A plant/fungal-type phosphoenolpyruvate carboxykinase located in the parasite mitochondrion ensures glucose-independent survival of Toxoplasma gondii

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ABSTRACT

Toxoplasma gondii is considered as one of the most successful intracellular pathogens, because it can reproduce in varied nutritional milieus, encountered in diverse host-cell types of essentially any warm-blooded organism. Our earlier work has demonstrated that the acute (tachyzoite) stage of T. gondii depends on cooperativity of glucose and glutamine catabolism to meet biosynthetic demands. Either of these two nutrients can sustain the parasite survival; however, what determines the metabolic plasticity has not been resolved yet. Here, we reveal two discrete phosphoenolpyruvate carboxykinase (PEPCK) enzymes in the parasite, one of which resides in the mitochondrion (TgPEPCKmt), whereas the other protein is not expressed in tachyzoites (TgPEPCKnet). Parasites with an intact glycolysis can tolerate genetic deletions of TgPEPCKmt as well as of TgPEPCKnet, indicating their nonessential roles for the tachyzoite survival. TgPEPCKnet can also be ablated in glycolysis-deficient mutant, whereas TgPEPCKmt is refractory to deletion. In accord, the lytic cycle of a conditional mutant of TgPEPCKmt in the glycolysis-impaired strain was aborted upon induced repression of the mitochondrial isoform, demonstrating its essential role for the glucose-independent survival of parasites. Isotope-resolved metabolomics of the conditional mutant revealed defective flux of glutamine-derived carbon into RNA-bound ribose sugar as well as metabolites associated with gluconeogenesis, entailing a critical nodal role of PEPCKmt in linking catabolism of glucose and glutamine with anabolic pathways. Our data also suggest a homeostatic function of TgPEPCKmt in cohesive operation of glycolysis and TCA cycle under normal glucose-replete milieu. Conversely, we found that otherwise-integrative enzyme pyruvate carboxylase (TgPyC) is dispensable not only in glycolysis-competent but also in glycolysis-deficient tachyzoites despite a mitochondrial localization. Last but not least, the observed physiology of T. gondii tachyzoites appears to phenocopy cancer cells, which holds promise for developing common therapeutics against both threats.
INTRODUCTION

*Toxoplasma gondii* is an obligate intracellular parasite of the protozoan phylum Apicomplexa, which is capable of infecting and replicating in most vertebrate organisms including humans. The disease acute toxoplasmosis is hallmarked by tissue necrosis, which is a consequence of incessant host-cell lysis following rapid intracellular proliferation of the tachyzoite stage (1). Previous work has demonstrated that glucose and glutamine are the two major carbon sources utilized during the lytic cycle of tachyzoites (2,3,4). Glucose is imported through a high-affinity glucose transporter (GT1) located in the plasma membrane of the parasite (2). It is subsequently catabolized to pyruvate in the cytosol (5,6), a major fraction of which is converted into lactate (3). A second pool of pyruvate enters the mitochondrion and is used to produce acetyl-CoA, which condenses with oxaloacetate to produce citrate to drive the TCA cycle (7). Oxaloacetate can be generated from either glucose-derived pyruvate and/or from glutamine catabolism through the TCA cycle (3).

One of the principal metabolic phenotypes observed in fast multiplying cells is an induction of aerobic glycolysis (*i.e.* Warburg effect), which is hallmarked by increased glucose catabolism and lactate synthesis (8). Besides glucose, glutamine is another major nutrient utilized as an anaplerotic substrate for the TCA cycle in proliferating cells (9). Glycolysis and TCA cycle together not only produce energy and reducing equivalents for cellular functioning, but also deliver carbon for the biogenesis of nucleotides, lipids, amino acids and intermediates of one-carbon metabolism (8). Likewise, while glucose is an important contributor to the cellular energy and carbon pools in tachyzoites, glutamine is another central carbon source, which feeds into the TCA cycle and contributes to the bioenergetic requirements (3,4). Interestingly, tachyzoites are quite resilient to genetic and biochemical perturbations of carbon metabolism (2,4). In particular, they can survive the genetic deletion of one and only glucose transporter, GT1. The Δtggt1 mutant displays an impaired glycolysis accompanied with a compensatory increase in glutamine catabolism and activation of otherwise-negligible gluconeogenesis. The mechanism by which glycolysis-deficient tachyzoites ensure their glucose-independent survival and reproduction in human host cells remains enigmatic, however.

In mammalian tissues, pyruvate can be converted back to glucose by gluconeogenesis when glucose supply becomes limited (10). The latter route is not a simple reversal of glycolysis; it involves additional reactions catalyzed by pyruvate carboxylase (PyC), phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase (FBP) and glucose 6-phosphatase. In the first step, pyruvate is carboxylated by PyC to produce oxaloacetate that is simultaneously decarboxylated and phosphorylated by PEPCK to yield phosphoenolpyruvate (PEP) in a GTP-dependent manner (10). PEP undergoes sequential catalysis by a series of glycolytic enzymes to produce fructose 1,6-bisphosphate, which is eventually hydrolyzed to fructose 6-phosphate by FBP enzyme. At last, glucose 6-phosphatase hydrolyzes glucose 6-phosphate (G6P), formed by isomeric conversion of fructose 6-phosphate, to produce glucose. PyC and PEPCK also function as typical anaplerotic and cataplerotic enzyme, respectively, ensuring a continual operation of TCA cycle during rapid biosynthetic activities. In tumor cells, glucose-dependent PyC-mediated anaplerosis allows cells to grow...
in the absence of glutamine (11). Conversely, cytosolic and mitochondrial isoforms of PEPCK (PEPCK-C/M) are required to support the growth of cancer cells from glutamine under glucose-starved condition (12,13).

The *T. gondii* genome harbors all gluconeogenic enzymes except for glucose 6-phosphatase (14). We have already characterized two isoforms of FBP in *T. gondii*, TgFBP1 and TgFBP2, only latter of which is involved in gluconeogenesis (15). TgFBP2 has another critical function (*i.e.* futile cycling), which makes the FBP activity essential even in tachyzoites with functional glycolysis. It has thus not been possible to test the significance of glutamine-derived carbon and gluconeogenesis in parasites with a defective glycolysis. In this work, we generated a set of mutants in glycolysis-competent and glycolysis-deficient backgrounds and discovered the mitochondrial PEPCK as a key regulator of metabolic plasticity in *T. gondii*. TgPEPCK<sub>mt</sub> mediates a metabolic shunt to counterbalance glycolytic defect, which furnishes carbon from glutamine to satisfy the bioenergetic demands of tachyzoites. Our results ascertain PEPCK<sub>mt</sub>-dependent metabolic reprogramming as a major mechanism by which tachyzoites manage in low-glucose environment.

**RESULTS**

*Pyruvate carboxylase is a mitochondrial protein but it is not expressed in tachyzoites*

Pyruvate carboxylase (PyC) is a member of the biotin-dependent enzyme family. It catalyzes physiologically-irreversible carboxylation of pyruvate to yield oxaloacetate in a broad range of prokaryotes and eukaryotes (16). Our bioinformatics search using the yeast and mammalian isoforms identified one putative PyC in the genome database of *T. gondii* (TGGT1_284190; www.ToxoDB.org). We cloned the complete ORF of TgPyC, which encodes for a protein of 1391 amino acids with a conserved catalytic domain. Ectopic overexpression of C-terminally-tagged TgPyC (TgPyC-HA) in intracellular tachyzoites revealed that the protein is localized in the mitochondrion that was confirmed by immunofluorescent staining with a known organelle marker, TgF1B-ATPase (17) (Fig 1A). Subsequently, we epitope-tagged the corresponding gene to gauge its native expression (Fig 1B). Unexpectedly, neither by immunofluorescence nor by immunoblot assays were we able to detect TgPyC-HA protein in parasites (Fig 1C-D).

