13}C glucose. Mass spectrometry analysis showed low but significant isotopic enrichment in +2 citrate, +2 α-ketoglutarate, +2 succinate, +2 malate, and +2 aspartate (a proxy for oxaloacetate, as it is normally in equilibrium with aspartate via transamination; Shen, 2005; Figure 1). The presence of +2 isotopomers in these samples is consistent with glucose-derived acetyl-CoA entering the TCA cycle. The abundance of glucose-derived carbon in TCA cycle

intermediates was much lower than glutamine-derived carbon, indicating that glucose is a minor contributor to TCA flux in asexual blood stages (Figure 1). Similarly, the low intensity of the +5 citrate signal in U-^{13}C-glucose-labeled samples indicated that anaplerotic carbon input from glucose (i.e., oxaloacetate from cytosolic PEPC reaction) was small. Our results are in general agreement with the recent publications (Cobbold et al., 2013; MacRae et al., 2013; Storm et al., 2014) showing that blood-stage *P. falciparum* parasites carry out an oxidative TCA metabolism.

Most TCA Cycle Enzymes Are Dispensable in Asexual Blood Stages

To determine whether TCA cycle enzymes are essential for parasite survival, we attempted to knock out all eight TCA cycle enzymes through double crossover homologous recombination in D10 parasite line. We successfully knocked out six TCA enzymes, including genes encoding α-ketoglutarate dehydrogenase E1 subunit (ΔKDH; PF3D7_0820700), succinyl-CoA synthetase α subunit (ΔSCS; PF3D7_1108500), SDH flavoprotein subunit (ΔSDH; PF3D7_1034400), CS (ΔCS; PF3D7_1022500), aconitase (ΔAco; PF3D7_1342100), and isocitrate dehydrogenase (ΔIDH; PF3D7_1345700; Figures S1 and S2). In addition, we also produced three double KO lines: (1) ΔKDH/ΔSCS, which should prevent glutamine-derived carbon from entering the canonical oxidative TCA cycle and block all of the biosynthetic routes to succinyl-CoA; (2) ΔKDH/ΔIDH, which should prevent utilization of glutamine-derived carbon in the TCA cycle; and (3) ΔSCS/ΔSDH, which should block oxidative turning of the cycle and substrate-level ATP generation in the mitochondrion (Figures S1 and S2). In contrast, we were unable to disrupt the genes encoding fumarate hydratase (FH; PF3D7_0927300) and malate quinone oxidoreductase (MQO; PF3D7_0616800), despite multiple trials using a variety of approaches (data not shown), suggesting that these two enzymes may be essential in the asexual blood stages.

To test the growth phenotypes of all nine KOs, we measured parasitemia over four or five generations (192–240 hr) relative to the WT (D10) parasites. Surprisingly, no significant growth defects were detected in any of the KO parasite lines when parasites were grown in complete RPMI-1640 medium (data not shown). To assess the possibility that growth defects may become apparent under nutritionally restrictive conditions, we also examined the growth phenotypes of the ΔKDH/ΔIDH and ΔAco lines under various nutritional stresses (e.g., glucose, glutamine, and aspartate starvation) but found no differences between the KO and WT parasites (data not shown). These results show that TCA metabolism is not essential in asexual blood stages in vitro.

We also examined possible transcriptional alterations that may accompany the disruption of the TCA cycle during the intra-erythrocytic development cycle (IDC) in the ΔKDH/ΔIDH double-KO line. A whole genome expression profile was determined through microarray analysis of RNA extracted from tightly synchronized parasite cultures sampled every 6 hr over a 48 hr period. There were only 37 genes that had a statistically significant change at every time point over the 48 hr IDC (overall p across time < 0.002; Table S3). Although these variations

were statistically significant, there were no clear coordinated changes in expression of TCA cycle or mitochondrial electron transport chain genes that could directly compensate for the genetic ablations of KDH and IDH.

