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Dissertation

Genetic Dissection of the Central Carbon Metabolism in the Intracellular Parasite *Toxoplasma gondii*

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I. Abstract

Toxoplasma gondii is a widespread protozoan parasite, infecting nearly all warm-blooded organisms. Asexual reproduction of the parasite within its host cells is achieved by consecutive lytic cycles, which necessitates biogenesis of significant energy and biomass. This work shows that glucose and glutamine are the two major physiologically important nutrients used for the synthesis of macromolecules (ATP, nucleic acid, proteins and lipids) in *T. gondii*, and either of them is sufficient to ensure the parasite survival. The parasite can counteract genetic ablation of its glucose transporter by increasing the flux of glutamine-derived carbon through the TCA cycle and by concurrently activating gluconeogenesis, which guarantee a continued biogenesis of ATP and biomass for host-cell invasion and parasite replication, respectively. In accord, a pharmacological inhibition of glutaminolysis or oxidative phosphorylation arrests the lytic cycle of the glycolysis-deficient mutant, which is primarily a consequence of impaired invasion due to depletion of ATP. Unexpectedly however, intracellular parasites continue to proliferate, albeit slower, notwithstanding a simultaneous deprivation of glucose and glutamine. Growth defect in the glycolysis-impaired mutant is caused by a compromised synthesis of lipids, which cannot be counterbalanced by glutamine, but can be restored by acetate. Consistently, supplementation of parasite cultures with exogenous acetate can amend the lytic cycle of the glucose transport mutant.

Furthermore, this work revealed two discrete phosphoenolpyruvate carboxykinase (PEPCK) enzymes in the parasite, one of which resides in the mitochondrion (*Tg*PEPCK$_{mt}$), whereas the other protein is not expressed in tachyzoites (*Tg*PEPCK$_{net}$). Parasites with an intact glycolysis can tolerate genetic deletions of *Tg*PEPCK$_{mt}$ as well as of *Tg*PEPCK$_{net}$, indicating their nonessential roles for the tachyzoite survival. *Tg*PEPCK$_{net}$ can also be ablated in glycolysis-deficient mutant, whereas *Tg*PEPCK$_{mt}$ is refractory to deletion. In accord, the lytic cycle of a conditional mutant of *Tg*PEPCK$_{mt}$ in the glycolysis-impaired strain was aborted upon induced repression of the mitochondrial isoform, demonstrating its essential role for the glucose-independent survival of tachyzoites. Isotope-resolved metabolomics of the conditional mutant revealed defective flux of glutamine-derived carbon into metabolites associated with gluconeogenesis and TCA cycle, which are required to safeguard biosynthetic and energy needs, respectively, when glycolysis is compromised. The data also entails a homeostatic function of *Tg*PEPCK$_{mt}$ in cohesive operation of glycolysis and TCA cycle under normal glucose-replete condition. Conversely, it was shown that the enzyme pyruvate carboxylase (*Tg*PyC) is dispensable not only in glycolysis-competent but also in glycolysis-deficient tachyzoites despite a mitochondrial localization.

Such plasticity in the parasite's carbon flux enables a growth-and-survival trade-off in assorted nutrient milieus, which may underlie the promiscuous survival of *T. gondii* tachyzoites in diverse host cells. Our results also indicate a convergence of parasite metabolism with cancer cells, which holds promise for developing common therapeutics against both threats.

Key words: *Toxoplasma gondii*, central carbon metabolism, glycolysis, gluconeogenesis

II. Zusammenfassung

Toxoplasma gondii ist ein weit verbreiteter einzelliger Parasit, der fast alle warmblütigen Organismen infizieren kann. Asexuelle Fortpflanzung des Parasiten in seiner Wirtszelle wird durch aufeinanderfolgende lytische Zyklen erreicht, was die Bereitstellung einer signifikanten Menge an Energie und Biomasse erforderlich macht. Diese Arbeit zeigt, dass Glukose und Glutamin die beiden wichtigsten physiologischen Nährstoffe für die Synthese von Makromolekülen (ATP, Nukleinsäure, Proteine und Lipide) in *T. gondii* sind. Die Verfügbarkeit einer der beiden Kohlenstoffquellen reicht aus, um das Überleben des Parasiten sicherzustellen. Der Parasit kann durch Erhöhen des Flusses von Glutamin-abstammendem Kohlenstoff durch den TCA-Zyklus und durch gleichzeitige Aktivierung der Gluconeogenese, eine stetige Biogenese von ATP und Biomasse zur Wirtszellinvasion und Replikation gewährleisten, bzw. der genetischen Deletion des Glukosetransporters entgegenwirken. Übereinstimmend führt eine pharmakologische Hemmung der Glutaminolyse oder oxidativen Phosphorylierung zum Arrest des lytischen Zyklus der Glykolyse-defizienten Mutante. Dies resultiert vor allem aus dem Absinken des ATP Spiegels, das in erster Linie die Invasion des Parasiten beeinträchtigt. Überraschenderweise replizieren jedoch bereits invadierte Parasiten, trotz eines gleichzeitigen Entzugs von Glukose und Glutamin, langsam weiter. Der Wachstumsdefekt in der Glykolyse-Mutante wird durch eine kompromittierte Synthese von Lipiden verursacht, die durch Glutamin nicht ausgeglichen werden kann. Die Zugabe von exogenem Acetat kann diesen Wachstumsdefekt allerdings kompensieren.

In dieser Arbeit konnten darüber hinaus zwei unterschiedliche Phosphoenolpyruvat-Carboxykinase (PEPCK) Enzyme im Parasiten identifiziert werden, von denen eines im Mitochondrium lokalisiert ist (*Tg*PEPCK$_{mt}$), während das andere Protein nicht in Tachyzoiten (*Tg*PEPCK$_{net}$) exprimiert wird. Parasiten mit intakter Glykolyse können die Deletion von *Tg*PEPCK$_{net}$, als auch die genetische Deletion von *Tg*PEPCK$_{mt}$ tolerieren, was ihre Redundanz für das Überleben der Tachyzoiten zeigt. *Tg*PEPCK$_{net}$ kann auch in der Glykolyse-defizienten Mutante deletiert werden, während *Tg*PEPCK$_{mt}$ für das Überleben des Parasiten in dieser Mutante essentiell ist. Dies zeigte sich durch ein konditionelles Knockdown von *Tg*PEPCK$_{mt}$, das zu einer Inhibierung des Wachstums des Parasiten führte. Die Metabolomics-Analyse der konditionellen Knockdown-Mutante zeigte einen defekten Fluss von Glutamin-abstammendem Kohlenstoff in Metabolite der Gluconeogenese und des TCA-Zyklus, die zur Sicherung des biosynthetischen und energetischen Bedarfs erforderlich sind. Die Daten suggerieren auch eine homöostatische Funktion von *Tg*PEPCK$_{mt}$ in der metabolischen Verknüpfung zwischen Glykolyse und TCA-Zyklus unter Bedingungen, in den Glukose nicht limitierend ist. Im Gegensatz dazu wurde gezeigt, dass das Enzym Pyruvat-Carboxylase (TgPyC) nicht nur in den Glykolyse-kompetenten Parasiten entbehrlich ist, sondern auch in den Glykolyse-defizienten Tachyzoiten.

Die demonstrierte hohe Plastizität von *T. gondii* in verschiedenen Nährstoffmilieus, schafft eine optimale Homöostase zwischen Wachstum und Überleben, die den Tachyzoiten ermöglicht, in verschiedensten Nährstoffbedingungen unterschiedlicher Wirtszellen zu überleben und optimal

zu replizieren. Die Ergebnisse dieser Arbeit weisen auch auf eine Konvergenz des Parasitenstoffwechsels mit dem von Krebszellen hin, was vielversprechend für die Entwicklung gemeinsamer Therapeutika gegen beide Bedrohungen ist.

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IV. Abbreviations

3PG	3-Phosphoglycerate
ATc	Anhydrotetracycline
ATP	Adenosine triphosphate
Bp	Base pair
CAT	Chloramphenicol acetyltransferase
cDNA	Complementary deoxyribonucleic acid
$CHCl_3$	Chloroform
cKO	Conditional knockout
DAPI	4',6-diamidino-2-phenylindole
DIC	Differential interference contrast
DHFR-TS	Dihydrofolate reductase thymidylate synthase
DMEM	Dulbecco's modified Eagle medium
dNTP	Desoxyribo nucleotide triphosphates
DNA	Deoxyribonucleic acid
DON	6-Diazo-5-oxo-L-norleucin
EDTA	Ethylendiamine tetraacetate
EtOH	Ethanol
F1B	ATPase subunit F1-β
FAS I/II	Fatty acid synthesis type I/II
FBP1/2	Fructose 1,6-bisphosphatase
FCS	Fetal calf serum
FUdR	5-Fluoro-2'-deoxyuridine
Fum	Fumarate
G3P	Glycerol 3-phostate
G6P	Glucose 6-phosphate
GAP45	Gliding-associated protein (45 kDa)
GC	Gas chromatography
gDNA	Genomic deoxyribonucleic acid
GFP	Green fluorescent protein
Glc	Glucose
Gln	Glutamine
GRA2	Dense granule protein 2
GSH	Glutathione (reduced)
HA	Hemagglutinin
h	Hour
HFF	Human foreskin fibroblast
Hsp90	Heat-shock protein (90 kDa)
HXGPRT	Hypoxanthine-xanthine-guanine phosphoribosyltransferase
ICM	Intracellular-type medium
IFA	Indirect immunofluorescence assay
IT	Insertional tagging
kb	Kilo bases
KO	Knockout
Mal	Malate
MeOH	Methanol
MOI	Multiplicity of infection
MPA	Mycophenolic acid
MS	Mass spectrometry
mt	Mitochondrion/mitochondrial
mTP	Mitochondrial targeting peptide
NADH	Nicotinamide adenine dinucleotide
OAA	Oxaloacetate
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEP	Phosphoenolpyruvate
PEPC	Phosphoenolpyruvate carboxylase
PEPCK	Phosphoenolpyruvate carboxykinase
PtdCho	Phosphatidylcholine
PtdEtn	Phopshatidylethanolamine
PtdIns	Phosphatidylinositol
PtdSer	Phosphatidylserine
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
Pyr	Pyrimethamine
RNA	Ribonucleic acid
rpm	Rotations per minute
qRT-PCR	Quantitative reverse-transcriptase PCR
Sag1	Surface antigen 1
Sc	Saccharomyces cerevisiae
SDS	Sodium dodecyl sulfate
Suc	Succinate
TaTi	Trans-activator trap identified
Tet	Tetracycline
Tg	*Toxoplasma gondii*
***Tg*PEPCK$_{mt}$**	*T. gondii* PEPCK mitochondrial
***Tg*PEPCK$_{net}$**	*T. gondii* PEPCK not expressed in tachyzoites
TLC	Thin layer chromatography
UPLC	Ultra-performance liquid chromatography
UPRT	Uracil phosphoribosyl transferase
UTR	Untranslated region