To corroborate the absence of TgPyC expression under normal glucose-replete condition, we utilized anti-biotin antibody staining the prosthetic group (Fig 1E). Yet again, we failed to detect immunostaining of the parasite mitochondrion. In contrast, we were able to detect another biotin-dependent enzyme acetyl CoA carboxylase (18) that localized with ferredoxin, a marker of the apicoplast in tachyzoites (19). Next, we tested the endogenous expression of TgPyC in the Δtggt1 strain to investigate whether PyC protein is produced under glycolysis-deficient environment (Fig 1E). Contrary to our notion, we did not see a detectable induction of TgPyC in the Δtggt1 strain. Taken together, several conclusions can be drawn from these unforeseen datasets. First, PyC is a mitochondrial protein, whose gene is transcribed but the transcript is not translated, which indicates its post-transcriptional regulation in parasites. An apparent lack of PyC expression implies its negligible contribution in proliferating (biosynthetically-active) parasites with functional glycolysis. Equally, metabolic importance of pyruvate carboxylase in the
absence of sugar transport can also be relegated.

Pyruvate carboxylase is dispensable for the lytic cycle of tachyzoites

In proliferating mammalian cells, one of the established functions of pyruvate carboxylase is to drive anaplerosis under normal conditions (16). A second equally important function is to power gluconeogenesis under glucose starvation.

To investigate the anaplerotic as well as the gluconeogenic significance of \( TgPyC \) in parasites, we engineered the knockout mutants in glycolysis-competent and glycolysis-deficient strains, respectively. The \( TgPyC \) gene was deleted by homologous recombination-mediated insertion of the DHFR-TS selection cassette (Fig S1A). Absence of \( TgPyC \) transcript in representative clones of the \( \Delta tgpyc \) and \( \Delta tgg1-\Delta tgpyc \) strains confirmed the genetic ablation of pyruvate carboxylase (Fig S1B). The successful making of the two mutants shows that \( TgPyC \) is not required for the survival of tachyzoites. The mutants were assessed for the overall growth fitness in plaque assays (Fig S1C). None of the knockout strains of \( TgPyC \) exhibited a noteworthy defect. The size as well as number of plaques formed by the \( \Delta tgpyc \) and \( \Delta tgg1-\Delta tgpyc \) mutants were comparable to respective progenitor strains. These results resonate with insignificant expression of pyruvate carboxylase in the tachyzoite stage (Fig 1C-E) and establish its physiological dispensability during the lytic cycle irrespective of glucose import.

The genome of \( T. gondii \) harbors two distinctive orthologs of PEPCK

A surprisingly nonessential nature of \( TgPyC \) prompted us to examine metabolic relevance of PEPCK for the asexual reproduction of tachyzoites in glucose-replete and glucose-deprived states. We identified two PEPCK paralogs, \( TgPEPCK1 \) (TGGT1_289650) and \( TgPEPCK2 \) (TGGT1_289930) in the parasite database (www.ToxoDB.org) encoding for 677 and 614 amino acids, respectively (Fig S2A). Sequence alignment with \textit{bona fide} orthologs confirmed the presence of a complete catalytic domain containing substrate, metal and nucleotide binding residues. MitoProt analysis predicted >95% probability of \( TgPEPCK1 \) being targeted to the mitochondrion via a mitochondrial targeting peptide (Fig S2B). Phylogenetic clustering demonstrated that \( TgPEPCK1 \) forms a distinct clade with other PEPCKs from the phylum apicomplexa and closely related to the ATP-dependent orthologs from fungi and plants (Fig S2C). \( TgPEPCK2 \) is markedly distinct from \( TgPEPCK1 \) and shows substitutions of otherwise-conserved residues in the nucleotide and substrate-binding sites. Quite notably, orthologs of \( TgPEPCK2 \) appear to be absent in most organisms; they could only be identified in selected coccidian parasites (\textit{e.g. Neospora caninum, Hammondia hammondii}).

Subsequently, we determined the endogenous expression and subcellular localization of the two PEPCK isoforms in tachyzoites. To this end, we performed crossover-mediated tagging of both genes with a 3'HA epitope (Fig 2A-B). Immunofluorescence staining established that C-terminally HA-tagged \( TgPEPCK1 \) (\( TgPEPCK1\text{-HA}_3' \)) was distributed in the mitochondrion, which was confirmed by its costaining with the organelle marker \( TgF1B\text{-ATPase} \) (Fig 2A). By contrast, \( TgPEPCK2\text{-HA}_3' \) was not detectable in tachyzoites despite a successful epitope-tagging of its gene (Fig 2B). Consistently, we were able to amplify transcript of \( TgPEPCK1 \) but not of \( TgPEPCK2 \), further corroborating the expression of only former isoform (see below in Fig 3C, S3B). Immunoblot analysis confirmed the
constitutive expression of TgPEPCK1-HA3'T (~76 kDa), while TgPEPCK2-HA3'T was not detectable (Fig 2C). Following these data, we renamed TgPEPCK1 to TgPEPCKmt (mitochondrial), and TgPEPCK2 to TgPEPCKnet (not expressed in tachyzoites). According to transcriptomics data (20), TgPEPCKnet is significantly induced during development of the parasite in the felid host (enteroepithelial stages; Fig 2D). Hence, it appears as though TgPEPCKmt and TgPEPCKnet serve different developmental stages in the asexual and sexual hosts, respectively, during the natural lifecycle of T. gondii.

Only TgPEPCKmt is required for an optimal lytic cycle under glucose-replete condition

We examined the biological importance of both PEPCKs in the glycolysis-competent as well as in glycolysis-deficient mutants. First, we generated the mutants of TgPEPCKnet in the standard (RHΔku80-TaTi) and its derivative Δtggt1 strain by exchanging the gene with a DHFR-TS selection cassette via double homologous recombination (Fig S3A). In agreement with aforementioned results (Fig 2C), neither the parental nor the Δtggt1 strain displayed the expression of TgPEPCKnet transcript (Fig S3B). Consequently, the resulting Δtgpepcknet and Δtggt1/Δtgpepcknet mutants also lacked the mRNA of TgPEPCKnet. No significant impairment in the plaque area or plaque number of the single and double mutants was evident when compared to respective progenitor strains (Fig S3C). It can therefore be concluded that TgPEPCKnet is not required during the lytic cycle of tachyzoites.

Using a similar approach, we examined the physiological requirement of TgPEPCKmt for the parasite in the presence or absence of glucose transport (Fig 3A). Indeed, we were able to knock out the TgPEPCKmt locus by introducing a DHFR-TS selection cassette, albeit only in the standard parental (RHΔku80-TaTi) strain. Pyrimethamine-resistant parasite clones were screened by genomic PCR, which displayed amplification of 5' and 3' recombination-specific bands in selected clones (Fig 3B). Sequencing of amplicons from a clone representing the Δtgpepckmt mutant confirmed the deletion of the TgPEPCKmt locus. As expected, the Δtgpepckmt mutant lacked the transcript expression, further endorsing the gene knockout (Fig 3C). The mutant formed smaller plaques (30% growth defect) when compared to the parental strain, while plaque numbers were not changed (Fig 3D). To ascertain the requirement of TgPEPCKmt in glucose-limiting conditions, we attempted to delete the gene in the Δtggt1 strain. However, our multiple efforts to make a Δtggt1/Δtgpepckmt mutant were futile. These data show that mitochondrial PEPCK is required for efficient growth in glucose-replete condition but nonessential for the parasite survival. It becomes refractory to knockout upon glycolytic defect, denoting a vital role of the enzyme under glucose-deprived condition.