Metabolic Consequences of TCA Cycle Disruptions in Asexual Blood-Stage Parasites

One possible explanation for the surprising absence of a growth phenotype in the KO parasite lines could be the presence of unannotated enzymes with redundant functions. To test this, we conducted a series of isotope-labeling experiments and used the diagnostic pattern of isotopomers to determine the metabolic capacity of the parasites using our nine KO lines. These experiments were conducted with U-^{13}C glutamine, because this amino acid is the main carbon source for the TCA cycle in WT parasites (Figure 1; Cobbold et al., 2013; MacRae et al., 2013). In general (but with some exceptions; see below), transgenic parasites incubated in U-^{13}C glutamine showed a consistent phenotype across the panel of TCA KO lines: metabolites upstream of the disrupted enzyme showed significant isotopic enrichment, whereas downstream metabolites showed significantly diminished levels of enrichment (Figure 2). The ΔSDH parasites, for example, accumulated +4 succinate (p < 0.01) but showed no appreciable production of +4 fumarate (p < 0.001) or +4 malate (p < 0.001). Similarly, the ΔKDH line, which interferes with the first committed step in TCA-related glutamine utilization, resulted in no detectable downstream labeling (p < 0.001 for all comparisons). These data show that *P. falciparum* does not contain redundant enzymes to bypass the deleted TCA enzymatic steps.

Although the majority of the parasite lines showed the anticipated metabolic accumulation upstream of the deleted enzymes, the ΔSCS and ΔIDH lines showed deviations from the overall pattern. In the case of the ΔSCS line, a reduced level of isotope labeling was observed in metabolites downstream of succinyl-CoA (Figure 2). Metabolic flux past the deleted enzyme could be attributable to the spontaneous conversion of succinyl-CoA to succinate (Simon and Shemin, 1953). In the ΔIDH line, parasites showed unexpectedly diminished levels of labeling in metabolites upstream of IDH (Figure 2), whereas the upstream flux in ΔCS and ΔAco lines was not affected (Figure 2). The mechanisms behind the diminished levels of TCA intermediates in ΔIDH line are unclear at this point and need further investigation.

Mixing of Glucose- and Glutamine-Derived Carbon in the Mitochondrion

Citrate is a diagnostic metabolite of TCA metabolism that is only generated in the parasite mitochondrion. Our ΔAco line is a convenient tool in this context because it accumulates citrate (Figure 2) and thus amplifies the mitochondrial signal. As shown in Figure S3, we incubated WT and ΔAco parasites in medium containing 2-^{13}C glucose (only one carbon at position 2 is labeled) plus U-^{13}C glutamine and analyzed the isotopomer pattern of citrate. Infected cells incubated in the dual glucose/glutamine-labeled medium showed significant accumulation of +5 citrate (p < 0.001), which arises when glutamine-derived +4 oxaloacetate condenses with glucose-derived +1 acetyl-CoA (Figure S3). Importantly, these data also suggest that the two

Figure 2. Metabolic Consequences of TCA Cycle Disruptions in the Asexual Blood Stages

(Top) A linearized depiction of oxidative TCA metabolism showing each of the expected isotopomers produced from U-^{13}C glutamine labeling. Among them, aspartate (Asp*) undergoes rapid exchange with oxaloacetate (oxaloacetate cannot be stably measured by the methods used in this work); +2 succinate (far right) is derived from a second round of the TCA cycle. Levels of isotopically enriched metabolites observed in extracts from uninfected RBCs, D10 WT, and nine different TCA KO lines are shown. For each line, data are averaged from at least three biological replicates, which were carried out in duplicate or triplicate. Each row shows a complete profile of the TCA cycle metabolites. Each column corresponds to the ratio of isotopomer in each KO line relative to the D10 WT. Orange circles show the positions of carbons labeled by U-^{13}C glutamine. Red triangles represent the ratio of each individual measurement relative to the D10 WT. Grey triangles indicate metabolites that are below the threshold of detection. Blue X's indicate the enzymatic steps that were disrupted in the KO lines. Abbreviations: αKG, α-ketoglutarate; Asp, aspartate; Cit, citrate; Fum, fumarate; Glu, glutamate; Suc, succinate; Mal, malate. Error bars indicate SEMs of at least three biological replicates. For genetic and phenotypic analyses of these KOs, see Figures S1 and S2 and Table S3.