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1. Introduction

1.1. Apicomplexan parasites

The *Apicomplexa* [lat. *apex* = tip, lat. *complexus* = complex], comprise a large group of obligatory intracellular parasites, which cause a number of serious diseases in humans and animals (e.g. *Plasmodium spp.*, *Eimeria spp.*, *Cryptosporidium spp.*, *Toxoplasma gondii*) (1). Parasitic diseases kill about one million people each year and effective treatments are not always available or pose severe threats to the patients (2). For example, *Plasmodium spp.*, causing malaria in humans, accounts for approximately 0.5 million deaths annually (3,4).

Most apicomplexan parasites have a complex life cycle, which strictly requires one or two host species (4). The majority of apicomplexan parasites show a high host specificity, of which *T. gondii* is a notable exception (5). It has the ability to infect almost all vertebrates, including mammals and birds. The infectious stages of *apicomplexan* parasites share a common structural feature: the apical complex, which comprises a microtubular corkscrew-shaped conoid and specialized secretory organelles (rhoptries and micronemes) and plays an essential role in the host-cell invasion by the parasite (6).

Most apicomplexan parasites harbor a plastid-like organelle, surrounded by four membranes, termed apicoplast, which was acquired by secondary endosymbiosis from a red algae and is essential to the parasite, as it harbors important metabolic pathways (e.g. heme biosynthesis, fatty acid biosynthesis II and isoprenoid synthesis) (7,8).

1.2. The apicomplexan parasite *Toxoplasma gondii*

1.2.1. Geographical distribution and clinical relevance

Toxoplasma gondii is undoubtedly one of the most successful pathogens in the world. It can infect almost any warm-blooded vertebrate and has a seroprevalence of around 30% in humans (9). There are three major lineages (type I-III) of *T. gondii* distributed in Europe and North America, while South America is dominated by various genotypes with a much higher genetic diversity (10,11). Intriguingly *Toxoplasma* is one of the few parasites, which has a high distribution in industrial countries, too (12). It is the causing agent of toxoplasmosis, a severe health threat for immunocompromised individuals and neonates. There is no available

vaccination, which confers immunity in humans. Medication for immunocompromised patients and infected pregnant women are limited and do have severe side effects. An attenuated strain (S48) is used for vaccination in ewes, but cannot prevent cyst development in muscle and brain tissue (13). Furthermore, the vaccine causes adverse effects, may revert to a pathogenic strain and is therefore not suitable for humans. Infection in domestic animals are a threat to public health via meat consumption. In livestock toxoplasmosis causes a great economic loss as it may lead to abortion or stillbirth (14).

Primary infection occurs mostly without diagnosis, as *T. gondii* causes flu-like symptoms and is controlled quickly by the humoral and adaptive immune response. A TH_1/IFN-γ mediated immune response clears the acute infection of the parasite within a couple of weeks (9,15). In immunocompromised people, the parasite can inflict ocular and cerebral toxoplasmosis by tissue lesions with potentially fatal outcome (9). If the infection occurs during pregnancy, the parasite can cross the placenta and can result in serious neurological damage, stillbirth or even death of the fetus (16). The severity of damage to the neonate is inversely proportional to the duration of pregnancy.

1.2.2. Life cycle

The life cycle of *T. gondii* consists of a sexual and asexual phase (Figure 1). The definite host are felids (17). Within the intestinal tissue the sexual phase of the life cycle occurs. Micro- and macrogametes form zygotes developing into oocysts, which are shed with the feces into the environment (9). In contact with oxygen, these oocysts sporulate and two sporoblasts develop within the oocyst, containing four sporozoites each, which are infectious for the intermediate host (18). These sporulated oocysts stay viable over years and endure harsh environmental conditions (19) and are transmitted by oral ingestion to the intermediate host (20,21), where the released sporozoites invade the epithelium of the gut and differentiate into the fast replicating tachyzoites.

Upon clearance of the acute infection by the host immune system, tachyzoites can convert into bradyzoites (Greek: *brady* = slow), which are surrounded by a protective cyst wall. These cysts have a tissue specific tropism for muscle and neuronal tissues and lead to the chronic infection of the host, as these cysts remain unchallenged by the immune system (22). These dormant cysts can be reactivated, if the immune system is compromised (e.g. AIDS patients or transplant recipients) and can cause a fetal disease (9). Once felids pray on infected animals, the

heteroxenous life cycle is completed (9). Another important factor for transmission and the high prevalence of the parasite is the possibility of horizontal transmission from intermediate to intermediate host by predation.

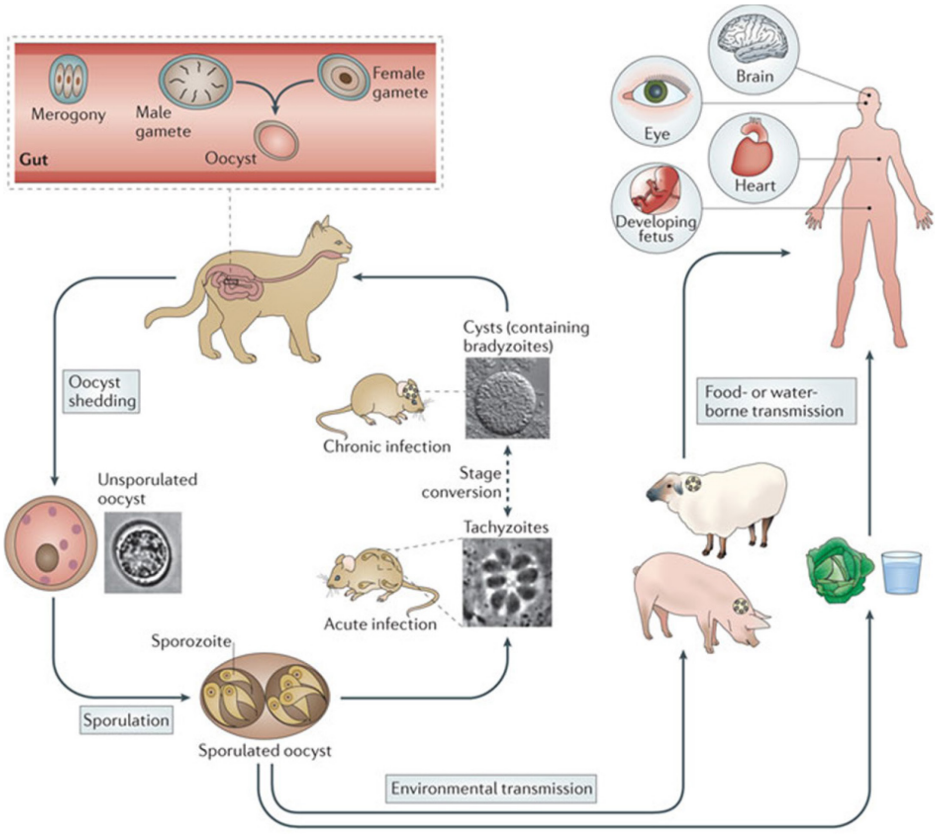


Figure 1. Schematic life cycle of *Toxoplasma gondii.*

The heteroxenous life cycle of *T. gondii* occurs between felids and the intermediate host. Oocysts are shed into the environment with the feces of the cats and sporulate upon contact with air. A short acute infection occurs in intermediate hosts, before the immune system forces the parasite to persist as slow replicating encysted bradyzoites, mainly in brain and muscle tissue. Upon feeding on infected pray, cats can become infected again and complete the life cycle. Adapted from (Christopher A. Hunter & L. David Sibley (23))

1.3. Acquisition of nutrients by intracellular parasites

The hallmark of a parasitic life style is the exploitation of host derived nutrients for replication and survival (4). This is even more predominant in the case of obligate intracellular parasites, which persist within one or more host species during their entire life cycle. The parasite has to scavenge nutrients and/or precursors from the host cell to generate energy and biomass. Apicomplexan parasites have lost or gained many metabolic pathways, while optimizing their life cycle within their host (24) and e.g. expanded certain metabolic gene families (25). *Apicomplexans* have developed various ways to acquire these nutrients from the host cytosol

or organelles and to manipulate host metabolism to ensure steady nutritional supply, which has been shown for *T. gondii, P. berghei* and *Trypanosoma cruzi* (26-28).

The fast replicating tachyzoites are highly dependent on an effective uptake of nutrients during the lytic cycle (29-36). The parasite has retained a relatively large set of metabolic pathways to sustain its metabolic needs in comparison to other apicomplexan parasites (24), but also acquires many nutrients from the host cell (37). As tachyzoites reside within a parasitophorous vacuole (PV), they have to ensure the diffusion or transport from the host cytoplasm over the PV and parasite membrane. It has been shown that the PVM serves as a molecular sieve for small molecules up to 1.4 kDa (38), which likely involves dense granule proteins (GRA17, GRA23) (39). Numerous active transporters have been identified in the parasite, which localize to the parasite membrane or the PVM (25,40-43), that may ensure an effective transport of host nutrients to the parasite's interior.

