# Mitochondrial Metabolism of Glucose and Glutamine Is Required for Intracellular Growth of *Toxoplasma gondii*

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## SUMMARY

*Toxoplasma gondii* proliferates within host cell vacuoles where the parasite relies on host carbon and nutrients for replication. To assess how *T. gondii* utilizes these resources, we mapped the carbon metabolism pathways in intracellular and egressed parasite stages. We determined that intracellular *T. gondii* stages actively catabolize host glucose via a canonical, oxidative tricarboxylic acid (TCA) cycle, a mitochondrial pathway in which organic molecules are broken down to generate energy. These stages also catabolize glutamine via the TCA cycle and an unanticipated γ-aminobutyric acid (GABA) shunt, which generates GABA and additional molecules that enter the TCA cycle. Chemically inhibiting the TCA cycle completely prevents intracellular parasite replication. Parasites lacking the GABA shunt exhibit attenuated growth and are unable to sustain motility under nutrient-limited conditions, suggesting that GABA functions as a short-term energy reserve. Thus, *T. gondii* tachyzoites have metabolic flexibility that likely allows the parasite to infect diverse cell types.

## INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular protozoan parasite of warm-blooded animals and may chronically infect one-third of the world's human population (Weiss and Dubey, 2009). While the majority of *T. gondii* infections are asymptomatic, severe disease and death can occur in prenatal infections and in immunocompromised individuals (Weiss and Dubey, 2009). Acute infections are mediated by the replicative tachyzoite stages that develop from ingested oocysts or tissue cysts and rapidly disseminate to extraintestinal tissues. While tachyzoites are effectively cleared in immunocompetent individuals, a small proportion of these parasites differentiate to bradyzoites that can form tissue cysts in the brain, liver, and muscle and sustain long-lived chronic infections (Dubey et al., 1998). Bradyzoites can spontaneously differentiate back to tachyzoites, leading to disease activation in immunocompromised patients.

Tachyzoites are capable of invading and proliferating in virtually any nucleated cell in mammalian hosts. In common with other intracellular pathogens, *T. gondii* tachyzoites must salvage carbon sources and other essential nutrients from their host cell. *T. gondii* tachyzoites reside within a unique parasitophorous vacuole in infected host cells that is surrounded by membrane that is freely permeable to many host metabolites (Schwab et al., 1994). Despite having access to a variety of carbon sources, *T. gondii* tachyzoites are generally thought to primarily utilize glucose as carbon source and to be dependent on glycolysis for ATP synthesis (Al-Anouti et al., 2004; Saliba and Kirk, 2001; Pomel et al., 2008; Starnes et al., 2009; Polonais and Soldati-Favre, 2010). While these stages encode all of the genes for a complete tricarboxylic acid cycle and a mitochondrial respiratory chain, they lack a mitochondrial isoform of pyruvate dehydrogenase (PDH) that normally links glycolysis with the TCA cycle via conversion of pyruvate to acetyl-CoA (Fleige et al., 2007; Mullin et al., 2006; Brooks et al., 2010; Nair et al., 2011; Saito et al., 2008; Crawford et al., 2006; Saliba and Kirk, 2001; Pomel et al., 2008; Starnes et al., 2009; Polonais and Soldati-Favre, 2010). The precise role of the TCA cycle in glucose catabolism, if any, therefore remains poorly understood. The possibility that *T. gondii* tachyzoites could catabolize other carbon sources in the mitochondrion has recently been suggested by the finding that genetic disruption of parasite glucose uptake had little effect on intracellular parasite growth in vitro and virulence in animal models (Blume et al., 2009). Egressed tachyzoites lacking the glucose transporter TgGT1/TgST2 exhibited restored gliding motility in the presence of glutamine, suggesting that these parasites can utilize this amino acid in response to glucose starvation (Blume et al., 2009). Glutamine
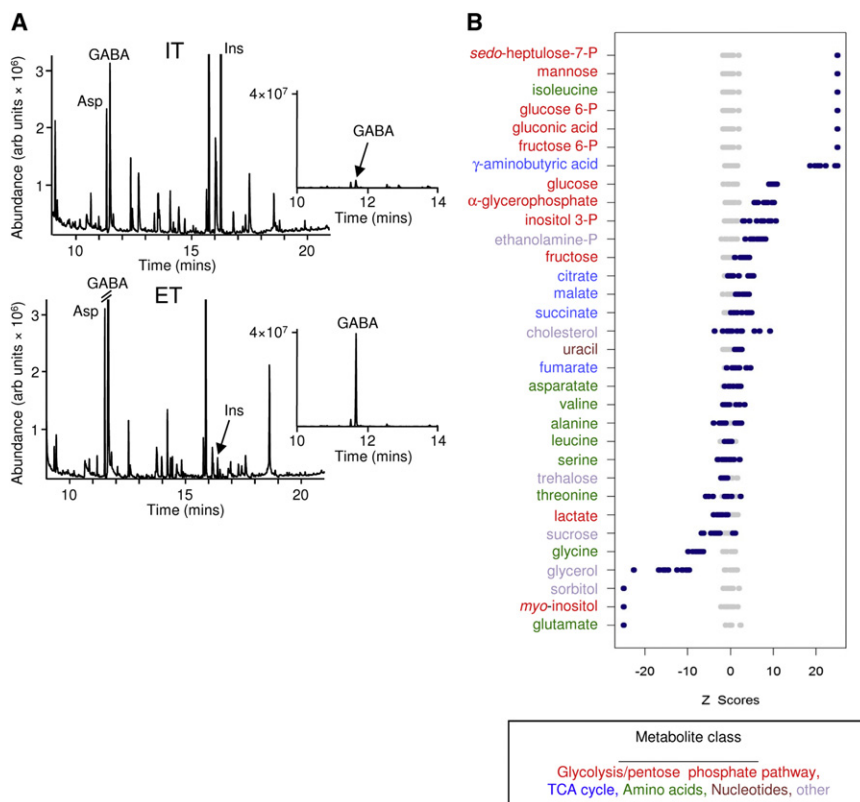
**Figure 1. Metabolomic Analysis of Intracellular and Extracellular Tachyzoites**

(A) GC-MS chromatograms of polar metabolite extracts from intracellular (IT) and extracellular (ET) tachyzoites ($2 \times 10^8$ cell equivalents). Insets show a section of these chromatograms (10–14 min) with 10-fold expansion of the y scale to show changes in γ-aminobutyric acid (GABA) levels in the two stages. Peaks corresponding to aspartate (Asp) and *myo*-inositol (Ins) that are either unchanged or decreased in extracellular tachyzoites are indicated.

(B) Z score plots of selected metabolites. Plotted Z scores show the mean and standard deviations of individual metabolites in replicate analyses following normalization to the intracellular tachyzoite sample set. Gray circles refer to metabolite levels in intracellular tachyzoites (which typically cluster within 5 SD of the mean), while blue circles refer to metabolite levels in extracellular tachyzoites. Metabolite levels that deviate from the mean by >5 SD are considered significant (note that the Z score plots are truncated at 25 SD for clarity). See also Figure S1.

show that a functional TCA cycle is essential for intracellular replication while the GABA shunt may allow accumulation of energy reserves. These findings provide important insights into *T. gondii* central carbon metabolism that may underlie the extraordinary capacity of these parasites to replicate within a wide variety of host cells.