ΔIDH, $\Delta KDH/\Delta IDH$, and ΔSDH) accumulated the intracellular products of PEPC (i.e., +3 aspartate and +3 malate) to levels 1.7–5.3 times higher than those seen in the WT (Table S4; p < 0.02 for all pairwise comparisons to WT; p = 0.06 for ΔAco). In addition, an analysis of metabolites excreted into the growth medium indicated that TCA KO parasites committed significantly more of their glucose-derived carbon to mitochondrial reactions (p < 0.05; Figure 3). As illustrated in Figure 3A, PEPC-derived metabolites can be divided into pre- and post-mitochondrial species, which can be differentiated on the basis of their isotopomer patterns. Pre-mitochondrial metabolites include +3 malate and +3 aspartate (as surrogate for +3 oxaloacetate), whereas post-mitochondrial metabolites include +5 citrate, +4/+5 α-ketoglutarate, and +4/+5 glutamate. The concentrations of these pre- and post-mitochondrial metabolites in the medium excreted by the D10 WT, ΔKDH, and $\Delta KDH/\Delta IDH$ lines are shown in Figure 3B. In WT parasites, 89% of the excreted PEPC-derived carbon pool was pre-mitochondrial (+3 malate and +3 aspartate; Figure 3C). Thus, the majority of this potentially anaplerotic carbon pool was excreted without having been committed to mitochondrial reactions. In contrast, the KO lines committed significantly more of this glucose-derived carbon to mitochondrial reactions. As shown in Figure 3C, the percentages of post-mitochondrial metabolites in ΔKDH and $\Delta KDH/\Delta IDH$ lines increased up to 3-fold in

carbon substrate, acetyl-CoA, is only derived from glucose (not from glutamine), most likely via the BCKDH reaction (Oppenheim et al., 2014).

Plasticity of TCA Metabolism in *P. falciparum*

Our glutamine labeling data showed that enzyme redundancy does not play a role in the survival of the TCA KO parasites (Figure 2). Another strategy that organisms can use to compensate for metabolic deficiencies is to increase the flow of carbon through alternative pathways. To test this possibility, we incubated parasites in U-^{13}C glucose and examined the isotope labeling of TCA-related metabolites. Glucose-derived carbon enters the TCA cycle via two classical mechanisms: (1) as two carbon acetyl-CoA units, which balance the two CO_2 molecules lost on each turn of the cycle, and (2) via anaplerotic reactions (i.e., PEPC reaction) that contribute four-carbon oxaloacetate or malate to the cycle (Cobbold et al., 2013; MacRae et al., 2013; Oppenheim et al., 2014; Storm et al., 2014).

Intracellular metabolites extracted from parasites grown in U-^{13}C glucose showed that TCA KO parasites (ΔAco, ΔKDH,

Fate of PEPC-derived carbon

(A) A schematic representation of the cytosolic and mitochondrial pathways used by PEPC-derived oxaloacetate. Blue arrows depict glucose utilization without mitochondrial participation (pre-mitochondrial flux), and red arrows indicate glucose utilization involving mitochondrial processes (post-mitochondrial flux).

(B) Extracellular concentrations of individual metabolites in D10 WT, ΔKDH, and ΔKDH/ΔIDH lines.

(C) Pre- and post-mitochondrial metabolites excreted into the medium by the D10 WT and various KO lines. The total excretion of glucose-derived carbon through PEPC is the sum of the concentrations of +3 malate and +3 aspartate (pre-mitochondrial, blue bars) and +5 citrate, +4/+5 α-ketoglutarate, and +4/+5 glutamate (post-mitochondrial, red bars). Data are derived from three biological replicates. Error bars indicate SDs of three biological replicates. The glucose labeling patterns in other TCA KO lines from the parasite pellet samples are shown in Figure S4 and Table S4.

the TCA cycle and mitochondrial electron transport chain in *P. falciparum*, we conducted metabolic analyses in parasites under conditions where the mitochondrial electron transport chain was inhibited at complex III by atovaquone (Fry and Pudney, 1992; Srivastava et al., 1997). Atovaquone-treated parasites are unable to recycle ubiquinol to ubiquinone and thus become functional KOs for all ubiquinone-requiring enzymes including SDH, MQO, and dihydroorotate dehydrogenase (DHOD). Because atovaquone is toxic to WT parasites, these experiments were conducted with a mitochondrial-electron-transport-chain-independent transgenic line that expresses the cytosolic ubiqui-

comparison to the WT. In ΔKDH parasites, post-mitochondrial PEPC-derived carbon was excreted primarily as +4/+5 glutamate, whereas ΔKDH/ΔIDH primarily excreted +5 citrate (Figure 3B). This excretion pattern is consistent with the intracellular labeling patterns of these KO lines (Figure S4). These data showed that (1) parasites can draw on either glucose or glutamine as significant carbon sources for the TCA cycle, (2) parasites can secrete a variety of mitochondrial metabolites into the medium, and (3) KO parasites with impaired glutamine utilization commit a significantly higher proportion of their glucose-derived carbon to mitochondrial TCA reactions.