Tachyzoites are able to synthesize many amino acids *de novo* but are also dependent on the uptake of essential amino acids from the host cell. *Toxoplasma* has retained the ability to generate most non-essential amino acids of humans and has acquired pathways to generate additional amino acids (e.g. lysine biosynthesis pathway) (Figure 2). It has been shown that fast replicating tachyzoites depend on the uptake of glucose and glutamine as carbon sources (35,44).

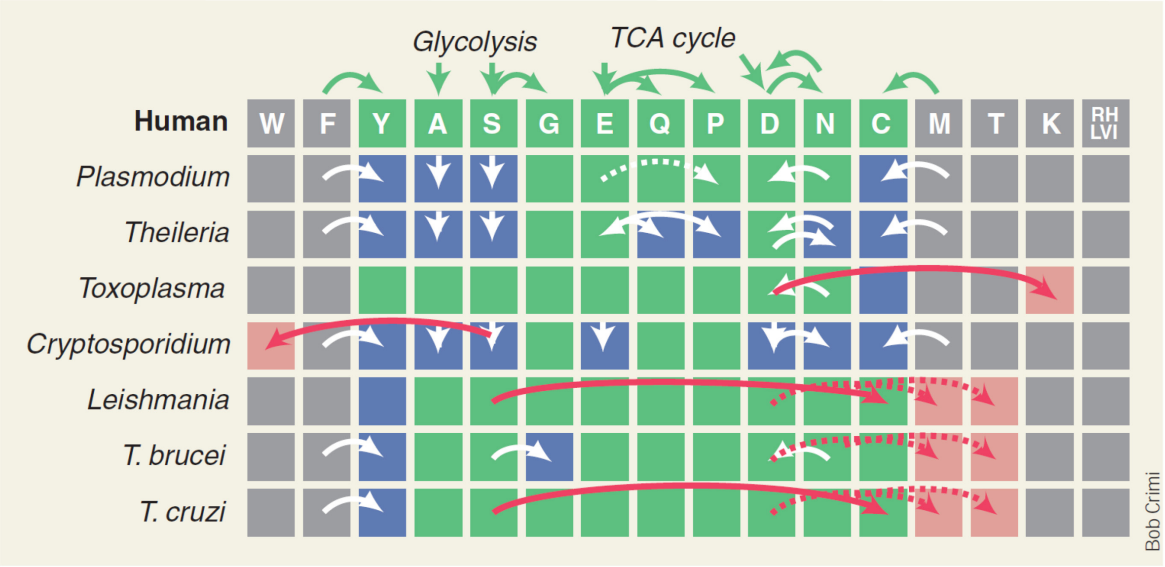


Figure 2. Amino acid metabolism in protozoan parasites and humans.

Comparative genomics show the metabolic diversity. Parasites are in need of uptake of essential amino acids (gray), but also have retained the ability to synthesize amino acids *de novo* (green) or to generate them from other non-essential amino acids (arrows). While in some species the capability of synthesizing some amino acids got lost (blue) others have gained additional biosynthesis pathways not present in humans (red) by acquiring these pathways by horizontal gene transfer (adapted from Chaudhary & Roos (37)).

1.4. Central carbon metabolism in apicomplexan parasites

1.4.1. Glycolysis

Glycolysis is one of the most conserved metabolic pathways in living organisms. It catabolizes glucose by sequential enzymatic reactions to pyruvate, which can either be converted to lactate or contributes to other metabolic pathways (Figure 3). In mammalian cells, glycolysis provides precursors for pentose phosphate pathway, GPI anchor biosynthesis, fatty acid biogenesis, glycine and serine synthesis. It also generates energy (2 ATP per molecule glucose) and pyruvate, which can be used to fuel TCA cycle (45).

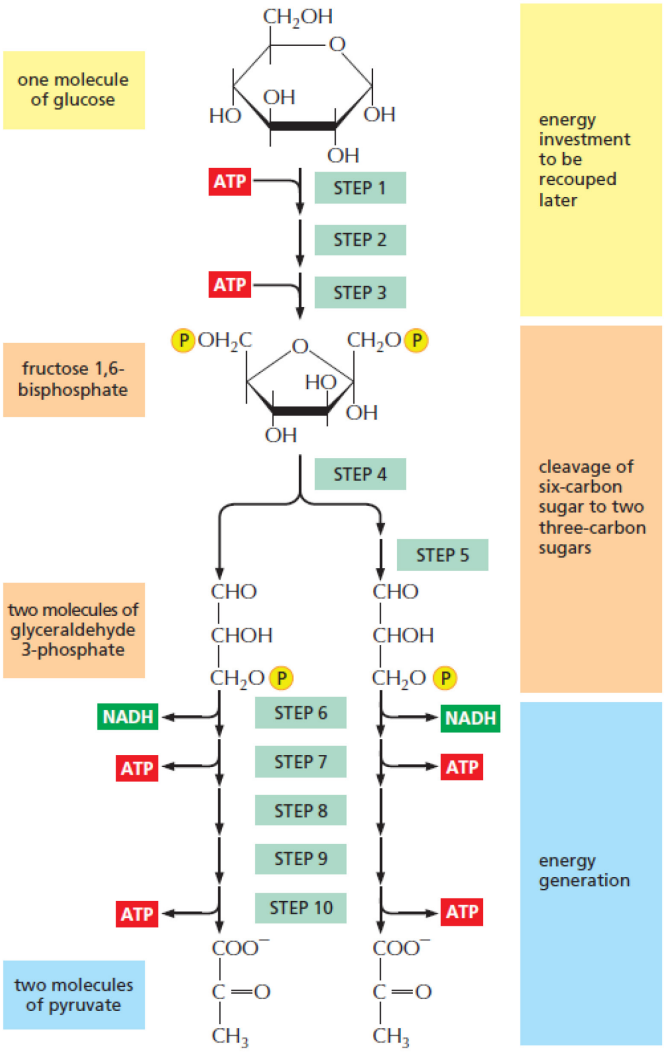


Figure 3. Glucose catabolism through glycolysis.

Glycolysis leads to degradation of one glucose molecule to two pyruvate molecules and net production of 2 ATP molecules. It is the central pathway of carbon metabolism and is present in all organisms. Each of the 10 steps shown is catalysed by a different enzyme (adapted from Alberts, 2010 (46)).

In Apicomplexa glycolysis is highly conserved across the entire phylum. Glucose transport seems to be essential for *Plasmodium spp.* red blood stages (47), but not for *T. gondii* tachyzoites (40,44,48). Nevertheless, glycolysis has been suggested as a major source of energy, the ATP-dependent gliding motility and host cell invasion by *T. gondii* tachyzoites (49). In *Plasmodium spp.* it ensures the maintenance of ATP-levels and pH homeostasis (50). Surprisingly, the apicoplast of *T. gondii* and *Plasmodium* harbors isoforms of glycolytic enzymes (e.g. phosphoglycerate kinase and pyruvate kinase) (51-53). Notably, many apicomplexan parasites (e.g. *Toxoplasma*, *Eimeria* and *Cryptosporidium*) possess amylopectin storages during their life cycle, which can serve as a source for glycolysis (54-56).

Surprisingly, in *T. gondii* the knockdown of the gluconeogenic enzyme fructose-1,6-bisphosphatase (*Tg*FBP2) leads to the loss of growth even under glucose replete conditions (44). This observation suggests an active role of *Tg*FBP2 in regulation of glycolytic flux by supporting a futile cycle. It was suggested that constitutive expression of *Tg*FBP2 may enable the parasite to adapt quickly to the availability of different carbon sources in the host cell without a need for transcriptional regulation (44).

1.4.2. TCA cycle

All aerobic organisms utilize the TCA cycle under aerobic conditions and it is one of the most conserved pathways. The TCA cycle is present in all Apicomplexan parasites with the exception of most *Crytosporidium* species, which have retained only a degenerated mitochondrion (mitosome) and have lost the electron transport chain (57,58). The TCA cycle is used by aerobic organisms to generate energy and regenerate NADH as a reducing agent. It is also a major hub of cellular metabolism (Figure 4). In *T. gondii* glucose-derived pyruvate enters the parasite mitochondrion and is converted to acetyl-CoA by the catalytic activity of BCKDH (branched-chain alpha-keto acid dehydrogenase). BCKDH substitutes for the PDH enzyme in *Toxoplasma gondii* and *Plasmodium falciparum*, which is located in the apicoplast (59). Acetyl-CoA is oxidized to carbon dioxide by the TCA cycle, generating NADH and GTP. TCA cycle intermediates are used for numerous pathways, such as heme biosynthesis, amino acid synthesis or the electron transport chain to generate ATP. Most enzymes of the TCA cycle are dispensable during *Plasmodium falciparum* blood stage development, while they become essential for the development of later stages, e.g. aconitase knockout mutant arrest as late gametocytes and α-ketoglutarate-dehydrogenase-deficient mutant fail to form oocysts in the mosquitoes (60). Such

a strict dependence on glycolysis and on the TCA cycle likely emerged due to excess supply of glucose in the blood. In contrast, in *T. gondii* the chemical inhibition of the TCA cycle enzyme aconitase with sodium fluroacetate completely inhibited tachyzoite replication *in vitro* and their capacity to induce infection in mice. These findings underline the importance of the TCA cycle as a central metabolic hub (29,59), though glycolysis and not electron transport chain seems to be necessary for invasion in *T. gondii* (61).