TgPEPCKmt is critical for glucose-independent growth of tachyzoites

An apparent lethality of the Δtggt1/Δtgpepckmt mutant prompted us to engineer an inducible knockdown of TgPEPCKmt in the Δtggt1 strain (Δtggt1/Δtgpepckmt). To achieve this, we first introduced an ATc-regulated and C-terminally HA-tagged ORF of TgPEPCKmt at the TgUPRT locus by FUdR selection and subsequently ablated the gene by DHFR-TS selection cassette (Fig 4A). As envisaged, TgPEPCKmt transcript was significantly repressed (not detectable) upon treatment of the mutant with ATc for 72 h (Fig 4B). Consistently, immunofluorescence and immunoblot
assays endorsed a marked inhibition of the protein in the Δtgg1i/Δtgpmt strain within a day of ATc treatment, which was not detectable upon prolonged exposure to the drug (see 48-72 h in Fig 4C-D). A chemically-repressible conditional mutant of TgPEPCKmt in the Δtgg1 strain enabled us to evaluate its importance for glucose-independent plaque growth of tachyzoites (Fig 5A-B). As expected, the precursor Δtgg1 mutant was not impacted by treatment with ATc, whereas the double mutant was severely impaired upon repression of TgPEPCKmt. The conditional mutant formed miniscule-size plaques even though their numbers were comparable with the Δtgg1 strain. Our measurements of growth rates by serial passage (Fig 5C) demonstrated a continued unaltered reproduction of the Δtgg1 mutant irrespective of ATc. Likewise, the double mutant behaved normally without ATc, but slowed progressively during successive passages and eventually ceased to grow upon drug exposure. The assay showed a continued but progressively slowing growth until day 7 (168 h), even though the protein was undetectable by western blot within 48 h of the drug treatment (Fig 4D). The observed yield pattern likely reflects a continuously declining residual activity of TgPEPCKmt in parasites. Taken together, these results demonstrate an essential role of the mitochondrial PEPCK in glycolysis-deficient tachyzoites.

**TgPEPCKmt regulates glutamine-derived gluconeogenic flux in tachyzoites**

To discern the underlying basis of observed phenotype in the Δtgg1i/Δtgpmt mutant, we performed stable isotope labeling assays. The mutant was cultured with or without ATc for 60-72 h, and then labeled in standard medium (+/- ATc), in which glutamine was exchanged by [U-13C]-glutamine. The viability of the parasite inoculum was >95% for both conditions. Label inclusion analysis of the central metabolites using GC-MS showed a notable flux of glutamine-derived carbon into metabolites associated with TCA cycle and gluconeogenesis in the on state, as can be judged by comparing the abundance of non-labeled compounds to the summed intensities of all their detected isotopomers (Fig 6). ATc treatment resulted in reduced inclusion of 13C from glutamine into select metabolites linked to gluconeogenesis (PEP, 3PG, G3P, R5P, Pyr) that was reflected in their abundance (Fig S4). We observed somewhat modest but evident decrease in labeling of certain TCA cycle intermediates (Cit, 2OG, Suc, Fum); although, the abundance of these metabolites did not mirror the isotope inclusion, probably due to anaplerosis of other substrates, which seems not to be the case for gluconeogenesis. A combined assessment of all glutamine-labeled metabolites confirmed a perturbed gluconeogenesis and TCA cycle when expression of TgPEPCKmt was turned off (Fig 7). Impairment of gluconeogenesis was more obvious than of the TCA cycle.

Interestingly, all six possible isotopomers of citrate were detected, confirming the occurrence of a whole TCA cycle fueled by glutamine only. The M+6 citrate isomeroter is derived from M+5 citrate after its cleavage by an ATP citrate lyase to form M+2 acetyl-CoA. The latter is further condensed with M+4 oxaloacetate to form M+6 citrate. The M+4 oxaloacetate comes from M+5 α-ketoglutarate via succinyl-CoA, succinate, fumarate and malate. Likewise, the occurrence of M+5 citrate indicates that the citric acid cycle steps between α-ketoglutarate and citrate are reversible via backward functioning of isocitrate dehydrogenase, as reported previously in mammalian cells (21,22). These findings underline TgPEPCKmt as a
A metabolic shunt connecting the mitochondrial and cytosolic flux of glutamine-derived carbon, and entail the presence of reductive carboxylation in *T. gondii* tachyzoites.

**A knockdown of TgPEPCK<sub>mt</sub> impairs the ribose biosynthesis in the \( \Delta \text{tggt1/\Delta tgpepck}_{\text{mt}} \) mutant**

Due to reversible nature of many reactions of the TCA cycle and glycolysis/gluconeogenesis as well as existence of multiple "branching points" in these pathways, the carbon flux cannot be quantitatively estimated on the basis of isotopic labeling of central metabolites presented above. Hence to obtain additional proof of the carbon transfer from glutamine to metabolites of gluconeogenesis, we determined the incorporation of glutamine-derived carbon into ribose moiety of RNA that can only occur via gluconeogenesis and subsequent pentose phosphate pathway. Our previous work has demonstrated the incorporation of \(^{14}\text{C}\)-glutamine into RNA of the GT1 mutant, suggesting glutamine’s role in biomass production via gluconeogenesis. Here, we inspected inclusion of \([U-^{13}\text{C}]\)-glutamine into RNA nucleosides to demonstrate that TgPEPCK<sub>mt</sub> plays a crucial role in biogenesis of macromolecules under glycolytic deficiency.

The \( \Delta \text{tggt1/\Delta tgpepck}_{\text{mt}} \) mutant was labeled with \(^{13}\text{C}\)-glutamine during its intracellular growth followed by extraction and hydrolysis of the parasite RNA. UPLC-MS analysis of hydrolyzed sample revealed labeling of adenosine, guanosine and uridine (Fig 8A). Uridine showed an ~80% inclusion of glutamine-derived carbon in the TgPEPCK<sub>mt</sub> mutant, while adenosine and guanosine were only modestly (~20%) labeled. A lower rate of label inclusion in purines as compared to uridine is consistent with the facts that tachyzoites are auxotrophic for purines (relying on host cell), whereas competent in synthesizing pyrimidines (23,24).

To discriminate whether the nucleoside labeling is primarily due to incorporation of glutamine carbon into the ribose moiety or into the base, we performed UPLC-MS/MS analysis of all the isotopomers of uridine and guanosine. We estimated the intensities of the unlabeled as well as of the labeled ribose fragments (Fig 8B), which confirmed a significant inclusion of \(^{13}\text{C}\) in uridine-bound ribose, but not in guanosine-bound sugar. Chemical repression of TgPEPCK<sub>mt</sub> by ATc treatment resulted in significant decrease of label incorporation into uridine-ribose. We recorded a decline across the individual isotopomers except for I+3 upon treatment with ATc (Fig 8C). In brief, these results indicate that pentose phosphate shunt is impaired upon knockdown of the mitochondrial PEPCK, endorsing a function of PEPCK<sub>mt</sub> in macromolecular synthesis via gluconeogenic pathway.