Mitochondrial Electron Transport Chain Inhibition Blocks Flux through the TCA Cycle

The mitochondrial electron transport chain is an established target of antimalarial drugs. To assess the connection between

none-independent *Saccharomyces cerevisiae* DHOD (yDHOD) (Ke et al., 2011; Painter et al., 2007). These parasites have a functional mitochondrial electron transport chain that can be inhibited by atovaquone but are able to grow in the presence of the drug because they generate pyrimidines via *yDHOD*. WT and y*DHOD*-transgenic parasite lines were treated with or without 100 nM atovaquone (~100 times EC$_{50}$) in medium containing U-^{13}C glucose for 4 hr. Labeling data showed that atovaquone-treated parasites did not assimilate glucose-derived carbon into TCA intermediates (Figure 4). The characteristic +2 isotopomers of TCA metabolites typically observed in control parasites were completely eliminated in atovaquone-treated parasites (Figures 4 and S5). Therefore, our data indicate that mitochondrial electron transport chain inhibitors prevent flux through the TCA cycle, as well as block the electron transport chain.

A

B

Figure 4. Mitochondrial Electron Transport Chain Inhibition Blocks the TCA Flux

(A) A schematic representation of carbon input from glucose into the TCA metabolites. Blue arrows/circles show anaplerotic input through PEPC reaction, whereas gold arrows/circles indicate flux from acetyl-CoA. Atovaquone (ATV) blocks the mitochondrial electron transport chain, thereby inhibiting MQO.

(B) Enrichment of each metabolite in *yDHOD* transgenic parasites in the presence or absence of ATV is shown. The +2 and +5 citrate isotopomers are diagnostic of flux through the citrate synthase reaction. Data are the average of three biological replicates. Error bars indicate SEMs of three biological replicates. A more comprehensive set of TCA cycle isotopomers is presented in Figure S5.

TCA Metabolism Is Essential for Malaria Transmission

All KO lines described above were derived from the *P. falciparum* D10 strain that is defective in sexual stage conversion. Therefore, we knocked out the *KDH-E1* and *Aco* genes, individually, in the gametocyte-producing NF54 strain. Disruption of *KDH* prevents glutamine-derived carbon from entering the TCA cycle, whereas *Aco* disruption blocks the full utilization of glucose as a TCA carbon source at an early step.

As observed for D10 lines, neither Δ*KDH* nor Δ*Aco* in the NF54 background exhibited growth defects in asexual stages (Figure S6A), suggesting that TCA metabolism is conserved among different parasite genetic backgrounds. When induced to generate gametocytes, NF54-Δ*KDH* and NF54-Δ*Aco* parasites behaved differently. NF54-Δ*KDH* was able to fully complete the gametocytogenesis process, forming mature stage V gametocytes in about 7–10 days (Figure S6B) with gametocytemia indistinguishable from that of WT NF54 (Figure 5A). In addition, there was no significant difference in the exflagellation profile of NF54-Δ*KDH* compared to the WT parasites (Figure 5B), sug-

gesting that disruption of the TCA cycle did not harm gamete formation. Although the NF54-Δ*Aco* line progressed normally until stage III, it failed to form mature stage V gametocytes (Figures S6C and S6D). Overall gametocyte production in NF54-Δ*Aco* line was largely diminished as well (Figure 5D). Due to its lack of stage V gametocytes, NF54-Δ*Aco* did not form gametes (Figure 5E). Our observations confirm the role of *Aco* in gameto-cytogenesis and are consistent with the results observed when parasites were treated with 10 mM sodium fluoroacetate (NaFAc), which indirectly inhibits Aco (MacRae et al., 2013).

To assess the direct requirement of TCA flux and mito-chondrial electron transport chain for exflagellation (gamete formation), mature WT NF54 parasites were incubated with atovaquone prior to exflagellation induction. As shown in Figure S6E, incubating mature WT gametocytes with 100 nM atovaquone for up to 24 hr had no effect on the parasite's exfla-gellation rate. These data indicate that mitochondrial electron transport chain and TCA fluxes are dispensable for male gamete formation by mature gametocytes. The absence of defects in NF54-Δ*KDH* parasites is also consistent with the conclusion that halting TCA metabolism is not harmful for gametocyte or gamete development. Thus, the defect in NF54-Δ*Aco* game-tocytes may be due to reasons other than disruption of the TCA cycle (see Discussion).