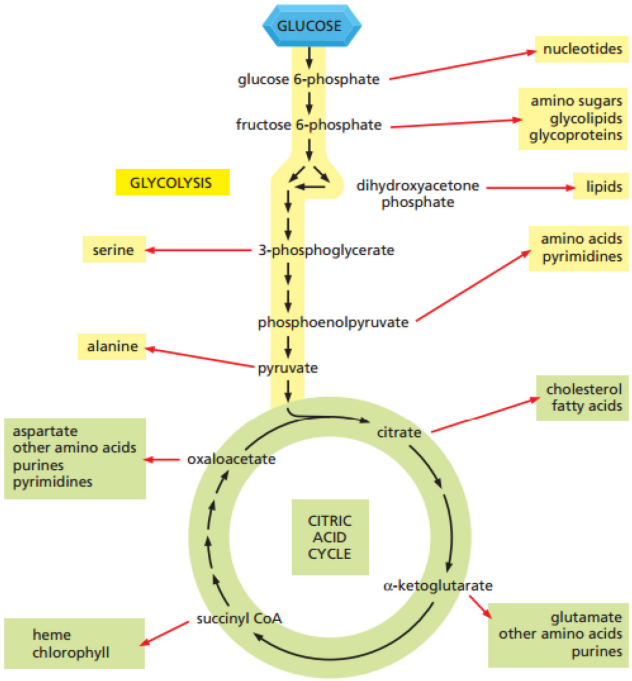


Figure 4. Schematic depiction of the TCA cycle and amino acid metabolism.

Glycolysis and the citric acid cycle provide the precursors needed to synthesize many important biological molecules. the amino acids, nucleotides, lipids, sugars, and other molecules—shown here as products—in turn serve as the precursors for the many macromolecules of the cell. each black arrow in this diagram denotes a single enzyme-catalyzed reaction; the red arrows generally represent pathways with many steps that are required to produce the indicated products (adapted from Alberts, 2010 (46))

1.5. Aim of this study

Tachyzoites encode all major pathways of central carbon metabolism including glycolysis, TCA cycle, pentose phosphate shunt and gluconeogenesis (2-4). These four routes of carbon metabolism constitute a metabolic hub to ensure the biomass, energy, and redox demands during cell proliferation as well as differentiation. The scope and extent to which these pathways satisfy the bioenergetic obligations during the intracellular and extracellular stages of *T. gondii*, is

poorly understood, however. In particular, the following queries remain to be resolved, (a) does the Δ*tggt1* mutant indeed lack a glycolytic flux, especially when intracellular with access to host-derived metabolites; (b) what underlies such a modest growth defect in the mutant given the extensive and diverse metabolic usage of glucose; (c) how important is glutamine-derived carbon for parasite growth and survival; (d) how is metabolism of the two nutrients balanced to accommodate the biogenesis of macromolecules; (e) what are the actual mechanisms that ensure parasite survival in different milieus; (f) what importance plays the gluconeogenic flux in glycolysis deficiency parasites?

2. Materials and methods

2.1. Materials

2.1.1. Biological resources

E. coli (XL-1blue)	Stratagene, Germany
Human Foreskin Fibroblasts	Carsten Lüder, University of Göttingen, Germany

***Toxoplasma gondii* strains**

RH Δ*ku80*-Δ*hxgprt*	Vern Carruthers, University of Michigan, Ann Arbor, USA
RH *hxgprt*$^-$	(40)
RH Δ*ku80*-TaTi	Boris Striepen, University of Georgia, USA, (62)

2.1.2. Antibodies

Alexa Fluor® 488 (goat-anti-mouse)	1:3.000	Thermo Fisher Scientific, Germany
Alexa Fluor® 594 (goat-anti-rabbit)	1:3.000	Thermo Fisher Scientific, Germany
α-biotin (mouse)	1:3000	Thermo Fisher Scientific, Germany
α-*Tg*F1B (mouse)	1:1000	P. Bradley, Univ. California, USA
α-*Tg*Gap45 (rabbit)	1:3.000	Soldati-Favre, Dominique (63)
α-HA (rabbit, mouse)	1:3.000, 1:10.000	Invitrogen, Germany
α-*Tg*Hsp90 (rabbit)	1:3.000	Angel, Sergio O. (64)
α-*Tg*Sag1 (mouse)	1:1.500	Dubremetz, Jean (65)
IRDye 680RD (goat-anti-rabbit)	1:20.000	Li-Cor, USA
IRDye 800CW (goat-anti-mouse)	1:20.000	Li-Cor, USA

2.1.3. Enzymes

Antarctic phosphatase	New England Biolabs, USA
Dream Taq Polymerase	Fermentas, Germany

Klenow	New England Biolabs, USA
Proteinase K	Sigma-Aldrich, Germany
Restriction Endonucleases	New England Biolabs, USA
T4-Ligase	New England Biolabs, USA
Q5 Polymerase	New England Biolabs, USA
Zymolase	Zymo research, USA

2.1.4. Vectors

Tab. 1: Vectors used in this study

Vector	Source
pTKO-HXGPRT	John Boothroyd, Stanford School of Medicine, USA
pTKO-UPKO	Modified *pTKO-HXGPRT*
pTET07-SAG1-UPKO	Modified *pNTP3* from Isabelle Coppens, Johns Hopkins University, USA
p2854-DHFR-TS	Dominique Soldati-Favre, University of Geneva, Switzerland

2.1.5. Chemical reagents

[^{13}C(U)]-D-glucose	EURISO-TOP, Germany
[^{13}C(U)]-L-glutamine	EURISO-TOP, Germany
[^{13}C(U)]-acetic acid	EURISO-TOP, Germany
[^{14}C(U)]-D-glucose	Hartmann Analytics, Germany
[^{14}C(U)]-L-glutamine	Hartmann Analytics, Germany
[14C(U)]-acetic acid	Hartmann Analytics, Germany
1,4-Dithiothreitol (DTT)	Roth, Germany
5-Fluoro-2'-deoxyuridine (FUdR)	Sigma-Aldrich-Aldrich, Germany
Acetic acid	Roth, Germany
Adenosintriphosphate (ATP)	Sigma-Aldrich-Aldrich, Germany
Ammonium molybdate	Applichem, Germany
Ampicillin	Applichem, Germany
Anhydrotetracycline hydrochloride (ATc)	Sigma-Aldrich-Aldrich, Germany

Ascorbic acid	Applichem, Germany
Bovine serum albumin fraction V (BSA)	Applichem, Germany
Bromophenol blue	Merck, Germany
Calcium chloride	Applichem, Germany
Chloramphenicol	Roth, Germany
Chloroform	Roth, Germany
Crystal violet	Sigma-Aldrich-Aldrich, Germany
Deoxynucleotide-triphosphate (dNTPs)	Rapidozym, Germany
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich-Aldrich, Germany
DNA marker (1kb ladder)	Thermo Scientific, Germany
Distilled water (HPLC-purified)	Roth, Germany
Dulbecco's modified eagle medium (DMEM)	Biochrom, Germany
Dulbecco's phosphate buffered saline (PBS)	Biochrom, Germany
Ethanol	Roth, Germany
Fluoromount G / DAPI	Southern Biotech, USA
α-D(+)-Glucose monohydrate	Applichem, Germany
Glycerol	Applichem, Germany
Hank's balanced salt solution (HBSS)	PAA, Austria
Iodine (anhydrous beads)	Sigma-Aldrich-Aldrich, Germany
Isopropanol	Applichem, Germany
Kanamycin sulfate	Applichem, Germany
L-Arginine	Sigma-Aldrich, Germany
L-Aspartic acid	Sigma-Aldrich, Germany
L-Glutamine	Sigma-Aldrich, Germany
L-Glutathione	Applichem, Germany
L-Histidine	Sigma-Aldrich, Germany
L-Lactic acid	FLUKA, Germany
L-Leucine	Sigma-Aldrich, Germany
L-Lysine	FLUKA, Germany
L-Methionine	Sigma-Aldrich, Germany
L-Phenylalanine	Sigma-Aldrich, Germany
L-Serine	Carl Roth, Germany
L-Threonine	Sigma-Aldrich, Germany
L-Tryptophan	Sigma-Aldrich, Germany

L-Tyrosine	Sigma-Aldrich, Germany
L-Valine	Sigma-Aldrich, Germany
Methanol	Roth, Germany
Mycophenolic acid	Applichem, Germany
MEM essential amino acids (50X)	Biowest, Germany
MEM non-essential amino acids (100X)	Biowest, Germany
Ninhydrin spray solution	Roth, Germany
Paraformaldehyde	Roth, Germany
Penicillin/Streptomycin	Biowest, Germany
Perchloric acid	Applichem, Germany
Potassium acetate	Roth, Germany
Potassium chloride	Roth, Germany
Rotiphorese gel 30 (Acrylamide)	Roth, Germany
Salmon sperm DNA (10mg/ml)	Invitrogen, Germany
Tris-HCl	Applichem, Germany
TRIzol® RNA Isolation Reagents	Thermo Scientific, Germany
Yeast extract	Roth, Germany
Yeast Nitrogen Base	Sigma-Aldrich, Germany

2.1.6. Instruments

Device	**Model**	**Source**
Camera system	E.A.S.Y. RH	Herolab, Germany
Centrifuge	5415C, 5417R, 5810R	Eppendorf, Germany
CO_2 incubators	FUNCTIONLine	Heraeus Instruments, Germany
	HERACELL 150i	Thermo Scientific, Germany
	APT.line C 150	Binder, Germany
Cryo container	Nalgene Mr. Frosty	Thermo Scientific, Germany
Electric pipetting aid	accu-jet Pro	Brand, Germany
Electrophoresis power supply	EPS 300	Pharmacia Biotech, Sweden