**DISCUSSION**

Our work suggests that TgPEPCK<sub>mt</sub> serves as a nodal enzyme, which coordinates glycolysis, gluconeogenesis and TCA cycle under glucose-rich condition to support anabolic activities in tachyzoites (Fig 9). In sugar-starved cells, TgPEPCK<sub>mt</sub> enables the production of glutamine-derived biosynthetic precursors and ensures a canonical TCA cycle. Consistent with the absence of glucose 6-phosphatase gene, tachyzoites do not produce glucose from \(^{13}\text{C}\)-glutamine, which signifies that the parasite engages in all steps of gluconeogenesis except for dephosphorylation of G6P, and fully consumes gluconeogenic intermediates to drive biosynthetic activities required for glucose-independent survival. Glutamine anaplerosis can more or less totally recompense for glucose anaplerosis and maintains the operation of TCA cycle as a
source for energy and a biosynthetic hub. The function of citric acid cycle is guaranteed at least in part via the synthesis of glutamine-derived pyruvate, which can be converted to acetyl-CoA for oxidation in the mitochondrion in a manner similar to glucose-deprived cancer cells (12,13). Even in glucose-replete condition, given the choice between glucose and glutamine-dependent anaplerosis, the latter should be favored because PyC is not expressed in tachyzoites. Also from an energetic perspective PyC uses 1 ATP per oxaloacetate molecule (16), whereas glutaminolysis does not need ATP; instead, glutamine catabolism yields reducing equivalents for oxidative phosphorylation and enzymatic reactions (25).

Although a continued operation of the TCA cycle and oxidative phosphorylation by glutamine may produce adequate energy and reductive power to drive invasion and sustain enzymatic catalysis, it cannot entirely account for the replication of glucose-starved tachyzoites because glycolytic metabolites are still needed to generate the macromolecules essential for the cell proliferation. For instance, glutamine-derived 3PG, G3P and R5P serve as precursors for the synthesis of amino acids, glycerophospholipids and nucleotides, respectively, in cancer cells (25). Our previous study has shown that glucose-derived carbon is incorporated into protein, glycerophospholipids and nucleotides (4). Indeed, inclusion of glutamine-derived carbon into 3PG, G3P and R5P is upregulated in the absence of glucose import (4) and declined upon repression of TgPEPCKnt, indicating a vital role of glutamine in biomass synthesis following glycolytic deficiency (Fig 9). As a result, decrease in level of macromolecule precursors upon repression of PEPCKnt impairs the biomass production and leads to eventual demise of the conditional mutant. It is also worth noting here that two carbon atoms in purines originate from glycine, and 2 one-carbon units are derived from N-10-formyl-tetrahydrofolate, which requires serine (25). Synthesis of both serine and glycine demands 3PG, initially made from glutamine in the Δtggt1 strain. Accordingly, addition of excess serine and glycine can bypass the knockdown of TgPEPCKnt in the Δtggt1/Δtgpepcknt mutant, as judged by a partial rescue of the phenotype (Fig S5). The fact that the lytic cycle was not restored entirely is consistent with additional bioenergetic routes served by glutamine that could not be satisfied by other nutrients present in the parasite culture.

In mammalian and yeast cells, other noncarbohydrate precursors of gluconeogenesis include pyruvate, lactate, glycerol, fatty acids and selected amino acids. It will now be interesting to investigate whether glutamine can supply biosynthetic precursors adequately enough to support the parasite survival or whether ancillary nutrients also play a significant role. None of the specified nutrients rescued the severe growth defect in the Δtggt1/Δtgpepcknt strain (not shown). The Δtggt1 mutant is unable to utilize exogenous pyruvate and lactate synthesis depends on the extent of glucose catabolism (2,3,4). Glycerol may enter gluconeogenesis at dihydroxyacetone phosphate, but consistent with its inability to restore the plaque growth, the enzyme glycerol kinase could not be found in the parasite genome. β-oxidation of fatty acids may provide yet another source for biosynthetic growth under glucose limitation (26), though there is no genetic or biochemical evidence of this pathway in T. gondii. Other anaplerotic nutrients capable of making oxaloacetate may include branched-chain amino acids (leucine, valine, isoleucine), which can
enter the TCA cycle through metabolism of propionyl-CoA, the metabolic relevance of this pathway in tachyzoites is not clear, however (27). Taken together, these data underpin the importance of glutaminolysis over alternative routes during the lytic cycle, which is further intensified by the fact that glutamine is the second most abundant nutrient in the parasite culture and in human blood plasma after glucose (28).

A role of PEPCK in promoting the entry of carbon from glutamine into biosynthetic precursors via PEP converges with cancer cells. Montal et al. (12) and Vincent et al. (13) found that certain tumor cells reprogram select metabolic pathways to meet their increased biosynthetic needs, particularly when glucose level are low in their microenvironment. They observed that activities of PEPCK-C and PEPCK-M are required to produce intermediates of nucleic acid from glutamine in glucose-limited environment. PEP fuels the pentose phosphate shunt and serine/glycine metabolism, both of which contribute to the synthesis of nucleotides required for cell proliferation. Utilization of glutamine and anabolic diversion of intermediates therefore appears to be a common strategy exploited by proliferating tachyzoites and tumor cells to counteract a dearth of glucose. PEPCK-M mRNA and protein are induced upon withdrawal of glucose in tumor cells (29). The catalytic activity of PEPCK enzymes also depends on GTP (30) and acetylation-deacetylation (31). Surprisingly, TgPEPCK_mt is constitutively expressed and appears to be ATP-dependent, which suggests a singular regulation of gluconeogenesis by acetylation-deacetylation mechanism in T. gondii. Indeed, acetylation of TgPEPCK_mt does occur in tachyzoites (32) and genetic depletion of acetyl-CoA is associated with activation of gluconeogenesis (7). We therefore postulate that a decrease in the mitochondrial acetyl-CoA in the Δtggt1 mutant causes deacetylation of TgPEPCK_mt, which turns on gluconeogenesis.

In conclusion, we show that the mitochondrial isoform of PEPCK embodies a main regulator linking catabolism of glucose and glutamine with anabolic routes in tachyzoites of T. gondii. Such a mechanism not only endows the parasite to concurrently assimilate two major carbon sources for biosynthetic activity but also sustains its survival when glucose becomes scarce. Moreover, this work strengthens the notion that metabolic flexibility facilitating the utilization of auxiliary nutrients provides the parasite with a selective advantage to meet its bioenergetic demands in a fluctuating nutritional milieu.

**MATERIAL AND METHODS**

**Biological reagents and resources**

Cell culture media and additives were purchased from Biochrom (Germany). Other common chemicals were procured from Appichem, Carl Roth and Sigma-Aldrich (Germany). [U-13C]-L-glutamine was received from Euriso-top (Germany). Anti-HA and anti-biotin antibodies were acquired from Sigma-Aldrich (Germany). Fluorophore-conjugated antibodies (Alexa488/594) and oligonucleotides were obtained from Life Technologies (Germany). DNA-modifying enzymes were bought from New England Biolabs (Germany). The RHΔku80-hxgprt (33,34) and RHΔku80-TaTi (35) strains of T. gondii were provided by Vern Carruthers (University of Michigan, USA) and Boris Striepen (University of Georgia, USA), respectively. The RHΔku80-TaTi-Δtggt1 mutant was achieved in our previous work. Primary antibodies against TgF1B, TgFd (19), TgGap45 (36) and TgHsp90 (37) protein were donated by Peter Bradley (University of California, Los Angeles, Los Angeles,
USA), Frank Seeber (Robert Koch Institute, Berlin), Dominique Soldati-Favre (University of Geneva, Switzerland) and Sergio Angel (National University of San Martín, Argentina), respectively.