To examine the role of TCA metabolism in mosquito stages, female *Anopheles gambiae* mosquitoes were fed with blood containing mature gametocytes derived from NF54 WT or NF54-Δ*KDH* parasites. The ability of parasites to mate success-fully and develop further in the insect was assessed by counting oocysts in each mosquito 8 days after the blood feed. We found that none of the mosquitoes fed on blood with NF54-Δ*KDH* gametocytes produced oocysts, whereas those fed on blood containing WT gametocytes generated normal numbers of oocysts (Figure 5C). As expected, there were no oocysts formed in mosquitoes fed on NF54-Δ*Aco* gametocytes (Figure 5F), which fail to progress to mature gametocytes or gametes (Figure S6D). Because *KDH* disruption had no effect on gametocyte development and gamete formation (Figures 5A and 5B), the inability to form oocysts in this KO suggests that a fully functional TCA metabolism is only essential for parasite development in mosquitoes subsequent to gamete formation.

DISCUSSION

By combining genetic manipulation and metabolic analysis in unprecedented detail, this study significantly clarifies our under-standing of TCA metabolism in the human malaria parasite, *P. falciparum*. The derivation of 11 KO lines also provides a resource for future investigations of the biological consequences of TCA cycle disruptions. Our study reveals several key findings, including (1) the TCA cycle is not essential for parasite survival in asexual blood stages but is required for parasite transmission; (2) the TCA metabolism can utilize different carbon sources and alter its pathway fluxes when the normal flux is disrupted; and (3) this metabolic plasticity is essential for parasites to meet bioenergetic demands in the multiple stages of their complex life cycle. A schematic representation of our principle findings is illustrated in Figure 6.

Figure 5. TCA Cycle Is Essential to Mosquito-Stage Development of *P. falciparum*

(A) Gametocytemia of NF54 WT and NF54-Δ*KDH* parasites on days 14–20 post-induction.

(B) Exflagellation percentage in NF54 WT and NF54-Δ*KDH* lines on days 14–20 post-induction.

(C) Infectivity of NF54 WT and NF54-Δ*KDH* parasites as measured by the number of oocysts per mosquito on day 8 after blood feeding.

(D–F) Results from NF54-Δ*Aco* line, corresponding to (A)–(C), respectively.

In (C) and (F), data are from two independent feeding experiments. Error bars indicate SEMs of three biological replicates. Morphological data are shown in Figure S6.

proceeding from glutamate to malate as in the WT (Figure 6A), in Δ*KDH* parasites, it proceeds from glucose-derived malate to glutamate, utilizing the *MQO* to *IDH* segment of the cycle (Figure 6C). This metabolic plasticity may allow multiple carbon sources to feed the TCA cycle at various times in the parasite's complicated life cycle.

A previous study in a related Apicomplexan parasite, *Toxoplasma gondii* (MacRae et al., 2012), provided evidence for a γ-aminobutyric acid (GABA) shunt, in which succinate is generated directly from α-ketoglutarate via the action of GABA α-ketoglutarate aminotransferase, glutamic acid decarboxylase, and succinic semialdehyde dehydrogenase enzymes. By extension, it has been proposed that a similar GABA shunt might also operate in *P. falciparum* (MacRae et al., 2013), although genes encoding all the requisite enzymes for the shunt cannot be detected in the *P. falciparum* genome. However, we found that the level of +4 succinate in the Δ*KDH* parasites labeled with U-^{13}C glutamine was very low (Figure 2), which would not be expected if a robust GABA shunt were to bypass the *KDH* reaction to feed the downstream TCA reactions. In light of the lack of evidence for GABA shunt enzymes in *P. falciparum,* we propose that the low level of +4 succinate in the Δ*KDH* line is likely a product of widely conserved α-ketoglutarate-dependent oxygenases, which generate succinate through iron- and oxygen-dependent decarboxylation of α-ketoglutarate (Schofield and Zhang, 1999). These enzymes carry out various protein hydroxylation and histone demethylation reactions, and genes encoding them are found in the *P. falciparum* genome (e.g., lysine-specific histone demethylase 1, PF3D7_1211600, and JmjC domain containing protein, PF3D7_0809900).