Electroporator	Amaxa Nucleofector	Lonza Switzerland
Fluorescence microscope	Axio Image.Z2	Zeiss. Germany
Freezer (-80°C)	UF85-360T	Colora, Germany
Gel electrophoresis equipment	Easy Phor EASY-CAST Electrophoresis System	Biozym, Germany Owl Scientific
Ice machine	ZBE 110-35	Ziegra, Germany
Incubators	Modell 500	Memmert, Germany
Infrared imaging system	Odyssey Fc	Li-Cor, USA
Inverted microscope	LABOVERT	Leitz, Germany
Microwave	M805 Typ KOR-6115	Alaska, Germany
Multi-channel pipette (50-200 µl)	Transferpipette-8/-12	Brand, Germany
NanoDrop spectral photometer	ND-1000	Peqlab, Germany
Counting chamber	Neubauer	Fuchs Rosenthal, Germany
PCR cycler	Flex Cycler	Analytik Jena, Germany
PerfectBlue semi-dry electro blotter	SEDEC M	Peqlab, Germany
Photometer	BioPhotometer	Eppendorf, Germany
Pipettes		Eppendorf, Germany
qRT-PCR cycler	Mastercycler realplex^2	Eppendorf, Germany
Safety work benches	HERA safe	Heraeus Instruments, Germany
Scales	PCB400-2	Kern & Sohn, Germany
	BP 110 S	Sartorius, Germany
SDS-PAGE system	SE 250 Mighty Small II	Hoefer, Germany
Shaking incubator	Innova 4000	New Brunswick, Germany
Steam-sterilizer	VARIOKLAV	Thermo Scientific, Germany
Thermo shaker	Thermomixer comfort	Eppendorf, Germany
UV-transilluminator	UVT-29M/W	Herolab, Germany
Waterbath	WB-4MS	Biosan, Latvia

2.1.7. Plastic ware

24-well-plates	Sarstedt
6-well-plates	Sarstedt
96-well-plates	Sarstedt
Cell culture dishes (60 x 15 mm)	Sarstedt
Cell-scraper (30 cm)	TPP, Switzerland
Cryotubes (1,8 ml)	Sarstedt
Disposable gloves (latex; nitrile)	Sempermed, Austria
Disposable injection syringes	Braun, Germany
Disposable pipettes (5 ml; 10ml; 25 ml)	Coster, USA
Disposable syringes	Braun, Germany
Electroporation cuvettes	Eppendorf, Germany
Falcon tubes (15 ml, 50 ml)	Greiner Bio-One, Germany
Filter tips	Biozym, Germany
Micro reaction tubes (0.2 ml, 0.5 ml, 1.5 ml, 2 ml)	Sarstedt, Germany
Nitrocellulose transfer membrane	Applichem, Germany
Object slides	Roth, Germany
PCR 8 tube strips	Biozym, Germany
Petri dish (94x16 mm)	Greiner Bio One, Germany
Pipette tips	Sarstedt, Germany
Rotilab blotting paper	Carl Roth, Germany
UV-cuvettes	Carl Roth, Germany

2.1.8. Commercial kits

PureLink® RNA Mini Kit	Thermo Scientific, Germany
innuPREP Plasmid Mini Kit	Analytik Jena, Germany
innuPREP Plasmid MIDI Direct Kit	Analytik Jena, Germany
Pierce™ BCA Protein Assay Kit	Thermo Scientific, Germany
BacTiter-Glo™ Microbial Cell Viability Assay	Promega, Germany

2.1.9. Buffers and media

Blocking buffer	5%	skimmed milk powder
		ad 1x TBS-T
Cytomix	120 mM	KCl
	25 mM	HEPES (pH 7.6)
	5 mM	$MgCl_2$
	2 mM	EDTA
	0.15 mM	$CaCl_2$
	10 mM	K_2HPO_4/KH_2PO_4 (pH 7.6)
		adjusted to pH 7.6
D10 medium	500 ml	DMEM (high glucose 5 g/l, w/o L-glutamine)
	50 ml	iFCS
	5.6 ml	non-essential amino acids (100x)
	5.6 ml	penicillin/streptomycin (100x)
	5.6 ml	sodium pyruvate (100 mM)
	5.6 ml	L-glutamine (200 mM)
LB medium	10 g	tryptone
	5 g	yeast extract
	10 g	NaCl
	15 g	agar (optional for plates)
		ad 1 l ddH_2O autoclaved
SDS-PAGE		
5% stacking gel	1.4 ml	H_2O
	0.33 ml	30% acrylamide
	0.25 ml	1M TRIS/HCl (pH 6.8)
	20 µl	SDS (10%)
	20 µl	APS (10%)
	10 µl	TEMED

12% resolving gel (5 ml)	1.6 ml	H_2O
	2.0 ml	30% acrylamide
	1.3 ml	1.5 M TRIS/HCl (pH 8.8)
	50 µl	SDS (10%)
	50 µl	APS (10%)
	3 µl	TEMED
2x SDS loading buffer	0.1%	bromophenol blue
	100 mM	DTT
	20%	glycerol
	5%	mercaptoethanol
	4%	SDS
	100 mM	TRIS/HCl (pH 6.8)
5xSDS running buffer	1.25 M	glycine
	0.5%	SDS
	0.125 M	TRIS
Semi-dry blot transfer buffer	38 mM	glycine
	20%	methanol
	0.0037%	SDS
	48 mM	TRIS/HCl (pH 8.3)
SOC-medium	20 g	tryptone
	5 g	yeast extract
	0.5 g	NaCl
	186 mg	KCl
	ad 1 l	ddH_2O
		adjusted to pH 7 and autoclaved
TAE buffer (1x)	40 mM	Tris-HCl (pH 8)
	1 mM	EDTA
	0.11%	acetic acid

10xTBS	2 g	KCl
	80 g	NaCl
	30 g	TRIS/HCl (pH 7.4)
		ad 1 l ddH_2O autoclaved
TBS-T (1x)	1/10 vol.	10xTBS
	0.2%	Tween20
T. gondii freezing medium	10%	DMSO
	20%	FCS
		ad D10

2.2. Methods – molecular cloning & DNA/protein analysis

2.2.1. Polymerase chain reaction (PCR)

5-300 ng of DNA was used as the template for PCR reactions. Standard PCR was performed using Dream Taq polymerase (Thermo Scientific) and Q5 polymerase (New England Biolabs). Programs and PCR samples were set up according manufacturers, primers and amplicon size.

2.2.2. Agarose gel electrophoresis

Agarose gel electrophoresis was performed with gels containing 0.8-2% agarose in TAE buffer. DNA samples were mixed with 1/6 vol. DNA loading dye. Gel electrophoresis was conducted at 90 V for 30-60 min. DNA fragments were visualized using the Herolab camera system and a UV transilluminator.

2.2.3. Purification of DNA

Bands with correct size were cut out of the gel and extracted using the innuPREP DOUBLEpure kit (Analytik Jena) according to the manual. Purified insert was eluted in sterile H_2O.

2.2.4. Endonuclease restriction

Inserts and vectors were digested with 3 units of restriction enzyme per µg DNA for 1 h at 25°C or 37°C in 1x enzyme specific reaction buffer, supplemented with 1x BSA, if necessary. Catalytic activities were inactivated subsequently by a 20 min incubation at 65°C and DNA was purified by the innuPREP DOUBLEpure kit (Analytik Jena).

2.2.5. Ligation

Digested vectors and inserts were ligated in a molar ratio of 1:3 (10 fmol of vector and 30 fmol of insert). The ligation mixture was incubated with 5 units of T4 DNA ligase in ligation buffer for 1 h at room temperature or at 14°C overnight.

2.2.6. Transformation of *E. coli*

100 µl of competent *E. coli* cells (XL-1blue) were thawed on ice and mixed immediately with the ligation mixture. Cells were incubated for 30 min on ice, and then heat shocked for 45 s at 42°C in a sterile water bath. Subsequently the mixture was cooled for 1 min on ice. 700 µl of pre-warmed SOC medium was added. Cells were incubated for 1 h at 37°C in a thermo shaker (600 rpm), pelleted at 8000*g* and resuspended in 100 µl SOC. The suspension was plated on a LB agar plate, containing ampicillin (0.1 mg/ml) and incubated overnight at 37°C.

2.2.7. Isolation of plasmid DNA

For plasmid miniprep the innuPREP plasmid mini kit (analytikjena) was used. 5 ml of overnight culture was pelleted and treated as stated in the manual. For midiprep 200 ml of overnight culture was used with the PureLink HiPure plasmid midiprep kit (Invitrogen). The DNA was eluted in 30 µl or 100 µl sterile H_2O, respectively.

2.2.8. Precipitation of DNA

DNA was precipitated by adding isopropanol (0.8 x sample volume). After centrifugation for 30 min at 4°C and 18.000*g* the pellet was washed with 1 ml of ice cold ethanol (70%) and

centrifuged again (15 min, 4°C, 18.000*g*). The supernatant was discarded and the DNA was dried under a sterile hood. The dried pellet was resuspended in 30 µl of sterile H_2O.

2.2.9. RNA extraction and cDNA synthesis

Syringe released parasites, which were washed with PBS and pelleted, were used for RNA extraction with the innuPREP RNA Mini Kit (analytikjena). The eluted RNA was dissolved in RNAase-free H_2O and subsequently used for cDNA synthesis using SuperScript III first strand cDNA synthesis kit (Invitrogen) and oligo-dT primers.

2.2.10. Isolation of genomic DNA from *T. gondii* tachyzoites

Freshly lysed out parasites were washed with PBS and centrifuged for 10 min at 2000*g*. The pellet was resuspended in 200 µl of lysis buffer and incubated in a thermo shaker at 55°C for 30 min. The DNA was precipitated by adding 400 µl of ice cold absolute ethanol. The suspension was stored at -20°C for 30 min. The DNA was pelleted by centrifuging at 15000*g* for 30 min and kept at 4°C. The DNA was washed with ethanol (70%) and after another centrifugation step (15000*g*, 4°C, 15 min), the pellet was air-dried and dissolved in 20 µl of sterile H_2O.