## RESULTS

### Extracellular Tachyzoites Accumulate High Levels of γ-Aminobutyrate

Polar metabolite levels in intracellular and egressed tachyzoite stages of *T. gondii* RH strain were determined by gas chromatography-mass spectrometry (GC-MS) (Figure 1A, see Figure S1 online). More than 200 metabolite peaks were detected, and the two stages were robustly separated by principal component analysis (Figure S1). Significant differences were observed in the normalized intracellular levels of many intermediates in central carbon metabolism (Figure 1B). Specifically, several intermediates involved in glycolysis, the pentose phosphate pathway, and inositol metabolism were highly elevated in extracellular tachyzoites (z score >20), suggesting that glucose uptake increases following tachyzoite egress and/or fluxes through the lower section of glycolysis decrease. In contrast, similar levels of TCA cycle intermediates were detected in both stages (Figure 1B), indicating the operation of a partial or complete TCA cycle throughout development. The levels of proteinogenic amino acids were generally similar in both stages, with the exception of isoleucine and glutamate, which were significantly (z score >20) increased or decreased in extracellular tachyzoites, respectively (Figure 1B). Unexpectedly, both tachyzoite stages contained high levels of the nonproteinogenic amino acid GABA (Figures 1A, inset; Figure 1B). GABA was the
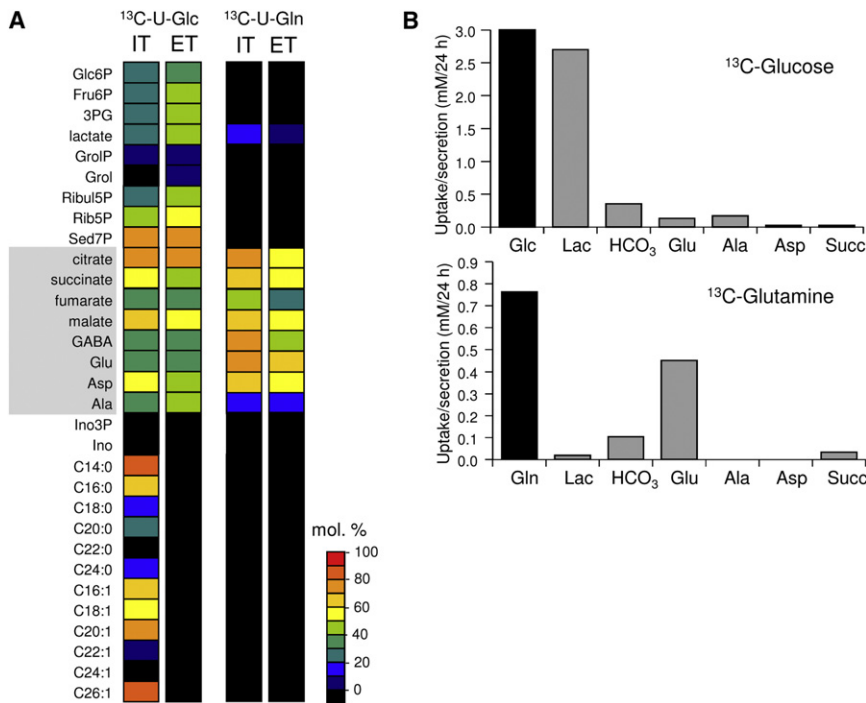
uptake and catabolism via an unusual bifurcated TCA cycle has been reported in asexual intraerythrocytic stages of the related apicomplexan parasite, *Plasmodium falciparum* (Olszewski et al., 2010), although the functional significance of mitochondrial TCA cycle enzymes in these stages remains undefined (Hino et al., 2012). In *T. gondii*, there are conflicting data as to whether the TCA cycle and glutaminolysis are important for ATP generation in intracellular tachyzoites under normal growth conditions. While an active respiratory chain is essential for normal intracellular replication (Lin et al., 2011b; Seeber et al., 2008; Srivastava et al., 1997; Vercesi et al., 1998), genetic deletion of the TCA cycle enzyme, succinyl-CoA synthetase, had only a modest effect on tachyzoite growth in vitro (Fleige et al., 2008).

Here we have used a combination of metabolite profiling and stable isotope labeling approaches to reassess *T. gondii* central carbon metabolism in intracellular parasites and in egressed tachyzoites. Egressed tachyzoites enter a growth-arrested state but maintain an active gliding motility that is thought to be powered by glycolytic ATP synthesis (Pomel et al., 2008). Intriguingly, egressed tachyzoites can remain motile for 1–2 hr in the absence of carbon sources, suggesting that they may utilize an as-yet-uncharacterized reserve material (Lin et al., 2011a). We find that both intracellular and egressed tachyzoites constitutively catabolize glucose and glutamine under normal growth conditions. Pyruvate was found to be converted to acetyl-CoA and fully catabolized in a complete TCA cycle, despite the absence of an annotated mitochondrial PDH, while glutamine enters the TCA cycle via either α-ketoglutarate or a previously unrecognized γ-aminobutyric acid (GABA) shunt. Furthermore, we

**Figure 2. *T. gondii* Tachyzoites Catabolize Glucose in a Complete TCA Cycle**

(A) Infected HFFs or egressed tachyzoites (ET) were suspended in medium containing either $^{13}C$-U-glucose or $^{13}C$-U-glutamine for 4 hr. Intracellular tachyzoites (IT) were isolated from host material prior to metabolite extraction. Incorporation of $^{13}C$ into selected polar metabolites and fatty acids (derived from total lipid extracts) was quantified by GC-MS, and levels (mol percent containing one or more $^{13}C$ carbons) after correction for natural abundance are represented by heat plots.

(B) Egressed tachyzoites were incubated in full medium containing either $^{13}C$-U-glucose (upper panel) or $^{13}C$-U-glutamine (lower panel) in place of naturally labeled glucose or glutamine, respectively. Culture medium was collected at 6, 12, and 24 hr and analyzed by $^{13}C$-NMR. Rates of utilization of each carbon source are shown in black, while rate of secretion of lactate (Lac), $CO_2$ (detected as $H^{13}CO_3$), glutamate (Glu), alanine (Ala), aspartate (Asp), and succinate (Suc) are shown in gray. See also Figure S2.

major amino acid in both stages, and levels increased dramatically (20-fold) following tachyzoite egress (0.3 ± 0.06 nmol to 7.3 ± 0.7 nmol/$10^8$ cell equivalents) (Figures 1A and 1B). GABA levels in uninfected host cells and in the culture medium were >100-fold lower than in the parasite extracts (data not shown). Collectively, these metabolite profiling studies indicate that both tachyzoite stages have an active mitochondrial metabolism that includes a partial or complete TCA cycle and a putative GABA shunt.

### Intracellular and Extracellular Tachyzoites Catabolize Glucose in a Complete TCA Cycle

To further define the metabolic capacity of *T. gondii* tachyzoites, infected human foreskin fibroblasts (HFFs) and egressed tachyzoites were metabolically labeled with $^{13}C$-U-glucose. $^{13}C$-U-glucose is internalized by infected HFFs and rapidly assimilated by intracellular tachyzoites, supporting the notion that these stages have direct access to host metabolites (Ramakrishnan et al., 2012). Label was incorporated into a range of glycolytic and pentose phosphate pathway intermediates, as well as the major long-chain fatty acids of cellular phospholipids (Figure 2A). We have recently shown that the labeling of fatty acid with $^{13}C$-glucose reflects the activity of the apicoplast FASII fatty acid synthase that utilizes acetyl-CoA derived from glycolytic intermediates (Ramakrishnan et al., 2012). Label derived from $^{13}C$-glucose was also incorporated into glycolytic and pentose phosphate pathway intermediates in egressed tachyzoites (Figure 2A). However, in contrast to the situation in intracellular tachyzoites, negligible labeling of fatty acids was observed in egressed tachyzoites (Figure 2A), indicating that de novo fatty acid synthesis is repressed in this nonproliferating stage.