In asexual blood stages, parasites are remarkably resistant to disruption of TCA metabolism; six of the eight TCA cycle enzymes can be deleted with no detectable growth defects

Our baseline metabolic data collected on WT parasites are in agreement with the current understanding of TCA cycle function in the parasite (Cobbold et al., 2013; MacRae et al., 2013; Oppenheim et al., 2014; Storm et al., 2014). Malaria parasites possess a canonical oxidative TCA cycle with the majority of TCA flux in asexual blood stages flowing from glutamine-derived α-ketoglutarate to malate (Figures 1, 2, and 6). Beyond these observations, we show that the parasite's mitochondrion can alter metabolic fluxes through alternative pathways when one route of carbon utilization is blocked. This metabolic plasticity was illustrated by our observations of substrate utilization in mutant lines lacking various TCA cycle enzymes (Figures 3, 6, and S4). Most TCA KO lines showed significant elevation in their utilization of glycolytically derived carbon in the remaining TCA reactions. In the extreme case of the *KDH* deletion, which prevents parasites from using glutamine as a carbon source for TCA reactions, parasites excrete glucose-derived glutamate into the medium (Figure 3). Thus, instead of the flow of carbon

Figure 6. Models for TCA Metabolism under Various Conditions
A schematic representation of TCA flux is shown for (A) WT, (B) ΔAco, (C) ΔKDH, and (D) atovaquone-treated parasites. Metabolic fluxes are depicted qualitatively as major (thick blue lines), minor (thin black lines), or zero (dotted lines). Some minor fluxes have been excluded for clarity of presentation. At the bottom of each panel, the progression of the parasite life cycle is indicated. IDC, intra-erythrocytic development cycle, is comprised of ring (R), trophozoite (T), and schizont (S). Gametocyte development progresses from stage I to stage V. Mosquito stages include gamete (G), zygote (Z), ookinete (OK), and oocyst (OC). Proposed reasons for developmental arrest at each of these stages are indicated by numbers within red octagons.

(data not shown). In the absence of redundancy (Figure 2), these data show that a full turning of the TCA cycle is not essential in asexual blood stages. The dispensability of TCA metabolism in blood stages extends to TCA-dependent pathways. For example, provision of succinyl-CoA via the TCA cycle to the heme biosynthetic pathway is not essential for blood-stage *P. falciparum* (Ke et al., 2014) or *P. berghei* (Nagaraj et al., 2013).

It was surprising to observe that disruption of the TCA cycle at the *KDH* step had no significant effect on sexual differentiation and gamete formation (Figure 5). Previous studies have shown

upregulation of the TCA enzymes in gametocytes (Young et al., 2005), suggesting functional importance of the TCA cycle in gametocytogenesis. However, our results with the ΔKDH line indicate that a complete turning of the TCA cycle is not essential for sexual differentiation. In contrast, the parasite line lacking aconitase failed to produce mature gametocytes and gametes (Figure 5). Our results now provide genetic evidence to confirm the importance of aconitase in gametocytogenesis shown by MacRae et al. (2013). The specific necessity for aconitase for later gametocyte development might be due to the following

possible reasons: (1) aconitase is required for the production of mitochondrial NADPH, which is crucial for maintaining the mitochondrial redox balance and defense against oxidative damage and sustaining NADPH-dependent biosynthetic enzymes and (2) aconitase converts citrate to downstream metabolites, preventing the accumulation of citrate to a potentially toxic level. The fact that asexual stages of ΔAco and ΔIDH parasites are not affected may suggest that the 48-hr life cycle is insufficient to produce lethal effects, whereas the 7–10 days required for gametocyte maturation results in accumulation of damage beyond the threshold of tolerability in the ΔAco parasite.

In stark contrast to the asexual blood stages, parasite development in mosquitoes was completely inhibited by TCA cycle disruptions (Figure 5C). These results clearly establish the evolutionary necessity for maintenance of the TCA cycle by P. falciparum, because inhibition of transmission through mosquitoes would render the parasite extinct. This observation also suggests a major switch in mitochondrial functions as the parasite transitions from its vertebrate to invertebrate host. The motile ookinete has to survive outside a host cell for 24 hr and invade the mosquito midgut epithelium. Whereas blood-stage parasites are able to thrive through substrate-level generation of a mere two ATP molecules per glucose via glycolysis, survival in mosquitoes may place a much greater value on more economical energy generation through oxidative phosphorylation powered by the TCA cycle. In the ΔKDH line, input of TCA-derived electrons into mitochondrial electron transport chain would be significantly reduced, thereby decreasing mitochondrial oxidative efficiency, potentially causing parasites to arrest due to energy insufficiency. Observations of defective mosquito stage development by P. berghei with gene KOs of SDH (Hino et al., 2012), NADH dehydrogenase (Boysen and Matuschewski, 2011), and BCKDH (Oppenheim et al., 2014) also lend support to this argument.