Parasite culture and plaque assays
Tachyzoites of *T. gondii* (RHΔku80-hxgprt, RHΔku80-TaTi and their derivative strains) were maintained by consecutive passage using human foreskin fibroblasts (HFFs) in a humidified incubator (37°C, 5% CO₂). Cells were cultured in Dulbecco's modified Eagle's medium augmented with fetal calf serum (10%), glucose (4.5 g/l), glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (100 µM of serine, glycine, alanine, asparagine, aspartic acid, glutamate, proline), penicillin (100 U/ml) and streptomycin (100 µg/ml). Parasites were cultured at a multiplicity of infection (MOI) of 3 on every second day. HFFs were harvested by trypsinization and grown to confluence in flasks/dishes. To isolate extracellular parasites, parasitized cultures (MOI, 3; 40-44 h infection) were washed with ice-cold PBS, scraped and extruded through 23G (1x) and 27G (2x) syringes. Free tachyzoites were further purified from remaining host cells by filtering (5 µm) and centrifugation (400g, 10 min, 4°C). The viability of extracellular tachyzoites was examined by staining them with 0.6% fluorescein diacetate and ethidium bromide for 5 min followed by fluorescence imaging (green, viable; red, dead). Our isolation procedure typically yielded viable parasites free of host-cell debris.

The growth of individual parasite strains was measured by plaque assays in standard culture medium. Plaques recapitulate successive rounds of lytic cycles (*i.e.* invasion, replication and egression), and therefore emulate the overall fitness of tachyzoites. Tachyzoites were used to infect confluent HFF monolayers in 6-well plates (150 parasites/well) and incubated for 7 days. Cells were washed with PBS, fixed with methanol (-80°C) and stained with crystal violet (15 min). Plaque images were analyzed using ImageJ software (National Institute of Health, USA). About 100-200 plaques were scored for their area (arbitrary unit) and numbers to evaluate the growth fitness of each parasite strain.

Ectopic and endogenous expression of epitope-tagged protein in tachyzoites
The parasite RNA was isolated by Trizol-based extraction method and reverse-transcribed into first-strand cDNA using commercial kits (Life Technologies). For ectopic expression, the epitope-tagged open reading frames of *TgPEPCKmt* and *TgPyC* were amplified from the tachyzoite-derived cDNA using PfuUltra II Fusion polymerase (Agilent Technologies) and matching primers (Table S1) followed by cloning into *pTETO7SAG1-UPKO* and *pTgGRA2-UPKO* plasmids at BspHl/Paci or NsiI/Paci sites, respectively. Both vectors allowed drug-selected insertion of the *TgPEPCKmt* and *TgPyC* expression cassettes at the uracil phosphoribosyltransferase (UPRT) locus using 5-fluorodeoxyuridine (FUDR) (38). The plasmid constructs (10 µg) were transfected into fresh extracellular tachyzoites (10⁷) of the RHΔku80-hxgprt or RHΔku80-TaTiΔtggt1 strain by electroporation. Parasites were transformed in filter-sterile cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl₂, 2 µM ATP and 2 µM glutathione, pH 7.4) using a BTX instrument (2 kV, 50Ω, 25 µF, 250 µs). Tachyzoites with a disrupted UPRT locus were selected by 5 µM FUDR (38).

The endogenous expression of *TgPyC*, *TgPEPCKmt* and *TgPEPCKnet* was
determined by genomic tagging of respective protein with an epitope in tachyzoites. To achieve this, a 1 kb to 1.5 kb long crossover sequence (COS) targeting the 3’-end of the gene of interest was amplified from tachyzoite-derived gDNA and restriction-cloned into a plasmid for 3’-insertional tagging (p3’IT-HXGPRT) (see primers in Table S1). The plasmids were linearized in the first half of the crossover sequence, as indicated in figures, and transfected into the RHΔku80-hxgprt strain. Stable transgenic parasites expressing hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) were selected using mycophenolic acid (25 µg/ml) and xanthine (50 µg/ml) (39). Genomic tagging was verified by recombination-specific PCR and sequencing. The eventual transgenic parasite strains harbored the epitope-tagged gene governed by native promoter and TgGRA2-3’UTR.

Genetic deletions of TgPyC, TgPEPCK\textsubscript{mt} and TgPEPCK\textsubscript{net} in tachyzoites

The deletion of TgPyC, TgPEPCK\textsubscript{mt} and TgPEPCK\textsubscript{net} genes was performed in the RHΔku80-TaTi (glycolysis-competent) and RHΔku80-TaTi-Dtgg1 (glycolysis-deficient) strains. A direct knockout of the TgPEPCK\textsubscript{net} gene in the RHΔku80-TaTi-Dtgg1 strain was lethal; henceforth we performed two-step conditional mutagenesis, which involved making of an intermediate merodiploid strain expressing anhydrotetracycline (ATc)-regulatable ORF of TgPEPCK\textsubscript{net}-HA governed by pTETO7SAG1 promoter, as described elsewhere (40). The knockout constructs were generated by cloning 5’ and 3’ UTRs (1-2 kb) flanking a selection cassette encoding for pyrimethamine-resistant dihydrofolate reductase thymidylate synthase (DHFR-TS) in a plasmid for knockout (pKO-DHFR-TS). 5’ and 3’ UTRs were amplified by genomic PCR using appropriate primers (Table S1). Parasites were selected with 1 µM pyrimethamine (41) and cloned by limiting dilution. The clonal mutants were identified by crossover-specific PCR screening of gDNA in conjunction with DNA sequencing.

Immunofluorescence and immunoblot assays

Confluent HFF cells grown on coverslips were infected with 4x10^5 tachyzoites for 24 h. Infected cultures were fixed with 4% paraformaldehyde (10 min). Samples were neutralized with 0.1% glycine/PBS for 5 min prior to permeabilization with 0.2% triton X-100/PBS for 20 min. Cells were stained with specified primary antibodies (mouse/rabbit α-HA, 1:3000; mouse α-biotin, 1:10000; rabbit α-TgGap45, 1:5000; rabbit α-TgHsp90, 1:1000; rabbit α-TgFd, 1:500; mouse α-TgF1B, 1:1000) and corresponding secondary antibodies (goat anti-mouse or anti-rabbit Alexa488/Alexa594, 1:10000) for 1 h. Samples were mounted in fluoromount G and DAPI followed by image acquisition using ApoTome microscope (Zeiss, Germany). For immunoblot analysis, protein samples isolated from fresh parasites (1-2x10^7) were resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane by semi-dry blotting. Blots were stained with designated primary (mouse α-HA, 1:3000; mouse α-biotin, 1:10000; rabbit α-TgHsp90, 1:1000) and secondary (IRDye® 680RD and IRDye® 800CW, 1:15000) antibodies for 1-2 h. Protein bands were visualized by Li-COR imaging system.

Stable isotope labeling and GC-MS measurement of central metabolites

Intracellular parasites growing within HFF cells were labeled in standard cell culture medium supplemented with dialyzed FCS, in which normal glutamine was substituted by 5 mM of [U-\textsuperscript{13}C]-glutamine. Our primary
Objective was to demonstrate perturbation in transfer of glutamine-derived carbon to glycolysis and gluconeogenic metabolites in the anhydrotetracycline (ATc)-repressible Δtgtt1/Δtgpepckmt strain of T. gondii. HFF were infected with control untreated and ATc-treated (60-72 h pre-culture in 1 μM ATc) tachyzoites of the conditional mutant at MOI of 2.5 and 6, respectively, followed by incubation for 30 h in normal medium without or with the drug, as applicable. The medium was then replaced by isotope-containing medium, and tachyzoites were labeled for indicated periods. Cultures were quenched by ice cooling (5 min), and tachyzoites were isolated from host cells by syringe extrusion and filtering as stated above. Subsequently, parasites were washed with ice-cold PBS (400g, 10 min, 4 ºC), counted and subjected to metabolomic analyses.

Inclusion of [U-13C]-glutamine-derived carbon into polar metabolites was determined by gas chromatography and mass spectrometry. Metabolites of extracellular parasites (1x10^8) were extracted in a mixture of chloroform, methanol and water (1:3:1 v/v) for 20 min at 60°C. Phase separation was induced by adding 200 μl H2O and 200 μl chloroform. The polar phase was dried under vacuum and further processed, essentially as reported previously (4). In brief, the dry residue was derivatized with methoxyamine and N-methyl-N-(trimethylsilyl)- trifluoroacetamide solution in pyridine, and 3 μl of the derivatized mix was used for GC-MS. Metabolites were identified by their retention index and fragmentation patterns. Fractional abundance of 13C atoms as well as of individual isotopomers is shown for those metabolites that were reproducibly detectable in independent assays.