Unexpectedly, label derived from $^{13}C$-U-glucose was efficiently incorporated into TCA cycle intermediates and interconnected amino acids, such as glutamate and aspartate, in both developmental stages (Figure 2A). The major mass isotopomers of citrate in $^{13}C$-glucose-fed tachyzoites contained two, four, or six labeled carbons, indicating the operation of a canonical TCA cycle (Figure 3). Similarly, the presence of +1 and +3 isotopomers could arise through the various decarboxylation reactions in the TCA cycle (consistent with production of $H^{13}CO_3$ [Figure 2B]) or via the direct conversion of $^{13}C_3$-pyruvate/$^{13}C_3$-phosphoenolpyruvate (PEP) to $^{13}C_3$-oxaloacetate by the anaplerotic enzymes, pyruvate carboxylase or PEP carboxykinase. To distinguish between these possibilities, tachyzoites were labeled with $^{13}C$-U-glucose in the presence of sodium fluoroacetate (NaFAc), a potent inhibitor of the TCA cycle enzyme aconitase (Saunders et al., 2011). Treatment of tachyzoites with NaFAc resulted in a 160-fold increase in citrate and aconitate levels, consistent with effective inhibition of the aconitase reaction (Figure S3). The accumulated citrate was almost exclusively labeled with +2 labeled carbons, while labeling of other TCA cycle intermediates was greatly reduced (Figure 3). GABA was similarly labeled to other TCA cycle intermediates (Figure 3), suggesting that the carbon backbone of this amino acid is derived in part from TCA cycle α-ketoglutarate and de novo synthesized glutamate. Consistent with this conclusion, the labeling of GABA in $^{13}C$-glucose-fed parasites was completely inhibited by NaFAc (Figure 3). Together, these findings demonstrate that glycolytic end products can be fully catabolized in a canonical TCA cycle in *T. gondii* tachyzoites and that this cycle can also be used to generate amino acids such as aspartate, glutamate, and GABA.

### Intracellular and Egressed Tachyzoites Coutilize Glutamine in Addition to Glucose

Egressed tachyzoites can switch to using glutamine as a carbon source when glucose uptake is inhibited (Blume et al., 2009). To
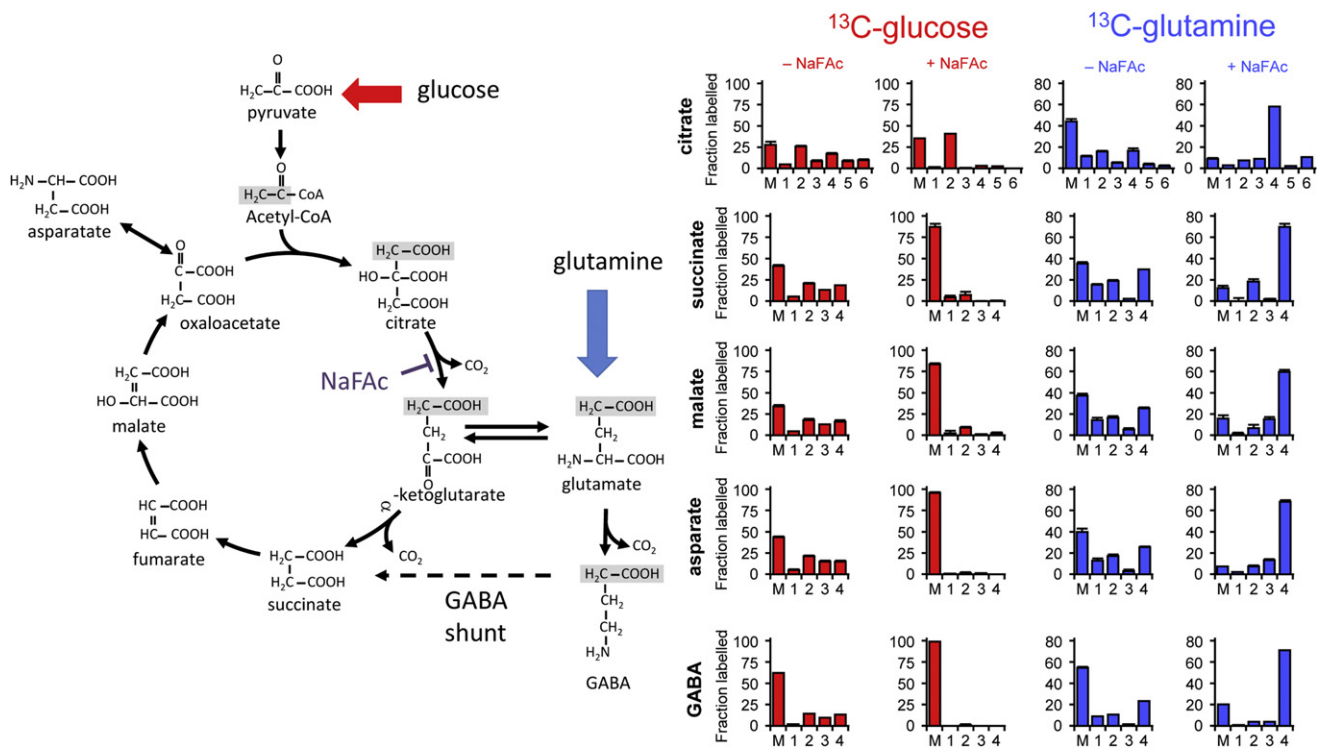
**Figure 3. Complete TCA Cycle and GABA Shunt in *T. gondii* Tachyzoites**

(Left panel) The diagram represents a reconstruction of a complete TCA cycle and GABA shunt in *T. gondii* tachyzoites inferred from isotopomer analysis. Acetyl-CoA generated from $^{13}$C-glucose is used to synthesize citrate. Gray boxes indicate the fate of carbons in the incoming acetyl group in early intermediates of the TCA cycle. Uniformly labeled citrate is generated through multiple rounds through the cycle. Inputs of 5-carbon and 4-carbon skeletons from glutamate or GABA comprise the major anaplerotic influxes. NaFAc leads to inhibition of the TCA enzyme, aconitase. (Right panel) Abundance of different TCA cycle isotopomers after labeling of egressed tachyzoites with $^{13}$C-U-glucose (red graphs) or $^{13}$C-glutamine (blue graphs) for 4 hr. Parasites were treated with or without NaFAc at the initiation of labeling. The numbers on the x axis indicate the number of labeled carbons in each metabolite. The y axis indicates the fractional abundance of each mass isotopomer. Data are represented as mean ± SEM, where n = 5. See also Figure S3.

investigate whether glutamine is used constitutively under normal growth conditions, infected HFFs and egressed tachyzoites were cultivated in glucose-replete medium containing $^{13}$C-U-glutamine. All of the TCA cycle intermediates were strongly labeled with generation of +2 and +4 isotopomers (Figure 3). Addition of NaFAc resulted in loss of +2 isotopomers and enrichment of +4 isotopomers in all labeled intermediates, consistent with entry at α-ketoglutarate and a block prior to citrate synthesis (Figure 3). GABA exhibited the same isotopomer labeling pattern as other TCA cycle intermediates, indicating that this metabolite can be derived from exogenous glutamine as well as de novo-synthesized glutamate, and the potential operation of a GABA shunt (Figure 3A). $^{13}$C-glutamine-fed tachyzoites also exhibited low but significant levels of labeling in lactate and alanine, reflecting the decarboxylation of oxaloacetic acid or malate to pyruvate (Figure 2A).

To further investigate the carbon source preference of egressed tachyzoites, these parasites were also labeled with $^{13}$C-acetate, $^{13}$C-leucine, or $^{13}$C-glycerol. With the exception of $^{13}$C-acetate (maximum labeling of citrate, 10%), negligible labeling of parasite metabolites was detected (Figure S2). These data suggest that egressed tachyzoites primarily utilize glucose and glutamine under nutrient-replete conditions. They also provide additional evidence that the acetyl-CoA used in the

TCA cycle is not derived from the single, cytoplasmically located acetyl-CoA synthetase (Mazumdar and Striepen, 2007).