Interestingly, we found that two of the TCA cycle enzymes, FH and MQO, could not be genetically ablated even in asexual blood stages. The inability to disrupt the MQO gene was surprising, because we have previously shown that biochemical activity of the enzyme could be functionally disrupted in yDHOD transgenic parasites (Ke et al., 2011; Painter et al., 2007). One possible reason could be an essential non-enzymatic structural function of MQO in mitochondrial biogenesis. This interpretation is supported by the observation that MQO is conserved among all apicomplexan parasites, including Cryptosporidium species, which have lost all other mitochondrial electron transport chain proteins (Abrahamsen et al., 2004). The essentiality of FH, on the other hand, could be explained by its role in a fumarate cycle serving the purine salvage pathway, as suggested by Bulusu et al. (2011). Plasmodium parasites are unable to synthesize purines and rely entirely on the purine salvage pathway. Two important enzymes in this pathway are adenylosuccinate synthetase and adenylosuccinate lyase. These enzymes utilize aspartate and generate fumarate in the process of converting inosine monophosphate to AMP. Mitochondrial FH could convert fumarate derived from purine salvage into malate in the mitochondrion, which could be transported to the cytosol and converted back to aspartate through successive reactions

with malate dehydrogenase and aspartate aminotransferase. The fact that we can completely eliminate TCA-derived malate in several KO lines (Figure 2) suggests that the role of FH in the TCA cycle is dispensable. The essentiality of FH is, therefore, likely related to the requirements of the purine salvage pathway. It is interesting to note that two mitochondrial enzymes critical for parasite survival in blood stages, DHOD and FH, appear to serve pyrimidine biosynthesis and purine salvage, respectively.

Storm et al. (2014) found that PEPC could be knocked out but only when the parasites were provided with malate or fumarate in the medium. These authors suggested that PEPC is a key enzyme in P. falciparum central carbon metabolism by providing anaplerotic carbon for the TCA cycle. However, our ΔAco, ΔCS, and ΔIDH lines grew normally despite their inability to incorporate anaplerotic carbon from glucose into downstream TCA reactions. Moreover, the survival of ΔIDH parasites indicates that mitochondrial NADPH production may not be essential in asexual blood stages, in contrast to the suggestion made by Storm et al. (2014). Consequently, we attribute the essentiality of PEPC to factors other than maintenance of the TCA cycle.

Our analysis of atovaquone-treated yDHOD transgenic parasites revealed another unexpected metabolic finding: oxaloacetate is evidently not transported into the mitochondrion, but malate is (Figures 4 and S5). This is surprising given that the parasite's mitochondrial dicarboxylate-tricarboxylate carrier (PfDTC) efficiently transports oxaloacetate in vitro (Nozawa et al., 2011). The absence of +2 and +5 citrate, despite normal levels of aspartate (observed as a surrogate for oxaloacetate), argues that PfDTC does not import oxaloacetate into the mitochondrion in vivo (Figures 4 and S5). Data from ΔKDH/ΔIDH line treated with atovaquone also support this argument (Figure S5); the residual signals (+2 and +5 citrate) were likely due to the delayed effect of atovaquone, which was added concurrently with ^{13}C-glucose to the culture during the 4-hr labeling. This observation also explains the parasite's propensity for secreting malate when using glutamine as a carbon source for TCA metabolism. Specifically, mitochondrial α-ketoglutarate/malate transport may be inherently linked through the action of an antiporter (presumably PfDTC).

It was interesting to note that, upon atovaquone treatment, the level of +3 succinate accumulated to a much-higher degree in D10 WT parasites when labeled with U-^{13}C glucose (Figure S5). Because input from glucose into the TCA cycle is inhibited under this condition, one potential source of +3 succinate could be a reverse reaction of SDH that reduces fumarate to succinate. Fumarate reductase activity was proposed by a previous study (Takashima et al., 2001). At this point, the nature of the electron donor for this reaction remains unclear, but it could be the high level of ubiquinol that would accumulate upon inhibition of the cytochrome bc_1 complex.