Stable isotope labeling and UPLC-MS/MS measurement of ribose in nucleosides
Fresh parasites labeled with 5 mM [U-13C]-glutamine for 12 h, as described above, were counted, and 20 mio tachyzoites were processed for the RNA extraction using innuPREP RNA Mini Kit (Analytic Jena AG, Germany). Hydrolysis of the parasite RNA was performed using a method modified from Bratty et al. (42). Briefly, 5 μg RNA was hydrolyzed in 250 μl of the digest mix containing 100 mM NaCl, 20 mM MgCl2, 20 mM Tris (pH 7.9), 300 U benzonase endonuclease (Sigma, E1014), 150 mU phosphodiesterase I (Sigma, P3134), 25 U alkaline phosphatase (Roche diagnostics, 10713023001), 10 μg EHNA hydrochloride (Sigma, E1114) and 3 mM deferoxamine mesylate (Sigma, D9533). Hydrolysis was performed at 37 ºC for 18 h. Hydrolyzed RNA was purified from enzymatic components using Amicon Microcon centrifugal column filter unit (10 kDa cut-off, Millipore UK Ltd.; 3000 g, 20 min).

RNA hydrolysates (≈150 μl each) were 3x concentrated by drying in a vacuum centrifuge and dissolving in 50 μl of water. The measurements were performed using a reversed phase ultra performance liquid chromatography device (Waters ACQUITY) coupled to a mass spectrometer (Thermo-Fisher QExactive) (sample injection volume: 3 μl). Chromatographic conditions and operation of the electrospray were as reported by Giavalisco et al. (43). All measurements were performed in negative electrospray ionization mode, and each sample was measured twice. First measurement was a DDA MS2 mode with Top3 ion selection and NCE set to 25eV, followed by a total ion current (TIC) scan. The DDA MS2 data were used to annotate individual nucleosides based on their fragmentation pattern by matching with the metaSysX reference library. Cytidine was not
detectable in our assays, presumably due to non-enzymatic deamination into uridine during the preparation of samples. The TIC data of the DDA MS2 analysis were used to estimate the inclusion of [U-\(^{13}\)C]-glutamine into intact nucleosides. Second sample measurement was an inclusion list-based UPLC-MS/MS of all isotopomers of guanosine (as a pyrimidine representative, detected as a proton loss anion) and uridine (a purine representative, detected as a proton loss anion) in order to establish the isotopomer profile of the ribose fragment (nominal \(m/z\) 110 for non-labeled fragment and \(m/z\) 111 to 115 for I+1 to I+5).

**Calculation of the label inclusion in the GC-MS and UPLC-MS/MS analysis**

For the GC-MS datasets, the \(m/z\) peaks of non-fragmented or poorly fragmented metabolites were used to measure the isotope inclusion by calculating intensities of the unlabeled analyte and all labeled isotopomers. Intensities were corrected taking into account both the natural enrichment for carbon and silicon, due to derivatization of samples producing TMS groups, as described in our previous work (4). For the LC-MS datasets, the data were corrected only for the natural abundance of \(^{13}\)C isotope. The label inclusion into the ribose was calculated as intensity of non-labeled sugar isotopomer versus the intensity of all labeled isotopomers. It should be stated here that the fate of the isotope label does not reflect the immediate fate of labeled metabolites (44). The conditions tested in the present work are close to equilibrium of label accumulation derived from [U-\(^{13}\)C]-glutamine and from unlabeled glucose, pyruvate and amino acids present in culture medium. Analysis of the individual isotopomer patterns as well as of concentrations of the precursors or of the final products would reflect the corresponding fluxes through the metabolites, which was not the aim of this study. However in case of a single passage point from TCA to glycolysis/gluconeogenesis pathway (as is the case for PEPCK\(_{mt}\) in tachyzoites), changes in the total equilibrium labeling of pertinent metabolites, without regard to particular isotopomer pattern, do reflect the difference in the carbon transit between the two metabolic pathways under conditions of on or off states of the mitochondrial PEPCK enzyme.

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**Conflict of interests:** The authors declare that there are no conflicts of interest.

**Author Contributions:** RN, ÖGE, MT and VZ designed, performed and analyzed experiments; NG conceived, designed and coordinated the study, and analyzed experiments; RN and NG wrote the paper. All authors reviewed and approved the manuscript.
REFERENCES


**FOOTNOTES**

*Data deposition:* Sequences reported here are accessible in GenBank™ through accession numbers: TgPyC, KX785383; TgPEPCKmt, KX785384; TgPEPCKnt, KX785385.
**Abbreviation:** IT, insertional tagging; ATc, anhydrotetracycline; GC/MS, gas chromatography and mass spectrometry; GT1, glucose transporter 1; HFF, human foreskin fibroblasts; PEP, phosphoenolpyruvate; Pyr, pyruvate; 3PG, 3-phosphoglyceraldehyde; G3P, glycerol 3-phosphate; G6P, glucose 6-phosphate; Glu, glutamate; R5P, ribose 5-phosphate; 2OG, 2-oxoglutarate; Suc, succinate, Fum, fumarate; Mal, malate; Cit, citrate

**FIGURE LEGENDS**

**Fig 1:** Ectopically overexpressed pyruvate carboxylase resides in the parasite mitochondrion; however, its endogenous expression is not detectable in the tachyzoite stage of T. gondii. (A) Schematics of the expression strategy and immunofluorescence images showing subcellular distribution of pyruvate carboxylase (PyC) in tachyzoites. A single copy of TgPyC tagged with a hemagglutinin epitope (HA) and regulated by the TgGRA2 elements was targeted at the TgUPRT locus via double homologous recombination in the RHΔku80-hxgprt- strain. Stable transgenic parasites were selected by FUdR for the loss of UPRT function and subjected to immunostaining with α-HA and α-TgF1B antibodies. (B) Genomic tagging of the endogenous TgPyC with a C-terminal HA epitope in tachyzoites. The construct for 3'-insertional tagging (3'IT) of the TgPyC gene contained a crossover sequence (COS) and HXGPRT expression cassette (E.C.). It was linearized by SacI enzyme and transfected in the RHΔku80-hxgprt- strain. Parasites expressing TgPyC-HA3IT under the control of native promoter and TgGRA2-3'UTR were drug-selected, screened by genomic PCR using recombination-specific primers (TgPyC-3'IT-Scr-F1/R1) and sequencing of the transcript. (C-D) Detection of TgPyC-HA3IT by immunofluorescence and western blot analysis. Intracellular parasites (24 h infection) were immunostained with α-HA and α-TgF1B antibodies to monitor the endogenous expression and location of TgPyC-HA3IT. For immunoblot, extracellular tachyzoites (10⁷) were subjected to SDS-PAGE, blotting and staining with α-HA and α-TgHsp90 (loading control) antibodies. (E) Detection of TgPyC in the parental (RHΔku80-hxgprt-) and Δttgt1 strains using α-biotin and α-TgFd antibodies. Note that only acetyl-CoA carboxylase co-localizing with ferredoxin in the apicoplast is visible, which confirms immuno-detection of the biotin prosthetic group.