## The TCA Cycle Is Required for Intracellular Growth of *T. gondii* Tachyzoites

Quantitative $^{13}$C-NMR analysis of the culture supernatant of $^{13}$C-U-glucose-fed egressed tachyzoites revealed that nearly all (95%) of the internalized $^{13}$C-glucose was catabolized to lactate and $H^{13}CO_3$ (a proxy for $CO_2$) or secreted as succinate, glutamate, alanine, and aspartate (Figure 2B), indicating minimal incorporation of glucose into biomass in these nondividing stages. $^{13}$C-glutamine uptake was ∼25% the rate of glucose uptake (Figure 2B) and was predominantly catabolized to $H^{13}CO_3$ and glutamate (Figure 2B). Assuming that the bulk of the $H^{13}CO_3$ production is associated with the TCA cycle and that the yield of ATP from glycolysis and oxidative phosphorylation is 2 and 36 mole/mole of glucose catabolized, respectively, these data suggest that egressed tachyzoites derive the bulk (>80%) of their ATP energy needs from the mitochondrial catabolism of glucose and glutamine.

To assess whether the TCA cycle is also important in intracellular stages, HFFs were infected with freshly egressed tachyzoites in the presence of NaFAc. HFFs have a highly glycolytic metabolism (Lemons et al., 2010), and NaFAc has no effect on
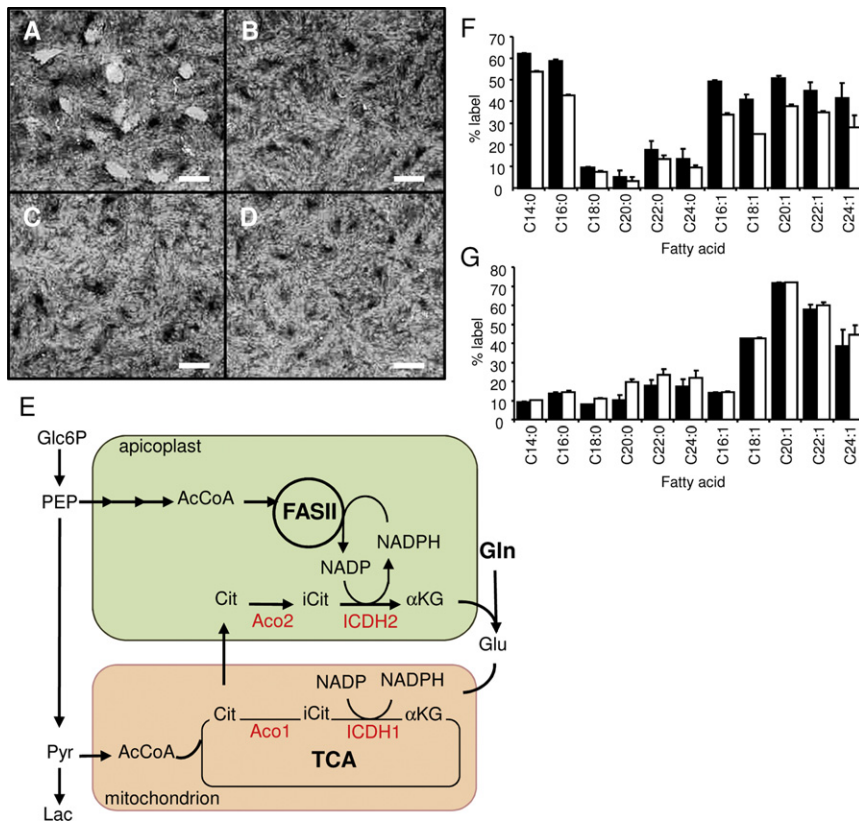
**Figure 4. *T. gondii* Tachyzoites Require a Functional TCA Cycle for Intracellular Growth**

(A–D) HFFs were infected with freshly egressed tachyzoites ($10^3$) in the presence or absence of 0.2 mM NaFAc. (A) Infection in the absence of NaFAc. (B and C) Tachyzoites were pretreated with NaFAc 4 hr prior to infection and infected cultures maintained in medium containing NaFAc. In (C), the medium was supplemented with an additional 13 mM glutamine. (D) NaFAc was added 2 hr after initiation of infection. The appearance of plaques was monitored by crystal violet staining after 6 days. Scale bars represent 15 mm.

(E) Schematic reconstruction of the proposed metabolic pathways and compartmentalization of glycolysis, the TCA cycle, and FASII fatty acid biosynthesis.

(F and G) (F) $^{13}$C-glucose and (G) $^{13}$C-acetate incorporation into tachyzoite fatty acids. Nomenclature Cx:y is shown where x is the number of carbons and y is the number of double bonds in the fatty acid chain. Error bars indicate standard deviation, where n = 6. Abbreviations are as follows: AcCoA, acetyl-CoA; αKG, α-ketoglutarate; Cit, citrate; G6P, glucose 6-phosphate; Glu, glutamate; Gln, glutamine; GABA, γ-aminobutyric acid; iCit, isocitrate; Lac, lactate; OAA, oxaloacetic acid; PEP, phosphoenolpyruvate; Pyr, pyruvate; Aco, aconitase; ICDH, isocitrate dehydrogenase. See also Figure S4.

HFF growth or their susceptibility to parasite infection (Figure S4). However, NaFAc completely inhibited plaque formation when added prior to infection or 2 hr after infection (Figure 4C), suggesting that a complete TCA cycle activity is required for parasite growth in these host cells. Significantly, growth inhibition was not reversed by addition of 13 mM glutamine (Figure 4D), suggesting that a defect in aconitase activity cannot be rescued solely by the supply of downstream TCA cycle metabolites.

**Inhibition of the TCA Cycle Results in Partial Inhibition of De Novo Fatty Acid Biosynthesis in the Apicoplast**

*T. gondii* express apicoplast-targeted isoforms of aconitase and an NADP$^+$-dependent isocitrate dehydrogenase (Pino et al., 2007). These enzymes could in principle convert mitochondrial-derived citrate to α-ketoglutarate, providing this organelle with a source of NADPH for FASII-fatty acid biosynthesis (Figure 4E). To investigate whether disruption of citrate synthesis impacts on apicoplast FASII synthesis, intracellular tachyzoites were labeled with $^{13}$C-glucose in the presence or absence of NaFAc. Addition of NaFAc resulted in a reproducible inhibition of FASII-dependent fatty acid biosynthesis (∼20%), as measured by incorporation of $^{13}$C-U-glucose into long-chain fatty acids (Ramakrishnan et al., 2012) (Figure 4F). Inhibition of the FASII pathway was not due to a global deficiency in ATP synthesis or accumulation of inhibitory fluoroacetate-CoA, as NaFAc treatment did not significantly affect the incorporation of $^{13}$C-acetate into total fatty acids (Figure 4G). The latter primarily reflects fatty acid biosynthesis by the ER-located fatty acid elongase system (Ramakrishnan et al., 2012). These data

support the presence of a shunt in which mitochondrial citrate is transported to the apicoplast and converted to α-ketoglutarate with regeneration of NADPH (Figure 4E). The incomplete inhibition of FASII-fatty acid biosynthesis in the presence of NaFAc likely reflects the presence of other mechanisms for regenerating NADPH in the apicoplast (Brooks et al., 2010).