2.3. Methods – cell culture and transfection

2.3.1. Propagation of human fibroblast host cells

HFF cells were maintained in Dulbecco´s modified Eagle's medium (DMEM) supplemented with fetal calf serum (10%), glucose (4.5 g/l), glutamine (2 mM), non-essential amino acids (required for minimum essential medium), penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified incubator (37°C, 5% CO_2).Cells were cultured in T-300 tissue culture flasks and subcultured by trypsinization into dishes or multi-well plates, as needed.
T. gondii tachyzoites were maintained by consecutive passages of freshly lysed out parasites (MOI: 3) in culture dishes, grown with confluent HFF monolayers.

2.3.2. Preparation of extracellular parasites

To prepare extracellular parasites for isotope labeling, infected host cells were grown in standard culture medium (MOI, 3; 40 hrs infection), and washed with ice-cold PBS. Cells were scraped and the parasites were released by extruding through 23G (1x) and 27G (2x) syringes and subsequently filtered (5 µm) and centrifuged (400*g*, 10 min, 4°C).

2.3.3. Transfection of *T. gondii* tachyzoites

10^6 freshly lysed out tachyzoites were used for transfection. Parasites were centrifuged for 10 min at 400*g* and resuspended in 100 µl of cytomix buffer. The mixture was supplemented with 2 µl ATP (100 mM), 2 µl GSH (250 mM) and 10 µg of linearized plasmid. The suspension was transferred to an electroporation cuvette and transformation was performed using the Amaxa electroporator (voltage: 1700 V, resistance: 50 Ω, pulse duration: 176 µs, number of pulses: 2, interval: 100 ms, polarity: unipolar). Parasites were subsequently pipetted into a dish with a confluent HFF monolayer. Transgenic parasites were selected with 25 µg/ml mycophenolic acid (MPA) and 50 µg/ml xanthine, 1 µM 5-Fluoro-2′-deoxyuridine (FUdR), 1 µM pyrimethamine or with 6.8 µg/ml chloramphenicol (66,67).

2.3.4. Making of clonal transgenic lines

To obtain clonal transgenic lines, stable, drug resistant parasites were diluted to 250 parasites ml^{-1}. 100 µl (25 tachyzoites) were added to a 96 well plate and diluted six times 1:2. The plate was incubated for 7 days unperturbed at 37°C, 5% CO_2. Wells containing single plaques were identified and used for downstream analysis.

2.4. Methods – phenotype and biochemical assays

2.4.1. Immunofluorescence assay

HFF cells growing on coverslips in 24 wells were infected with tachyzoites at a multiplicity of infection of 3 (MOI: 3) for 24 h (37°C, 5% CO_2). Infected HFF cells were washed twice with PBS and fixed with 4% PFA for 15 min. Fixation was terminated by neutralizing cells in 1 M

glycine/PBS for at least 5 min. Permeabilization was achieved by adding 500 µl 0.2% triton X-100/PBS for 20 min. To prevent unspecific antibody binding, cells were treated with 500 µl 2% BSA/0.2% triton X-100/PBS for additional 20 min.

Primary antibodies were applied for 1 h at room temperature in 2% BSA/0.2% triton X-100/PBS, followed by 3 times washing steps with 0.2% triton X-100/PBS. The incubation with secondary antibodies (α-rabbit Alexa 488 and α-mouse Alexa 594 1:3000 in 2% BSA/0,2% triton X-100/PBS) was carried out in darkness for 45 min. After three washes with PBS the coverslips were mounted using 10 µl Fluoromount + DAPI onto a microscopic slide. Imaging was done by ApoTome.

2.4.2. SDS-PAGE and western blot

Approximately 10^7 parasites were pelleted and suspended in 40 µl SDS sample buffer (2x) and heated at 95°C for 5 min in a thermo shaker.

For SDS-PAGE, 15 µl of the sample was loaded in each lane onto a 5% stacking gel, and separated by a 12% resolving gel (100 mV, 2 h). The gel was stained with coomassie brilliant blue R250 dye for 1 h on a shaker and then rinsed overnight with destaining solution (20% ethanol, 10% acetic acid).

Alternatively, proteins were blotted onto a nitro cellulose membrane for western blot analysis. The blotting was carried out at 85 mA for 90 min in a semi-dry chamber. The membrane was blocked overnight at 4°C with TBS-tween20/5% skimmed milk powder. Primary antibodies were diluted in TBS-tween20/5% skimmed milk powder and the membrane was incubated for 2 h, followed by three washing steps with TBS-tween20. The membrane was incubated with secondary antibodies (IRDye® Li-Cor, 1:20000 in TBS-tween20/5% skimmed milk powder) for 45 min. Protein was visualized by Li-Cor Odyssey® Fc imaging system.

2.4.3. Plaque assay

Plaque represent the lytic cycle, which comprises invasion, replication, egress and motility. HFF cells in 6 well plates were infected with 250 parasites and incubated undisturbed for 7 d (37°C, 5% CO_2). The medium was carefully removed and cells were fixed with -80°C methanol for 2 min, and stained with crystal violet. Plaque numbers were counted and plaque sizes of 50-

100 plaques were measured using an inverted microscope (Leica) and ImageJ software (National Institutes of Health, Bethesda, MD).

2.4.4. Replication and yield assays

Parasites replication rates were analyzed by counting parasites per vacuole after 24 h and 40 h of infection. Parasites were fixed with 4% paraformaldehyde and stained with α-*Tg*Gap45 antibodies. 100 vacuoles per sample were analyzed. Yield of parasite replication was measured for 7 d, culturing parasites in culture dishes and counting absolute numbers every 24 h. Intracellular parasites were syringe released prior to counting. 10^6 parasites were passaged each time into a HFF confluent dish.

2.4.5. Invasion assay

Fresh syringe-released parasites were added onto HFF cells growing on glass coverslips in indicated medium ($4x10^5$, 1 h, 37°C, 5% CO_2). Samples were fixed with 4% paraformaldehyde for 15 min. Parasitized cells were stained with mouse α*Tg*Sag1 antibody (1:1500) before permeabilization, and incubated with rabbit α*Tg*Gap45 antibody (1:3000) after treatment with 0.2% triton X-100 in PBS. Samples were then treated with goat anti-mouse Alexa488 and anti-rabbit Alexa594 antibodies (1:3000) prior to mounting in DAPI/fluoromount G solution. Immunofluorescence imaging was performed using the ApoTome microscope equipped with AxioVision software (Zeiss, Germany). Intracellular and extracellular parasites were identified by differential staining with *Tg*Gap45 only, or with *Tg*Sag1/*Tg*Gap45 antibodies, respectively.
Synchronized invasion assays were performed following Kafsack *et al*. (68). Briefly, HFF cells grown on glass clover slips were infected with fresh syringe-released parasites ($4x10^5$) either in standard culture medium (control) or high potassium buffer (44.7 mM K_2SO_4, 10 mM $MgSO_4$, 106 mM sucrose, 5 mM glucose, 20 mM Tris, 3.5 mg BSA/ml, pH 8.2). Parasites were allowed to settle for 30 min before medium was carefully replaced by standard culture medium (0.005 mM potassium) with or without 2 mM glutamine. Invasion assay was performed for 1 hour.

2.4.6. Stable isotope labeling of tachyzoites

Tachyzoites were labeled with [U-^{13}C]-glucose or [U-^{13}C]-glutamine. Extracellular parasites were purified from late-stage cultures (40 h p.i.) by syringe-release and filtering, followed by washing with ice-cold PBS twice. Purified parasites (10^8) were suspended in ^{12}C-glucose and ^{12}C-glutamine-free culture medium supplemented with 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or with 5 mM glucose and 2 mM [U-^{13}C]-glutamine. Parasites were labeled for 4 h in a humidified incubator (37°C, 5% CO_2). Metabolism was quenched by rapid cooling on ice followed by centrifugation (400*g*, 10 min, 4°C) and washing of parasites with ice-cold PBS twice. Metabolites were extracted and analyzed by GC-MS. For intracellular labeling, parasites replicating in host cells (MOI, 3; 40 h infection) were incubated in glucose and glutamine free cell culture medium supplemented with either 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or 5 mM glucose and 2 mM [U-^{13}C]-glutamine. Labeling was carried out for 4 h at 37°C, 5% CO_2. Parasites were then cooled on ice and purified by syringe release and filtering.

2.4.7. Metabolite extraction and metabolomics

Parasite pellets ($1x10^8$) were suspended in ice-cold mixture of chloroform:methanol:water (1:3:1 v/v) and metabolites were extracted for 20 min at 60°C (29). After induction of phase separation with 200 µl H_2O and 200 µl $CHCl_3$, the polar phase was dried under vacuum and derivatized in two steps. First with 2 µl of 4% solution of methoxyamine in pyridine (90 min, 30°C) followed by addition of 18 µl MSTFA (30 min, 37°C). 3-10 µl of the derivatized solution were injected onto the column for GC-MS analysis with Pegasus IV instrument (Leco Corporation) (69). Data extraction was performed using Leco ChromaTOF software. Metabolites were identified by fragmentation pattern using reference standards from the Golm metabolome database (http://gmd.mpimp-golm.mpg.de) and by matching the retention index to the standard library of metaSysX GmbH. The inclusion of stable isotopes was calculated (70). The *m/z* peak corresponding to the intact or poorly fragmented derivatized analyte was identified. Centroid intensity of the unlabeled peak (M) or intensities of the labeled peaks with isotope inclusion from M+1 to M+*n* (where *n* corresponds to the number of carbons in underivatized metabolite) were used to quantify the incorporation of ^{13}C-glucose and ^{13}C-glutamine. The correction for the natural abundance of the stable isotopes present in the unlabeled precursors and in the trimethylsilyl derivatization group was performed assuming

that M+1 analyte includes input from the natural isotopes in M (^{13}C with a rate natural occurrence equal to 1.1%, or from ^{29}Si with a rate of natural occurrence equal to 4.7%). Likewise, M+2 analyte includes input from M (^{30}Si with a rate of natural occurrence equal to 3.02%) or from M+1; and M+3 analyte includes input from M+1 or from M+2, and so forth. Inclusion of isotopes was determined by calculating the percentage of the sum of intensities of the labeled peaks to the total intensity of all detected isotopomers for a given metabolite. Only those metabolites that were reproducibly detectable in independent assays are shown. A minor contamination of host-cell metabolites, if any, cannot be excluded.