**Fig 2:** TgPEPCKmt (TgPEPCK1) localizes in the mitochondrion, whereas TgPEPCKnet (TgPEPCK2) is not expressed in the tachyzoite stage of T. gondii. (A) 3'-insertional tagging (3'IT) of the TgPEPCKmt gene with a HA tag in tachyzoites and detection of TgPEPCKmt-HA3IT protein by immunofluorescence. The PstI-digested plasmid construct with a crossover sequence (COS) targeting the 3'-end of the TgPEPCKmt gene was transfected into the RHΔku80-hxgprt- strain. Tachyzoites encoding TgPEPCKmt-HA3IT under the control of its native promoter and TgGRA2-3'UTR were selected with indicated drugs, screened by genomic PCR using recombination-specific primers (TgPEPCKmt-3'IT-Scr-F1/R1) and sequencing. Intracellular parasites (24 h infection) were immunostained with α-HA and α-TgF1B antibodies to determine the subcellular location of TgPEPCKmt-HA3IT. (B) Genomic tagging of the TgPEPCKnet gene and fluorescent detection of TgPEPCKnet-HA3IT in tachyzoites. Stable transgenic parasites were generated and immunostained with α-HA and α-TgHsp90 antibodies, as stated in panel A. (C) Immunoblot showing the natural expression of TgPEPCKnet-HA3IT, TgPEPCKnet-HA3IT and TgHsp90 in transgenic tachyzoites from panel A-B. Extracellular parasites (10⁷) of the indicated strains were subjected to protein isolation followed by SDS-PAGE and immunostaining using α-HA and α-TgHsp90 (loading control) antibodies. Parental strain served as a negative control for α-HA staining. (D) Comparative levels of TgPEPCKnet RNA in the tachyzoite and merozoite stages of T. gondii. The graph is
reproduced from the parasite database (www.ToxoDB.org). Tachyzoites were grown in vitro; merozoites were isolated from enterocytes of the cyst-infected cats on specified days. Y-axis denotes transcript levels of fragments per kilobase of exon model per million mapped reads (FPKM), as measured by standard paired-end Illumina sequencing of the parasite mRNA.

**Fig 3:** *TgPEPCK<sub>mt</sub>* promotes the lytic cycle of glycolysis-competent tachyzoites. (A) Scheme depicting double homologous recombination-mediated knockout of the *TgPEPCK<sub>mt</sub>* gene by DHFR-TS in tachyzoites. The construct was transfected into indicated parental strains and pyrimethamine-resistant clonal parasites were screened for 5' and 3' crossover events using applicable primer pairs (*TgPEPCK<sub>mt</sub>-KO-5'Scr-F1/R1 or *TgPEPCK<sub>mt</sub>-KO-3'Scr-F1/R1). (B) Genomic PCR screening showing 5' and 3' recombination in selected parasite clones generated according to the scheme described in *panel A*. The specificity of the *TgPEPCK<sub>mt</sub>* knockout in recombination-positive clones was confirmed by sequencing of PCR amplicons. (C) Validation of the Δtgpepck<sub>mt</sub> mutant by transcript analysis. A representative clonal mutant was examined for the presence or absence of *TgPEPCK<sub>mt</sub>* and *TgFBP2* (control) transcripts using ORF-specific primer sets. Parental strain was included as a positive control. (D) Plaque assays revealing comparative growth of the Δtgpepck<sub>mt</sub> mutant with respect to its parental strain. Shown are the area (arbitrary units) and number of plaques (mean ± SE, n=3 assays). Significance was tested using Student’s *t*-test (***, *p*<0.001).

**Fig 4:** Conditional mutagenesis enables a tetracycline-regulated knockdown of *TgPEPCK<sub>mt</sub>* in glycolysis-impaired tachyzoites. (A) Scheme for generating a tetracycline-inducible mutant of *TgPEPCK<sub>mt</sub>* (iΔtgpepck<sub>mt</sub>) in a GT1-knockout strain with impaired glycolysis (RHΔku80-TaTi-Δtggt1). In the first step, a ATc-repressible ORF of *TgPEPCK<sub>mt</sub>-HA* was integrated at the *TgUPRT* locus by F UdR selection and the *TgPEPCK<sub>mt</sub>* gene was replaced by DHFR-TS. The eventual Δtggt1/iΔtgpepck<sub>mt</sub> mutant was identified by genomic PCR using 5' and 3'-crossover-specific primers (*TgPEPCK<sub>mt</sub>-KO-5'Scr-F1/R1 and *TgPEPCK<sub>mt</sub>-KO-3'Scr-F1/R1*). (B) PCR confirming the regulation of *TgPEPCK<sub>mt</sub>* transcript by ATc in the Δtggt1/iΔtgpepck<sub>mt</sub> mutant. Total parasite RNA was used to amplify *TgPEPCK<sub>mt</sub>*-HA and *TgFBP2* (control for RNA integrity) using ORF-specific primers. (C) Immunostaining of the Δtggt1/iΔtgpepck<sub>mt</sub> strain showing ATc-regulation of *TgPEPCK<sub>mt</sub>*-HA protein. The untreated control and drug-treated parasites (24 h infection) were stained using α-HA and α-*TgGap45* (a marker of inner membrane complex) antibodies. (D) Immunoblot depicting the ATc-mediated repression of *TgPEPCK<sub>mt</sub>*-HA in the conditional mutant. Parasites (10<sup>7</sup>) were subjected to immunoblot analyses using α-HA and *TgHsp90* (loading control) antibodies.

**Fig 5:** *TgPEPCK<sub>mt</sub>* is essential for the lytic cycle of tachyzoites with impaired glycolysis. (A-B) Growth phenotype of the ATc-regulatable Δtggt1/iΔtgpepck<sub>mt</sub> strain. The *TgPEPCK<sub>mt</sub>* mutant and parental (RHΔku80-TaTi-Δtggt1) strains were evaluated by plaque area (arbitrary units) and numbers in the absence or presence of ATc. Statistics was done by Student’s *t*-test (***, *p*<0.001; mean ± SE, n=3 assays). (C) Cell doublings of the indicated strains over a period of 4 serial passages. Tachyzoites cultured with or without ATc (MOI, 1) were syringe-released from host cells to calculate the yield and determine the replication rates (n=3 assays).

**Fig 6:** Fractional abundance of the select isotopomers in the Δtggt1/iΔtgpepck<sub>mt</sub> tachyzoites labeled with [U-<sup>13</sup>C]-glutamine. Isotope inclusion was evaluated by metabolic labeling of intracellular tachyzoites with [U-<sup>13</sup>C]-glutamine in standard culture medium (4 h, 37°C, 5% CO<sub>2</sub>). Polar metabolites were isolated from isotope-labeled parasites and subjected to GC-
MS analysis. ‘M’ represents unlabeled fractions, whereas the ‘Sum’ shows the collective abundance of all $^{13}$C-containing isotopomers of a given metabolite. Only fragmented analytes were detectable for G6P and R5P. Note that our experimental design of metabolomics had to make a reasonable trade off between getting sufficient amount of purified tachyzoites for the metabolite measurements and maximum growth inhibition (a proxy for the enzyme activity). This trade-off was best met between d3-d4 of ATc treatment, on which the parasite growth is still nearly the half-maximum (see Fig 5C). The obvious explanation for the residual labeling in the off-state mutant is that knockdown of PEPCK mt is incomplete in the conditions used to prepare samples for metabolomic analysis. In particular, when we infer enzymatic activity from reduction in PEP labeling (a direct product of PEPCK mt), we observed a decline of ≈50%, which is consistent with the growth (half-maximum) at the time of sample collection. Statistical significance was determined separately for each group (+/-ATc) using student’s t-test (n = 4 assays; *, p<0.05; **, p<0.01; ***, p<0.001).