**Identification of Enzymes Involved in GABA Synthesis**

The *T. gondii* and *P. falciparum* genomes encode a putative lysine decarboxylase (TGME49_080700 and PFD0285c, respectively; Table S1) which shares conserved domains with members of the bacterial amino acid decarboxylase superfamily (Cook et al., 2007) that include glutamate decarboxylase (GAD, EC 4.1.1.15), the first enzyme in the GABA shunt. However, cadaverine, the product of lysine decarboxylation, was not detectable in our metabolomic analyses, raising the possibility that these genes encode the glutamate decarboxylase. To functionally characterize the putative *Tggad* gene, a null mutant was generated in the RHΔKu80 parasite line (Huynh and Carruthers, 2009) (Figure S5). A *Tggad* deletion clone (Δ*gad*) was identified by PCR (Figure S5) and successful ablation of the gene verified by Southern blot analysis (Figure S5). The Δ*gad* tachyzoites lacked detectable levels of GABA and contained highly elevated levels of glutamate (Figure 5A), along with significant changes in abundance of a number of other metabolites involved in central carbon metabolism (Figure S6). Cell lysates of Δ*gad* also lacked GAD activity, as measured by incubation of parasite cell lysates with $^{13}$C-glutamate and analysis of the products by GC-MS (Figure 5B). Intriguingly, GAD activity in wild-type parasites
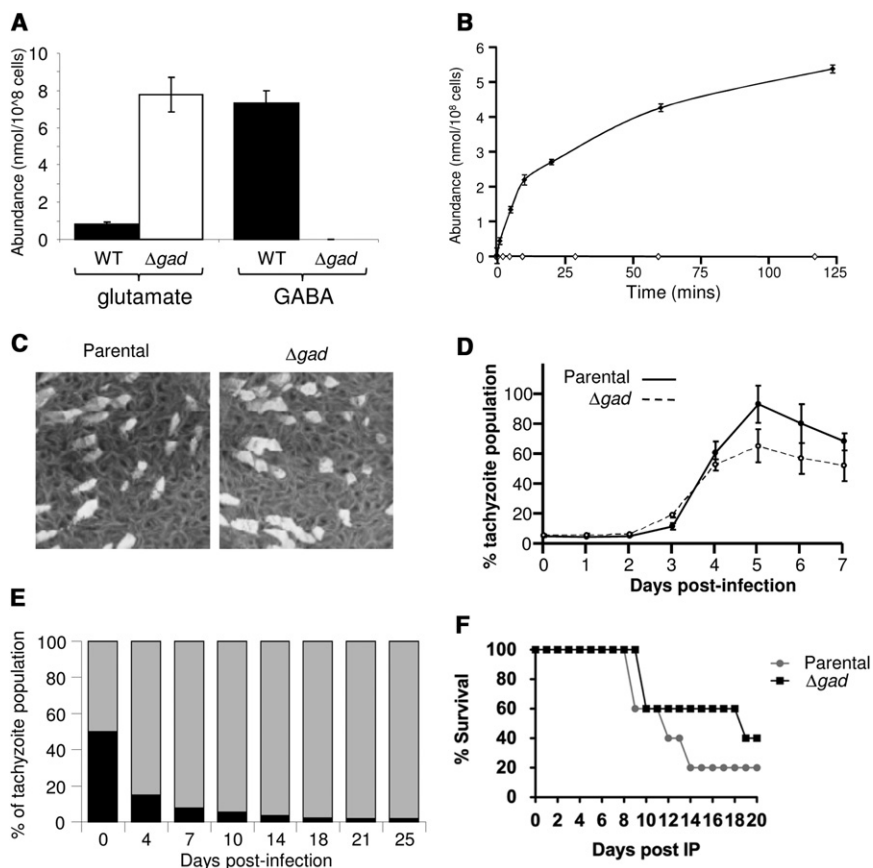
**Figure 5. Loss of GAD Results in Complete Ablation of GABA Biosynthesis and Partial Attenuation of Parasite Propagation**

(A) Intracellular GABA and glutamate levels in parental and Δ*gad* tachyzoites. Error bars represent standard deviation where n = 4.

(B) Cell-free extracts of *T. gondii* parental (filled circles) and Δ*gad* tachyzoites (open circles) were incubated with 10 mM $^{13}$C-U-glutamate in the presence of 1 mM ATP. The synthesis of GABA at indicated time points was measured by GC-MS. Error bars represent standard deviation, where n = 3.

(C) Parental and Δ*gad* parasite lines are both capable of generating plaques in HFF monolayers.

(D) Fluorescence growth assays showing similar growth rates (no significant difference) for parental (solid line, closed circles) and Δ*gad* (dotted line, open squares) strains. Each data point represents the mean of six wells, and the error bars indicate standard deviation where n = 6.

(E) Equal numbers of wild-type parasites (gray bar) expressing the dTomato fluorescent protein and Δ*gad* mutant tachyzoites (black bar) were used to infect HFF. The recovery of fluorescent and nonfluorescent parasites was determined by FACS analysis of 2 × 10$^6$ cells at indicated days. The Δ*gad* parasite line was rapidly outcompeted by the WT line.

(F) Swiss Webster mice were infected with type 1 parental and Δ*gad* tachyzoites (ten parasites/mouse) and mouse survival observed over 20 days. See also Figure S5.

was only observed when lysates were supplemented with ATP (Figure S6). In the absence of ATP, the major product synthesized was α-ketoglutarate. No GABA production was observed in cell lysates prepared from the Δ*gad* knockout line (Figures 5A and 5B). These studies confirm that TGME49_080700 encodes the only functional *Tg*GAD and that this enzyme is directly or indirectly activated by ATP.

The *T. gondii* genomes also contain putative genes for other enzymes in the GABA shunt. These include the glutamate transamidase (GDC, EC 2.6.1.19), a succinic-semialdehyde dehydrogenase (SSDH, EC 1.2.1.16) that was localized to the mitochondrion (Figure S5), and a GABA transporter (Table S1). Homologs for most of these genes were also identified in the genomes of *Plasmodium falciparum* and *Neospora caninum* (Table S1), consistent with the operation of a similar pathway in these parasites.

### The GABA Shunt Impacts on Tachyzoite Fitness In Vivo and In Vitro but Is Not Essential

Both the parental and Δ*gad* mutant lines induced similar-sized plaques in HFF monolayers when assayed independently (Figures 5C and 5D). However, the Δ*gad* mutant displayed a clear attenuation of growth in a competition infection experiment with its parental strain (Figure 5E). This selective advantage was also evident in infections in vivo, where BALB/c mice infected with wild-type succumb to infection faster than those infected with the mutant (Figure 5F). The *Tggad* gene was also deleted in the less virulent type II Prugniaud strain (Fox et al., 2009).

The Pru∆Ku80∆*gad* mutant lacked the capacity to synthesize GABA but was able to form plaques in complete growth medium in vitro (Figure S5) and able to differentiate to bradyzoites in an in vitro assay (Fux et al., 2007) (Figure S5). As with RH∆*gad*, this mutant exhibited a modest reduction in virulence in mice compared to the parental strain based on survival, time to death, or weight loss (Figure S5). These data suggest that the GABA shunt increases parasite fitness but that this pathway is not essential for survival.