2.4.8. Lipidomics analysis

For lipidomics analysis, the parasite pellet ($5x10^7$) was suspended in ice-cold mixture (1.425 ml) of methyl tert-butyl ether and methanol (3:1, v/v). Samples were sonicated in a water-bath (10 min, 4°C) and then incubated on ice for 8 hours with vortex-mixing every hour. Phase separation was achieved by adding 0.542 ml of cold H_2O followed by incubation on ice for an additional 2 h and centrifugation (5000*g*, 5 min). The upper organic phase was collected, dried under vacuum, dissolved in 120 µl of isopropanol and acetonitrile (3:7, v/v) and analyzed by ACQUITY UPLC (C8 column, Waters Inc.) coupled to MS/MS (QExactive Orbitrap, Excalibur suite (Thermo Scientific) (71). The electrospray ionization (ESI) source was operated under standard conditions, and data were acquired in DDA mode with CID fragmentation of precursor ions at 40 eV. Acyl composition of triacylglycerol species was determined by the [Acyl+NH_4] neutral loss pattern of the precursor in positive ionization mode. Areas of chromatographic peaks of the selected lipid species were used to quantify the relative amount of lipids in the indicated parasite strains using Genedata Refiner 7.5.

2.4.9. Radiolabeling and isolation of biomass

Extracellular parasites ($0.5\text{-}1x10^8$) were incubated (4 h, 37°C, 5% CO_2) in defined labeling medium (33) containing either [U-^{14}C]-glucose (0.5 µCi, 0.1 mM) and 2 mM glutamine, or [U-^{14}C]-glutamine (0.5 µCi, 0.1 mM) and 2 mM glucose, or the precursors of major phospholipids, such as [U-^{14}C]-choline (2 µCi, 50 µM), [U-^{14}C]-serine (2 µCi, 90 µM), [U-^{14}C]-ethanolamine (1 µCi, 25 µM), or [U-^{14}C]-acetate (2 µM, 0.2 mM), or a mix of ^{35}S-cysteine and ^{35}S-methionine (2 µCi, 0.2 mM each). Intracellular parasites (MOI, 3; 40 h infection) were labeled in standard

cell culture medium containing the specified isotopes for 4 h, and then syringe-released and filtered to yield host-free parasites. Radiolabeled parasites were washed three times in ice-cold PBS to remove excess radioactivity and subjected to nucleotide, protein or lipid extractions, as appropriate. Briefly, total RNA was isolated using TRIzol and PureLink Kit (Life Technologies). Total proteins were extracted from the parasite pellets suspended in H_2O (1 ml) and trichloroacetic acid (250 µl) followed by washing twice with ice-cold acetone (15000*g*, 10 min, 4°C) and drying at 95°C. The eventual protein pellet was dissolved in 1% SDS. Lipids were isolated by methanol-chloroform extraction (72). Chloroform phase containing lipids was dried, suspended in 100 µl of chloroform/methanol (9:1) for measuring radioactivity and/or for two-dimensional thin layer chromatography on silica 60 plates in chloroform/methanol/ammonium hydroxide (84.5:45.5:6.5) and chloroform/acetic acid/methanol/water (80:12:9:2). They were visualized by iodine staining and identified by their co-migration with standards. Incorporation of radioactivity was determined by liquid scintillation spectrometry of individually isolated biomass fractions.

2.4.10. Quantification of biomass and ATP

The parasite RNA was quantified by UV-absorption spectroscopy using the Beer-Lambert law. Total proteins were measured by bicinchoninic acid assay (Pierce) using BSA as the internal standard (73). Phospholipids were scraped off the silica plate and measured by chemical phosphorus assay (74). To determine the cellular ATP, freshly syringe-released parasites were filtered and washed 3x in ice-cold PBS. Parasites pellets ($5x10^7$ cells) were suspended in 250 µl of boiling water, cooled on ice and then centrifuged (15000*g*, 5 min, 4°C) to generate the supernatant. ATP levels in the supernatant were measured using a commercial kit (Promega BacTiter-Glo™).

2.5. Oligonucleotides

Tab. 2: Primers used for cloning in this study

Primer name (Restriction site)	Primer sequence (Restriction site underlined)	Purpose (Plasmid name)
	***Tg*GT1**	
*Tg*GT1-3UTR-KO-F1 (*Spe*I)	CTCATCACTAGTTTGAGCGACATTTTGTCTGTC	Cloning of 3'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*GT1-3UTR-KO-R1 (*Not*I)	CTCATCGCGGCCGCACAAAAACAAGAGAGAAGCACG	
*Tg*GT1-5UTR-KO-F1 (*Apa*I)	CTCATCGGGCCCCGTCCAGCATCCAGTGAAC	Cloning of 3'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*GT1-5UTR-KO-R1 (*Apa*I)	CTCATCGGGCCCAACGCACGTCTCTCGGAA	
*Tg*GT1-compl-F1 (*Spe*I)	CTCATCATGCATAATCCGCATGCAGCTTCC	*Tg*GT1 complementation (*pTgGRA2-UPKO*)
*Tg*GT1-compl-R1 (*Not*I)	CTCATCGCGGCCGCGCGCTTGGACACAAAAACAA	
	***Tg*ACS**	
*Tg*ACS-ET-F1	CTCATCCCACCGGTCACCTGGAGTGGAAATGAAATCGAAGGG	*Tg*ACS-HA endogenous tagging (*pTKO-HXGPRT*)
*Tg*ACS-ET-R1	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTATGGGTAAGCTTTCGCAAGAGAGCCC	
	***Tg*PyC**	
*Tg*PyC-ORF-F1 (*Nsi*I)	CTCATCATGCATATGATCATGGCACCTCTGACT	Ectopic overexpression (*pTgGRA2-UPKO*)
TgyC-ORF-HA-R1 (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGGTATAGAATGCGGACTAGCAAGTCA	
*Tg*PyC-KO-5'UTR-F1 (*Apa*I)	CTCATCGGGCCCTGTACTGAGACAGCAAACGAAAA	Cloning of 5'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PyC-KO-5'UTR-R1 (*Apa*I)	CTCATCGGGCCCTTCAACGCTACATGACACGC	
*Tg*PyC-KO-3'UTR-F1 (*Spe*I)	CTCATCACTAGTTAGAGCAGCGATTCAAGGACA	Cloning of 3'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PyC-KO-3'UTR-R1 (*Not*I)	CTCATCGCGGCCGCTTAATACAAGAACACGGTGATCCA	
*Tg*PyC-KO-5'Scr-F1	TACGACGCAGGGCAAATC	Screening of 5' recombination at the gene locus (*pDrive*)
*Tg*PyC-KO-5'Scr-R1	ATCGAGTAAGCACACTACTCCACG	

*Tg*PyC-KO-3'Scr-F1	GGGATCAGGGAGAGTGCC	Screening of 3' recombination at the gene locus (*pDrive*)
*Tg*PyC-KO-3'Scr-R1	TATATTCTTTCACAGTTGTTTCTCCG	
*Tg*PyC-3'IT-COS-F1 (*Hpa*I)	CTCATCGTTAACTTTATCTTCGGTCCCTGAGTTTC	Cloning of crossover sequence for 3'HA tagging (*p3'IT-HXGPRT*)
*Tg*PyC-3'IT-COS-HA-R1 (*EcoR*I)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTA TGGGTATAGAATGCGGACTAGCAAGTCA	
*Tg*PyC-3'IT-Scr-F1	GAAAAACTTCGGAGATGTTTCG	Screening of 3' crossover at the gene locus (*pDrive*)
*Tg*PyC-3'IT-Scr-R1	CTGATCGGCTTTGTAGACTTCTC	
***Tg*PEPCK$_{net}$**		
*Tg*PEPCK$_{net}$-KO-5'UTR-F1 (*Apa*I)	CTCATCGGGCCCGCTACGGATTGATGATATGATCC	Cloning of 5'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PEPCK$_{net}$-KO-5'UTR-R1 (*Apa*I)	CTCATCGGGCCCTTCGGGAACATGCTACGG	
*Tg*PEPCK$_{net}$-KO-3'UTR-F1 (*Spe*I)	CTCATCACTAGTAGAGGGCGTTTGAGTGTAGG	Cloning of 3'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PEPCK$_{net}$-KO-3UTR-R1 (*Not*I)	CTCATCGCGGCCGCAGACAAAAAGAAGGAGGGCC	
*Tg*PEPCK$_{net}$-KO-5'Scr-F1	CCTTCAGTGCAGTGTCGTGT	Screening of 5' recombination at the gene locus (*pDrive*)
*Tg*PEPCK$_{net}$-KO-5'Scr-R1	ATCGAGTAAGCACACTACTCCACG	
*Tg*PEPCK$_{net}$-KO-3'Scr-F1	GGGATCAGGGAGAGTGCC	Screening of 3' recombination at the gene locus (*pDrive*)
*Tg*PEPCK$_{net}$-KO-3'Scr-R1	ATGCCATAGCGGGTAGTCAC	
*Tg*PEPCK$_{net}$-3'IT-COS-F1 (*Hpa*I)	CTCATCGTTAACTCTGATTTGTATTGCATGCCTG	Cloning of crossover sequence for 3'HA tagging (*p3'IT-HXGPRT*)
*Tg*PEPCK$_{net}$-3'IT-COS-HA-R1 (*EcoR*I)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTA TGGGTAGGAAGGGAGACTGTGGGTG	
*Tg*PEPCK$_{net}$-3'IT-Scr-F1	TGGTGATTTTCGGTACATGC	Screening of 3' crossover at the gene locus (*pDrive*)
*Tg*PEPCK$_{net}$-3'IT-Scr-R1	CTGATCGGCTTTGTAGACTTCTC	
***Tg*PEPCK$_{mt}$**		
*Tg*PEPCK$_{mt}$-ORF-F1 (*BspH*I)	CTCATCTCATGAATTATACAATGCACTTGTCTCGCA	