Fig 7: TgPEPCK mt regulates the flux of glutamine-carbon through gluconeogenesis and TCA cycle. (A-B) Violin plots showing the impairment of glutamine flux into metabolites involved in or directly associated with gluconeogenesis and TCA cycle in the Δtggt1/Δtgpepck mt mutant. Intracellular parasites were labeled as described in Fig 6 (see methods for additional details). Fractional abundance of $^{13}$C into selected metabolites is depicted (n=4 assays). The extent of variation in the contour of ATc-treated samples is proportional to the degree of perturbation in aggregate carbon flux via a given pathway. PEP, phosphoenolpyruvate; Pyr, pyruvate; 3PG, 3-phosphoglycerate; G3P, glycerol 3-phosphate; G6P, glucose 6-phosphate; R5P, ribose 5-phosphate; 2OG, 2-oxoglutarate; Suc, succinate, Fum, fumarate; Mal, malate; Cit, citrate.

Fig 8: Glutamine-derived carbon is utilized for synthesis of RNA in the Δtggt1/Δtgpepck mt strain. (A) Inclusion of $^{13}$C in adenosine, guanosine and uridine nucleosides of RNA labeled with [U-$^{13}$C]-glutamine (12 h, 37°C, 5% CO2) in the presence or absence of ATc. The figure shows general labeling based on the total ion current data, which does not discriminate between the label in ribose or base. (B) Stable isotope labeling of ribose in nucleosides, as deduced by MS/MS. The labeling of ribose in adenosine was undetectable, and in guanosine was >10%. The minor labeling of purines appears to be a consequence of isotope inclusion in the base, likely through glycolysis and CO2 derived from glutamine’s usage through the TCA cycle. (C) Fractional abundance of the $^{13}$C atoms in all isotopomers of the ribose moiety of uridine. Percentage fractions of the unlabeled (Ribose 0) and labeled isotopomers (I+1 to I+5) are displayed for the drug-treated and control samples. Statistics was done using Student’s t-test (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001; mean ± SE, n=4 assays).

Fig 9: A prototype model featuring the function of TgPEPCK mt as a metabolic shunt in central carbon metabolism of tachyzoites. Schemes are constructed based on this study and earlier work referred in the main text. In cells with an intact glycolysis (left panel), the mitochondrial PEPCK maintains a homeostatic bidirectional flow of glucose and glutamine-derived carbon, which may enable integrated use of both nutrients and rapid metabolic rewiring in response to nutritional oscillations within and/or outside host cells. Neither glucose nor glutamine alone produces significant amounts of fully labeled citrate, suggesting co-usage of both nutrients to operate the TCA cycle (depicted by green/red colors). A minor amount of (+6)-citrate that is produced from glucose requires a pool of sugar-derived oxaloacetate likely generated by TgPEPCK mt, since TgPyC and TgPEPCK mt are not expressed and expendable during the
lytic cycle. On the other hand, in glycolysis-impaired cells (right panel), $Tg_{PEPCK_{mt}}$ allows glutamine-fueled gluconeogenic flux to ensure the biosynthetic activities and thereby parasite survival. Likewise, $Tg_{PEPCK_{mt}}$-derived PEP and ensuing recycling of pyruvate can sustain a glutamine-fueled TCA cycle that is critical to produce energy and reducing equivalents. The work also implicates the presence of a PEP transporter in the membrane of mitochondrion.
**Fig 3**

A)

Parental strain (RHΔku80-TaTi or RHΔku80-TaTi-Δtggt1)

Knockout construct (pTgPEPCKmt-KO-DHFR-TS)

Transgenic strain (Δtgpepckmt or Δtggt1/Δtgpepckmt)

---

B)

5' recombination-specific screening (TgPEPCKmt-KO-5' Scr-F1/R1)

Clones 1 2 3 4 5

1.5 kb

3' recombination-specific screening (TgPEPCKmt-KO-3' Scr-F1/R1)

1.8 kb

1.2 kb

2.0 kb

---

C)

**Parental**

<table>
<thead>
<tr>
<th>TgFBP2</th>
<th>TgPEPCKmt</th>
</tr>
</thead>
</table>

**Δtgpepckmt**

<table>
<thead>
<tr>
<th>TgFBP2</th>
<th>TgPEPCKmt</th>
</tr>
</thead>
</table>

---

D)

**Plaque area (a.u. x 10^5)**

Parental  Δtgpepckmt

***

**Plaque numbers**

Parental  Δtgpepckmt
(1) Integration of Atc-regulatable TgPEPCK_{mt}-HA ORF at TgUPRT locus (negative selection by FUDR)

(2) Deletion of the TgPEPCK_{mt} gene locus by DHFR-TS (positive selection by pyrimethamine)

**Fig 4**

**A**

**B**

(+) ATc

TgFBP2, TgPEPCK_{mt}-HA

(-) ATc

TgFBP2, TgPEPCK_{mt}-HA

Transcript PCR (Δtggt1/iΔtgpepck_{mt})

Immunofluorescence (Δtggt1/iΔtgpepck_{mt})

**C**

**D**

ATc-treated (1 μM)

Control 24 h 48 h 72 h

TgHsp90

TgPEPCK_{mt}-HA

Immunoblot (Δtggt1/iΔtgpepck_{mt})
Fig 5

A

Plaque area (a.u. x10^5)

-ATc
+ATc

Parental (Δtggt1)  Δtggt1/Δtgpepck_{mt}

B

Plaque numbers

-ATc  +ATc

Parental (Δtggt1)  Δtggt1/Δtgpepck_{mt}

C

Cell doublings

Parental (Δtggt1) (-ATc)  Δtggt1/Δtgpepck_{mt} (-ATc)
Parental (Δtggt1) (+ATc)  Δtggt1/Δtgpepck_{mt} (+ATc)

Passage 1  Passage 2  Passage 3  Passage 4
Fig 6

Analysis of metabolite levels in different conditions:

- **Glucose 6-phosphate (G6P)**
- **3-phosphoglycerate (3PG)**
- **Phosphoenolpyruvate (PEP)**
- **Pyruvate (Pyr)**
- **Ribose 5-phosphate (R5P)**
- **Glycerol 3-phosphate (G3P)**
- **Citrate (Cit)**
- **2-oxoglutarate (2OG)**
- **Succinate (Suc)**
- **Fumarate (Fum)**
- **Malate (Mal)**
- **Glutamate (Glu)**

For each metabolite, data are presented for two conditions:

- **Δtgt1Δtgpepck<sub>mt</sub> (-ATc)**
- **Δtgt1Δtgpepck<sub>mt</sub> (+ATc)**

The graphs show the fraction labeled (%) for each condition across different time points (+1, +2, +3, +4) and a sum column. Significant differences are indicated by asterisks: * for p < 0.05, ** for p < 0.01, *** for p < 0.001.
A

Gluconeogenic

\[ \frac{\Delta tgg1/i\Delta tggpeck_{mt}}{-ATc} \]

-ATc  +ATc

Label incorporation (%)

- 0  
- 20  
- 40  
- 60

B

TCA cycle

\[ \frac{\Delta tgg1/i\Delta tggpeck_{mt}}{-ATc} \]

-ATc  +ATc

Label incorporation (%)

- 40  
- 50  
- 60  
- 70  
- 80
Fig 8

A

Bar graph showing nucleoside labeling (%).

B

Bar graph showing ribose labeling (%).

C

Pie charts showing nucleoside labeling distributions.

Legend:
- Ribose 0
- Ribose I+1
- Ribose I+2
- Ribose I+3
- Ribose I+4
- Ribose I+5
A plant/fungal-type phosphoenolpyruvate carboxykinase located in the parasite mitochondrion ensures glucose-independent survival of Toxoplasma gondii
Richard Nitzsche, Özlem Günay-Esiyok, Maximilian Tischer, Vyacheslav Zagoriy and Nishith Gupta

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