Our demonstration that the antimalarial drug atovaquone eliminated anaplerotic carbon input from glucose into TCA metabolism has implications for the potent efficacy of mitochondrial electron transport chain inhibitors in blocking transmission of parasites to mosquitoes (Fowler et al., 1994; Nilsen et al., 2013). Greater demand for mitochondrial contribution to bioenergetics in insect stages would make the mitochondrial electron transport chain, and the TCA cycle that primes it with reducing

equivalents, more critical for parasite survival. Our results show that mitochondrial electron transport chain inhibitors not only affect parasite respiration but also interfere with the TCA cycle (Figures 4 and S5). This assault on mitochondrial functions is the likely reason for the exquisite sensitivity of the mosquito stage development of parasites exposed in the mammalian host to mitochondrial electron transport chain inhibitors (Nilsen et al., 2013). Whereas our results clearly argue against potential TCA cycle inhibitors as antimalarial drug leads, because they are unlikely to inhibit blood-stage parasite growth, such compounds could serve as potent transmission-blocking agents. One TCA cycle enzyme with potential to be an attractive drug target is FH, inhibition of which would be effective at both vertebrate and invertebrate stages of the parasite. Selectively toxic compounds could be envisioned because the parasite possesses a type I FeS-dependent FH, which is substantially different from the human type II enzyme (Woods et al., 1988).

In summary, this study describes a comprehensive analysis of TCA cycle function in the human parasite *P. falciparum*. As shown in Figure 6A, malaria parasites maintain an oxidative TCA cycle with the main flux supplied by glutamine. In Δ*Aco* parasites (Figure 6B), maturation of gametocytes is prevented, perhaps due to an accumulation of damage related to the loss of mitochondrial NADPH production and/or high levels of citrate. In the Δ*KDH* line (Figure 6C), blood-stage parasites metabolize glucose into glutamate, whereas the insect-stage parasites fail to survive. Compounds targeting the mitochondrial electron transport chain, such as atovaquone, completely block the flux of metabolites through the TCA cycle (Figure 6D). Our study also reveals that the parasites have a flexible carbon metabolism, which may be important for making the transitions between the different environments encountered during the life cycle. In addition, the availability of 11 different KO lines described here provide an important resource for investigating biological consequences of disrupting the TCA cycle under various conditions, such as nutritional restrictions, in *P. falciparum*.

EXPERIMENTAL PROCEDURES

Gene KO Protocol
WT D10 and NF54 *P. falciparum* parasites were transfected with each KO construct and subjected to positive and negative selections. Deletion of the target gene was mediated via double crossover recombination.

Metabolite Labeling and HPLC-MS
Uninfected RBCs from heparinized blood were sedimented through 65% Percoll to remove any contaminated reticulocytes, platelets, and white blood cells prior to parasite culturing. Mycoplasma-free parasite cultures were tightly synchronized and grown to late trophozoite stage, and infected RBCs were isolated by density centrifugation using a Percoll step gradient (35%, 60%, and 65%). Purified parasites (late trophozoite to schizont stages) were incubated with the labeling medium for 4 hr. All of the isotopes used in this study (U-^{13}C glutamine, U-^{13}C glucose, and 2-^{13}C-glucose) were 99% pure and were purchased from Cambridge Isotope Laboratories.

Methanolic metabolite extracts were dried under a stream of N$_2$ gas and reconstituted in 200 μl (four times the original extraction volume) of HPLC-grade H$_2$O. High-resolution MS data were collected on a Thermo Scientific Exactive mass spectrometer in negative mode using ion-pairing C18 chromatography following previously published methods (Lu et al., 2010). Metabolite data were analyzed using MAVEN (Melamud et al., 2010), and isotopomers

were corrected for naturally occurring ^{13}C using established methods (Fan et al., 2014). Data from technical replicates were averaged, and error bars report error across biological replicates. All p values were calculated by two-tailed t test. Raw data for all metabolites are provided in Table S5 (for U-^{13}C glutamine labeling of the wild-type and KO lines) and Table S6 (for multiple stable isotope labeling of the wild-type and selected KO lines).

For all other procedures, please see the Supplemental Experimental Procedures.

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