### The GABA Shunt Is Required to Maintain Tachyzoite Motility under Nutrient-Limited Conditions

*T. gondii* tachyzoites are able to sustain normal motility and virulence for at least 1 hr after egress in the absence of exogenous carbon sources, suggesting that they may have a short-term energy reserve (Lin et al., 2011a). To investigate whether GABA is catabolized under nutrient-limiting conditions, egressed tachyzoites were suspended in PBS with or without exogenous carbon sources (Figure 6A). Intracellular GABA levels were maintained at very high levels when suspended in PBS containing exogenous glutamine. In contrast, GABA levels were rapidly depleted when tachyzoites were suspended in PBS or PBS containing glucose (Figure 6A). GABA levels were further depleted by the addition of 2-deoxyglucose, an inhibitor of glycolysis (Figure 6A). These results suggest that the large intracellular pool of GABA in egressed tachyzoites is utilized when either carbon or amino acid sources are limiting.
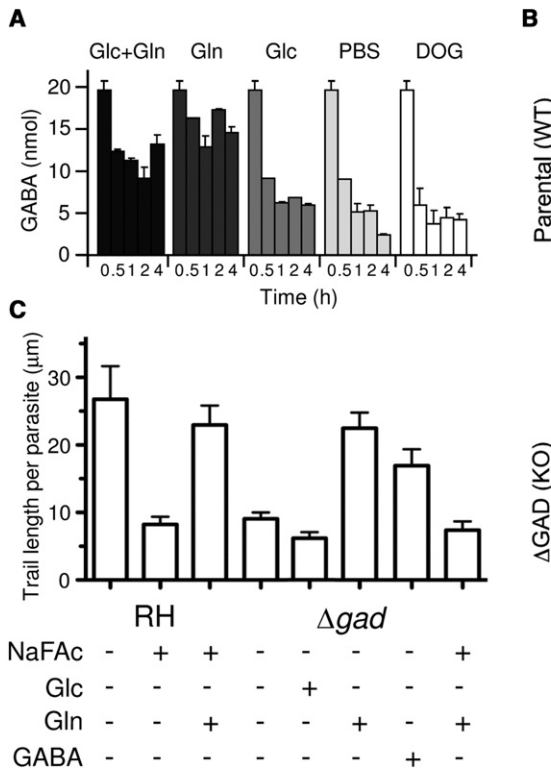
**Figure 6. GABA Can Be Used to Sustain Tachyzoite Motility under Nutrient-Limited Conditions**

(A) Wild-type (RH) tachyzoites were suspended in medium containing glucose and/or glutamine as carbon source, PBS, or PBS containing 2-deoxy-glucose (DOG). Intracellular GABA levels were measured at the indicated time points over 4 hr. GABA was rapidly depleted in the absence of glutamine. Error bars indicate standard deviation, where n = 2 and results are representative of four biological replicates.

(B) Parental and Δ*gad* tachyzoites were suspended in medium lacking carbon sources and allowed to glide on poly-L-lysine-coated coverslips after addition of 2 μM ionophore to stimulate motility. Parasites were fixed, and the resulting gliding trails were visualized using α-SAG1 antibodies. The Δ*gad* mutant displays a clear defect in gliding motility under these conditions.

(C) Parental and Δ*gad* tachyzoites were incubated in HBSS-HEPES with or without NaFAc, and different carbon sources, as indicated. The average trail length/parasite was documented in ten fields (>100 parasites). Counts are of representative frames from two independent experiments. Data are represented as mean ± SEM. See also Figure S6.

Tachyzoites are dependent on gliding motility to reach and invade new host cells. To determine whether the TCA cycle and GABA shunt are required for gliding motility, egressed tachyzoites of the wild-type and Δ*gad* strains were suspended in PBS containing NaFAc or different carbon sources and motility monitored by fluorescence microscopy. Consistent with previous reports, wild-type parasites retained active gliding motility in the absence of exogenous carbon sources for several hours (Figures 6B and 6C) (Lin et al., 2011a). Parasite motility was completely blocked by NaFAc treatment, although motility in the presence of NaFAc was largely restored by addition of glutamine (Figure 6C), suggesting that operation of a partial TCA cycle is sufficient to sustain the energy needs of parasite gliding. Strikingly, the Δ*gad* mutant had a comparable defect in gliding motility to that of wild-type parasites treated with NaFAc (Figure 6C). The motility of the Δ*gad* mutant was largely restored by addition of glutamine or GABA, although in contrast to the situation in wild-type parasites, addition of exogenous glutamine did not restore motility when the Δ*gad* mutant was treated with NaFAc (Figure 6C). Collectively, these data suggest that the GABA shunt plays a key role in regulating the catabolism of glutamine/glutamate in the TCA cycle and that this pathway cannot be bypassed by the direct conversion of glutamate to α-ketoglutarate under the conditions tested.

## DISCUSSION

Metabolite profiling and stable isotope labeling have been used to identify pathways of carbon metabolism in intracellular and egressed *T. gondii* tachyzoite stages. We show that these parasite stages constitutively utilize both glucose and glutamine as major carbon sources and that the energy metabolism of these stages is dependent on mitochondrial metabolism and oxidative phosphorylation. The highly efficient and flexible energy metabolism of *T. gondii* tachyzoites may underlie the extraordinary capacity of these parasites to proliferate within a wide range of host cells.

Previous studies have suggested that intracellular tachyzoites are primarily dependent on glucose uptake and glycolysis for ATP synthesis. Free glucose is present at relatively high concentrations in the cytosol of mammalian cells (1–6 mM) (Behjousiar et al., 2012) and is likely to be passively transported across the limiting membrane of the parasitophorous vacuole. $^{13}$C-glucose internalized by infected HFF was rapidly internalized by intracellular tachyzoites and incorporated into intermediates in the glycolytic and pentose phosphate pathways. Unexpectedly, intermediates in the TCA cycle were also labeled with $^{13}$C-glucose, and contained an isotopomer fingerprint consistent with the operation of a canonical oxidative TCA cycle. Chemical inhibition of the parasite aconitase with NaFAc effectively blocked tachyzoite growth in HFF monolayers, indicating that the mitochondrial catabolism of glucose is essential for growth of intracellular tachyzoites. This phenotype is more severe than that observed following the inducible downregulation of the TCA cycle enzyme, succinyl-CoA synthetase (Fleige et al., 2008), which is now explained by the presence of the GABA shunt that bypasses the steps catalyzed by α-ketoglutarate dehydrogenase and succinyl-CoA synthetase (Figure 7). The primary function of this cyclical TCA cycle is the generation of reducing equivalents for oxidative phosphorylation, as genetic or chemical inhibition
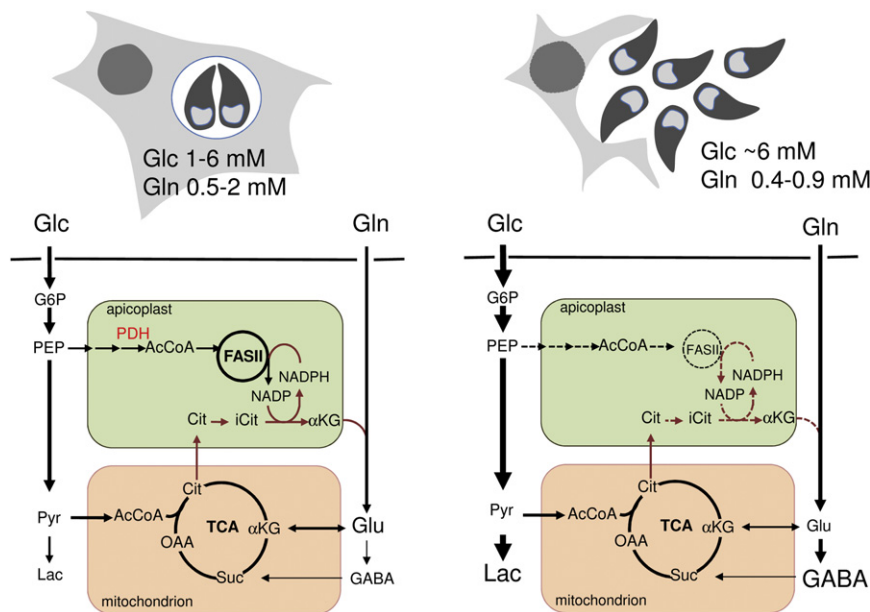
**Figure 7. Role of the TCA Cycle in Intracellular and Egressed *T. gondii* Tachyzoites**

Intracellular tachyzoites coutilize glucose and glutamine scavenged from the host, and both carbon sources are catabolized in mitochondria via a canonical oxidative TCA cycle (left panel). Glucose is also used to fuel the FASII-dependent pathway of fatty acid biosynthesis in the apicoplast. Inhibition of tachyzoite aconitase (comprising mitochondrial and apicoplast isoforms) results in a reduction of FASII biosynthesis, providing evidence that a citrate shunt between the mitochondrion and apicoplast (brown lines) is at least partially required for regeneration of reducing equivalents for apicoplast fatty acid synthesis. Tachyzoites are likely exposed to elevated glucose levels following host cell egress (right panel). However, these stages continue to coutilize glutamine and are dependent on operation of the TCA cycle for most of their ATP synthesis and normal gliding motility. The GABA shunt identified in this study involves at least three enzymes and a putative mitochondrial transporter (data not shown). GABA accumulated under glutamine-replete conditions may function as a short-term energy reserve under nutrient limiting conditions. For abbreviations, see Figure 3.

of the *T. gondii* mitochondrial respiratory chain, including the unusual Type II NADH dehydrogenases, inhibits intracellular growth (Lin et al., 2009, 2011b). However, as shown here and proposed elsewhere, the TCA cycle may also generate anabolic precursors for fatty acid and heme biosynthesis (Mazumdar et al., 2006; Ramakrishnan et al., 2012).

The mechanism by which pyruvate is converted to acetyl-CoA remains to be established, as *T. gondii* and other apicomplexan parasites lack a mitochondrial PDH complex (Seeber et al., 2008; Polonais and Soldati-Favre, 2010; Danne et al., 2012). In principle, acetyl-CoA generated by the apicoplast PDH could be transported to the mitochondrion. However, organellar membranes are impermeable to acetyl-CoA, and interorganellar transport typically involves a carnitine/acetyl-carnitine shuttle, for which there is no precedent in the case of plastid membranes. Moreover, exogenous ^{13}C-acetate was only minimally incorporated into TCA cycle intermediates, indicating that acetyl-CoA generated by the single cytoplasmic acetyl-CoA synthetase (Mazumdar and Striepen, 2007) is not a major source of mitochondrial acetyl-CoA. Alternatively, pyruvate could be converted to acetyl-CoA by the mitochondrial-localized branched chain α-keto acid dehydrogenase (BCKDH) complex (Polonais and Soldati-Favre, 2010), which in some cases can utilize pyruvate as substrate (Heath et al., 2007; Pettit et al., 1978). In support of this hypothesis, all members of the Apicomplexa retain the subunits of the BCKDH complex, even when other enzymes required for catabolism of branched chain amino acids have been lost (Polonais and Soldati-Favre, 2010). We also found little evidence for catabolism of branched chain amino acids in *T. gondii*, further supporting the possibility that the BCKDH is primarily used for mitochondrial pyruvate catabolism. The establishment of an alternative mechanism for coupling glycolysis with TCA cycle appears to have evolved prior to the divergence of apicomplexa from related protist

groups and the evolution of obligate parasitic lifestyle (Danne et al., 2012).

The finding that intracellular tachyzoites coutilize glutamine under glucose-replete conditions further highlighted the metabolic flexibility of these parasites and the potential importance of mitochondrial metabolism. Glutamine is present at high concentrations (0.5–2 mM) in the cytosol of mammalian cells (Behjousiar et al., 2012), and the uptake and catabolism of this amino acid by intracellular tachyzoites appears to be sufficient to sustain oxidative phosphorylation in the absence of glucose uptake (Blume et al., 2009). Glutaminolysis could also be required to top up TCA cycle intermediates exported from the mitochondrion for anabolic processes. In particular, we provide evidence for a citrate shunt involving apicoplast-located isoforms of aconitase and the NADP-dependent isocitrate dehydrogenase (Denton et al., 1996; Pino et al., 2007). This shunt could regenerate apicoplast pools of NADPH that are required for FASII-dependent fatty acid biosynthesis in intracellular tachyzoites. However, other mechanisms for regenerating NADPH in the apicoplast must also exist, as inhibition of citrate synthesis only partially blocked fatty acid synthesis (Fast et al., 2001).

Anabolic pathways, such as fatty acid biosynthesis, are repressed in egressed tachyzoites, and most of the glucose and glutamine taken up by this stage is used to generate ATP for gliding motility and host cell invasion. ^{13}C-NMR analysis of the secreted end products of egressed tachyzoites indicated that glucose is primarily catabolized to lactate, while ~20% is oxidized in the TCA cycle. Given the relative yield of ATP from glycolysis and oxidative phosphorylation, these data suggest that oxidative phosphorylation could account for >90% of ATP synthesis in egressed tachyzoites. Consistent with this conclusion, chemical inhibition of aconitase resulted in a marked decrease in gliding motility, which was largely restored by

addition of exogenous glutamine. While previous studies have highlighted the role of glycolysis in supplying ATP for motility (Lin et al., 2011a; Pomel et al., 2008), disruption or inhibition of glycolytic enzymes would also lead to reduced TCA cycle flux. Thus, TCA cycle fluxes appear to be important for both intracellular and egressed tachyzoites.

The identification of a functional GABA shunt in *T. gondii* tachyzoites highlighted the utility of metabolomic approaches for discovering parasite metabolic pathways that are not anticipated from genome annotations. Genes encoding a putative glutamate decarboxylase, a glutamate deaminase, a succinic semialdehyde dehydrogenase, and a GABA transporter were subsequently identified in the *T. gondii* genomes. Bioinformatic analyses indicate that this shunt is present in some other apicomplexa, including *P. falciparum* (Figure S5, Table S1), consistent with the reported presence of GABA in asexual red blood cell stages (Teng et al., 2009). *T. gondii* mutant strains lacking GAD exhibited reduced fitness both in vitro, in competition with the parental strain, and in vivo, in murine infections. The relatively modest growth phenotype of the Δ*gad* mutant is reminiscent of the mild growth phenotype of *T. gondii* glucose transporter mutants (Blume et al., 2009) and underlies the remarkable flexibility of these parasites to adapt to altered fluxes in central carbon metabolism. This robust flexibility would allow tachyzoites to adapt to major fluctuations in nutrient supply (during, for example, tachyzoite egress) without the need for transcriptional adjustment. A central role of allosteric or metabolic regulation of TCA cycle fluxes was suggested by the finding that GAD activity was strongly activated when cell lysates were charged with ATP. The conversion of glutamate to GABA under ATP-replete conditions may be required to prevent overproduction of NAD(P)H and leakage of reactive oxygen species from the mitochondrial respiratory chain (Wellen and Thompson, 2010; Murphy, 2009). GABA accumulated under these conditions could subsequently be used as a short-term energy reserve material. The presence of such an internal reserve has been posited based on the ability of egressed tachyzoites to retain full motility, ATP levels, and invasiveness for at least 1 hr in the absence of any exogenous carbon sources (Lin et al., 2011a). We show that the accumulated GABA is utilized under these conditions and that the Δ*gad* mutant has a severe motility defect in the absence of carbon sources. Interestingly, glutamine did not restore the motility of the Δ*gad* mutant in the presence of NaFAc, suggesting that an active GABA shunt may be required to maintain a high flux through a partial TCA cycle. The GABA shunt may thus have an important role in regulating carbon metabolism under both nutrient-replete and starvation conditions.

Finally, the finding that nonproliferating tachyzoites stages can both synthesize GABA and utilize exogenous GABA is of interest given the strong tropism that these parasites display for the central nervous system during chronic infections. While our current studies suggest that GABA is not actively secreted, it is possible that release of GABA from dead or slow-growing bradyzoites stages could underlie the changes in behavior, mood, and mental health of *T. gondii*-infected humans and mice (Webster, 2007; Lamberton et al., 2008; Webster and McConkey, 2010).

## EXPERIMENTAL PROCEDURES

### Parasite Culture and Construction of Mutants

*T. gondii* RH (wild-type), RHΔKu80 and RHΔKu80Δ*gad*, PruΔKu80, and PruΔKu80Δ*gad* tachyzoites were maintained by passage through HFFs or in hTERT-BJ1 (Clontech) cells in Dulbecco's modified Eagle's medium supplemented with 5% (for type I) and 10% (type II) fetal bovine serum (FBS, Invitrogen) at 37°C with 5% CO$_2$ (Mazumdar et al., 2006). Parasite cloning and plaque assays were performed in HFFs. Mutant lines lacking the TGME49_080700 (GAD) locus, and lines stably expressing RFP, were generated as described in the Supplemental Experimental Procedures. Bradyzoite differentiation was performed by growth in alkaline pH as previously described (Fux et al., 2007). The phylogenetic analysis of GABA genes is described in the Supplemental Experimental Procedures.

### Metabolite Extraction of *T. gondii* Tachyzoites

Confluent HFF cultures in 175 cm$^2$ flasks were infected with freshly egressed tachyzoites and harvested when >90% of the HFF contained >64 tachyzoites. Host cell and parasite metabolism was quenched by placing culture flasks on ice and the overlying medium removed by aspiration. Host cells were washed twice with ice-cold PBS (pH 7.4), scraped into 5 ml ice-cold PBS, and lyzed by passage through a syringe needle. Released tachyzoites were pelleted by centrifugation (4,000 rpm, 25 min, 0°C) and washed in ice-cold PBS prior to extraction. Extracellular tachyzoites were obtained after 60%–80% of infected host cells had lyzed. Egressed tachyzoites were transferred to a 50 mL centrifuge tube and metabolically quenched by immersion of the tube in a dry ice/ethanol bath (Saunders et al., 2011). Parasite pellets were washed three times with ice-cold PBS prior to metabolite extraction.

Intracellular tachyzoites and egressed tachyzoites (2 × 10$^8$ cell equivalents) were extracted in chloroform/methanol/water (1:3:1 v/v containing 1 nmol *scyllo*-inositol as internal standard) for 20 min at 60°C. Polar and apolar metabolites were separated by phase partitioning and polar metabolites derivitized by methoximation and trimethylsilylation (TMS) and analyzed by GC-MS as previously described (Saunders et al., 2011). Apolar metabolites were derivitized by MethPrep II (Grace) and analyzed by GC-MS (Supplemental Experimental Procedures). Culture supernatants (540 mL) from $^{13}$C-glucose/$^{13}$C-glutamine-fed egressed tachyzoites were diluted with 70 μl D$_2$O containing D$_6$-DSS (5 mM), $^{13}$C-U-glycerol (2.1 mM), imidazole (2.1 mM), and NaN$_3$ (0.2%) and $^{13}$C-spectra collected at 200 MHz using an 800 MHz Bruker-Biospin Avance fitted with a cryoprobe (Saunders et al., 2011) (Supplemental Experimental Procedures). Data were processed with Bruker TOPSPIN 2.0 and spectra assigned by reference to authentic standards (Supplemental Experimental Procedures).

### Stable Isotope Labeling of *T. gondii* Tachyzoites

For polar metabolites, infected HFF or freshly egressed tachyzoites were resuspended in DMEM in which the unlabeled glucose or glutamine was replaced with 8 mM $^{13}$C-U-glucose or $^{13}$C-U-glutamine (Ramakrishnan et al., 2012). Parasites were harvested after 4 hr and metabolites extracted as above. The aconitase inhibitor, sodium fluoroacetate (NaFAc), was added at 2 mM at the same time as the $^{13}$C-glucose/$^{13}$C-glutamine. Changes in the mass isotopomers of key intermediates in central carbon metabolism were assessed by GC-MS analysis (Supplemental Experimental Procedures). The level of labeling of individual metabolites was estimated as the percentage of the metabolite pool containing one or more $^{13}$C atoms after correction for natural abundance.

### GAD In Vitro Enzyme Assay

Metabolically quenched extracellular tachyzoites were prepared as described above and lyzed in hypotonic buffer (double-distilled water containing 4% EDTA-free protease inhibitors (Roche) with regular sonication (0°C, 10 min), followed by a freeze-thaw in liquid nitrogen. Cell lysates (50 mL, 5 × 10$^7$ cells equivalents) were incubated with 50 ml assay buffer (20 mM pyridoxine/0.4% [v/v] β-mercaptoethanol ± 2 mM ATP in PBS [pH 6.8]) containing 10 mM $^{13}$C-glutamate at 37°C. The reaction was quenched at indicated times by boiling for 5 min, and $^{13}$C-labeled polar metabolites were recovered and detected by GC-MS.

### Lytic and Plaque Viability Assays

Freshly egressed tachyzoites ($10^3$) were pretreated with or without 2 mM NaFAc for 4 hr before being used to infect host HFF. After 6 days, HFFs were stained with crystal violet (Sigma) and cell growth assessed by presence of intact HFFs. In variations of the same experiment, NaFAc was added 2 hr postinfection. As a control, HFFs were treated with 2 mM NaFAc for 6 days prior to infection. The growth of parental and Δ*gad* lines (Huynh and Carruthers, 2009), expressing a tandem red fluorescent protein (RFP-RFP) transgene, was measured using a real-time fluorescence assay (Sheiner et al., 2011). For competition experiments, parental parasites expressing the RFP-RFP transgene were mixed with an equal number of nonfluorescent Δ*gad* parasites and used to infect HFF. The number of fluorescent and nonfluorescent parasites was scored by fluorescence-activated cell counting every time the cultures lysed.

### Gliding Assay

Freshly egressed tachyzoites were 3.0 μm filter purified and resuspended in Hank's balanced salt solution supplemented with 20 mM HEPES (HBSS-H). Parasites were layered on poly-L-lysine-coated coverslips, and parasites were allowed to adhere for 15 min at RT. The coverslips were overlaid with HBSS-H containing 2 mM ionomycin, a strong inducer of parasite motility, and incubated for 15 min at 37°C. Parasites were fixed with 4% paraformaldehyde/0.005% glutaraldehyde (15 min, RT) and trails visualized by immunofluorescence using the α-SAG1 antibody (Dubremetz et al., 1985). For gliding experiments using different carbon sources, HBSS-H was supplemented with the corresponding compound (0.2 mM glutamine, 4.5 mg/ml glucose, 2 mM GABA, 0.2 mM NaFAc), and washes, adherence, and induction were performed in the resulting medium.

### Nutrient Stress Analysis

Egressed tachyzoites were preincubated in PBS containing glucose (6 mM), glutamine (6 mM), and 0.1% fatty acid free BSA (37°C, 2 hr) prior to being resuspended in PBS with or without glucose (6 mM), glutamine (6 mM), or 2-deoxyglucose (6 mM). Parasites were sampled at the indicated time points and polar metabolites levels determined by GC-MS.

### ACCESSION NUMBERS

The GeneDB accession numbers for the *T. gondii gad* and *SSDH* sequences reported in this paper are TGME49_080700 and TGME49_057480, respectively. The GeneDB accession number for the *P. falciparum gad* sequence reported in this paper is PF3D7_0405700.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables, six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at http://dx.doi.org/10.1016/j.chom.2012.09.013.

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