*Tg*PEPCK$_{mt}$-ORF-HA-R1 (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCG TATGGGTAAAAAACCGGACCAGCAGC	Tetracycline-regulated expression (*pTETO7SAG1-UPKO*)
*Tg*PEPCK$_{mt}$-KO-5'UTR-F1 (*Apa*I)	CTCATCGGGCCCGGCAAGGAATGATACAAAAGTGA	Cloning of 5'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PEPCK$_{mt}$-KO-5'UTR-R1 (*Apa*I)	CTCATCGGGCCCTTTATGTAGACAACCACCCCG	
TgPEPCK$_{mt}$-KO-3'UTR-F1 (*Spe*I)	CTCATCACTAGTGCACTTCGAAAAGTCATCGTG	Cloning of 3'UTR for the gene knockout (*pKO-DHFR-TS*)
*Tg*PEPCK$_{mt}$-KO-3'UTR-R1 (*Not*I)	CTCATCGCGGCCGCAACTGGCCAGGTAAGGCAG	
*Tg*PEPCK$_{mt}$-KO-5'Scr-F1	GTGATGCATGCACTTCTGCT	Screening of 5' recombination at the gene locus (*pDrive*)
*Tg*PEPCK$_{mt}$-KO-5'Scr-R1	ATCGAGTAAGCACACTACTCCACG	
*Tg*PEPCK$_{mt}$-KO-3'Scr-F1	GGGATCAGGGAGAGTGCC	Screening of 3' recombination at the gene locus (*pDrive*)
*Tg*PEPCK$_{mt}$-KO-3'Scr-R1	GGTGCCCTAACATCAACGTC	
*Tg*PEPCK$_{mt}$-3'IT-COS-F1 (*Xcm*I)	CTCATCCCACCGGTCACCTGGGGTCTGATTTAGTGG CGCTAAG	Cloning of crossover sequence for 3'HA tagging (*p3'IT-HXGPRT*)
*Tg*PEPCK$_{mt}$-3'IT-COS-HA-R1 (*EcoR*I)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTA TGGGTAAAAAACCGGACCAGCAGC	
*Tg*PEPCK$_{mt}$-3'IT-Scr-F1	GAATTACATGCGCCCAGC	Screening of 3' crossover at the gene locus (*pDrive*)
*Tg*PEPCK$_{mt}$-3'IT-Scr-R1	CTGATCGGCTTTGTAGACTTCTC	

3. Results

3.1. Glucose, glutamine and acetate are major nutrients used by tachyzoites

3.1.1. Genetic ablation of the sugar transport impairs glycolytic flux and activates glutamine-dependent gluconeogenesis

It was previously shown that the Δ*tggt1* mutant shows a negligible transport of glucose to the parasite interior (40). It is unclear however whether a defective sugar transport translates into a compromised glycolysis and TCA cycle. To answer this, first the Δ*tggt1* mutant in the RHΔ*ku80*-TaTi strain was generated, as reported before for the RH *hxgprt*$^-$ parental strain (40). Then labeling of extracellular parasites was performed with ^{13}C-glucose in defined medium supplemented with glutamine and other pertinent nutrients and monitored the inclusion of stable isotopes into primary metabolites of the central carbon metabolism employing GC-MS. As shown elsewhere (29,59), a panel of metabolites of glycolysis, pentose phosphate shunt and TCA cycle, as well as glycerol-3-phosphate (a precursor for lipid synthesis) and glutamate were significantly labeled in the parental strain (Figure 5, Figure S22, Figure S23). Usually, glycolytic and pentose phosphate shunt metabolites showed a higher (60-80%) fraction of ^{13}C pool compared to the TCA cycle and other intermediates, which showed only 20 to 40% labeling with glucose (Figure 5). The Δ*tggt1* mutant strain displayed an evidently lower inclusion of glucose-derived carbon in most of the metabolites, as shown by fractional abundance of stable isotope in the indicated metabolites (Figure 5, Figure S22) as well as in their respective isotopomers (Figure S23). The results suggested an impaired flux of glucose-derived carbon through glycolysis, pentose phosphate shunt and TCA cycle. As anticipated, ectopic expression of a functional glucose transporter in the complemented strain largely restored the carbon flux through all mentioned pathways.

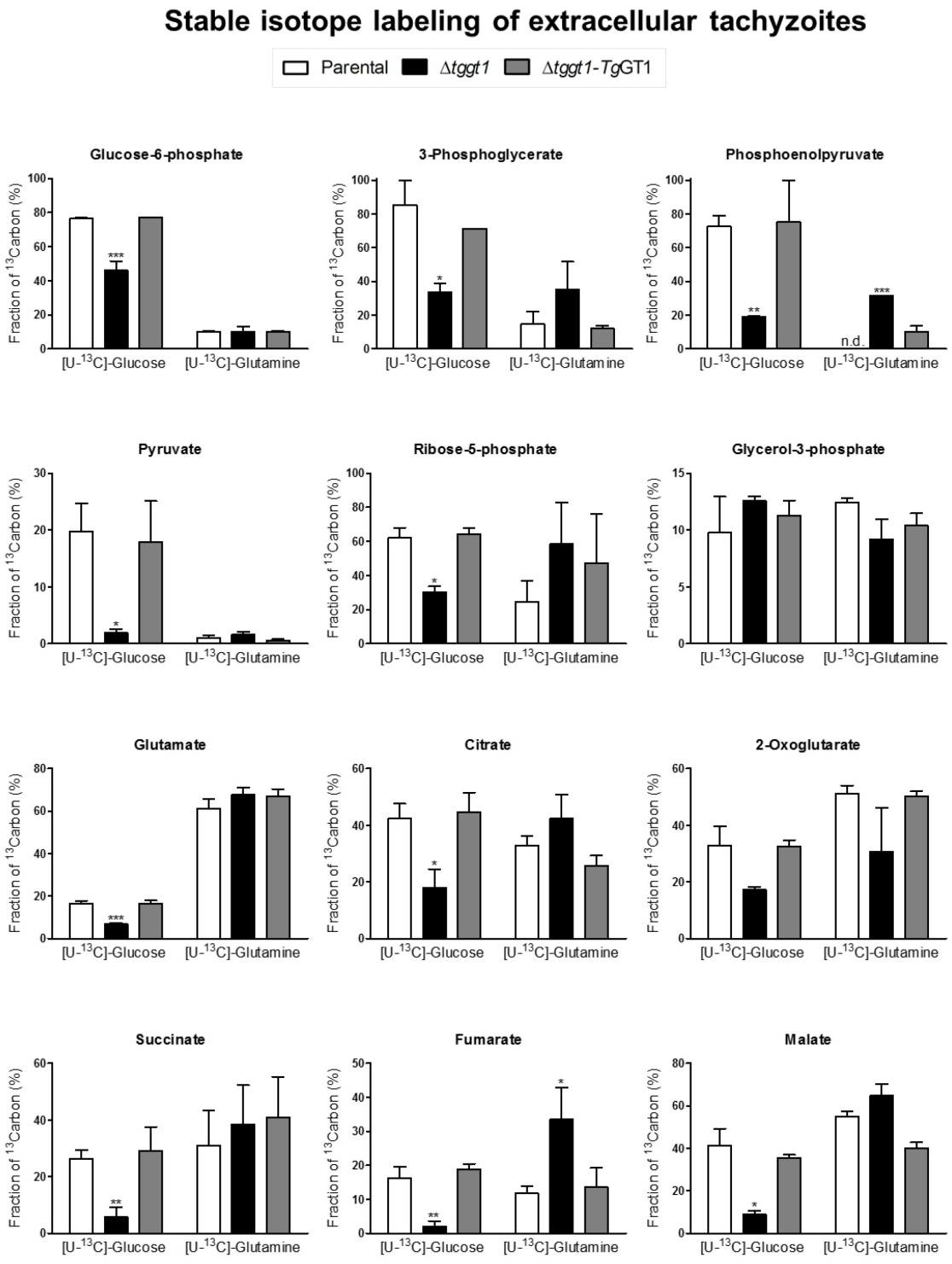


Figure 5. The Δ*tggt1* strain is deficient in utilizing glucose-derived carbon, whereas glutamine metabolism is constitutively active.

Fractional abundance of ^{13}C atoms in the carbon pool of selected metabolites from the indicated parasite strains labeled either with [U-^{13}C]-glucose or [U-^{13}C]-glutamine. Extracellular parasites were incubated with 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or with 2 mM [U-^{13}C]-glutamine and 5 mM glucose for 4 h (37°C, 5% CO_2), and then subjected to metabolomics analysis by GC-MS, as described in methods (mean +/- SEM, n=4 assays). Statistical significance was measured separately for each group compared to the parental strain using student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Abbreviation: n.d., (label) not detectable.

Because intracellular parasites may access the metabolic intermediates generated by host metabolism of glucose, the extent to which glycolysis and TCA cycle are operative in parasites replicating intracellular was examined. The parental, mutant and complemented strains replicating within human host cells were labeled in cultures containing ^{13}C-glucose as a carbon source (Figure 6, Figure S24, Figure S25). The labeling patterns of the specified metabolites across the strains were similar to those seen in the extracellular parasites. Yet again, the Δ*tggt1* mutant exhibited a significant decline of sugar flux into all three pathways, and the *Tg*GT1-complemented strain displayed an expected reversal of metabolic phenotype similar to the parental parasites (Figure 6, Figure S24, Figure S25). These data confirmed an impaired glycolysis in the Δ*tggt1* strain, which in turn reduces the carbon flux *via* pentose phosphate shunt and TCA cycle. The Δ*tggt1* strain also offered an effective model to study the role of an isolated TCA cycle.

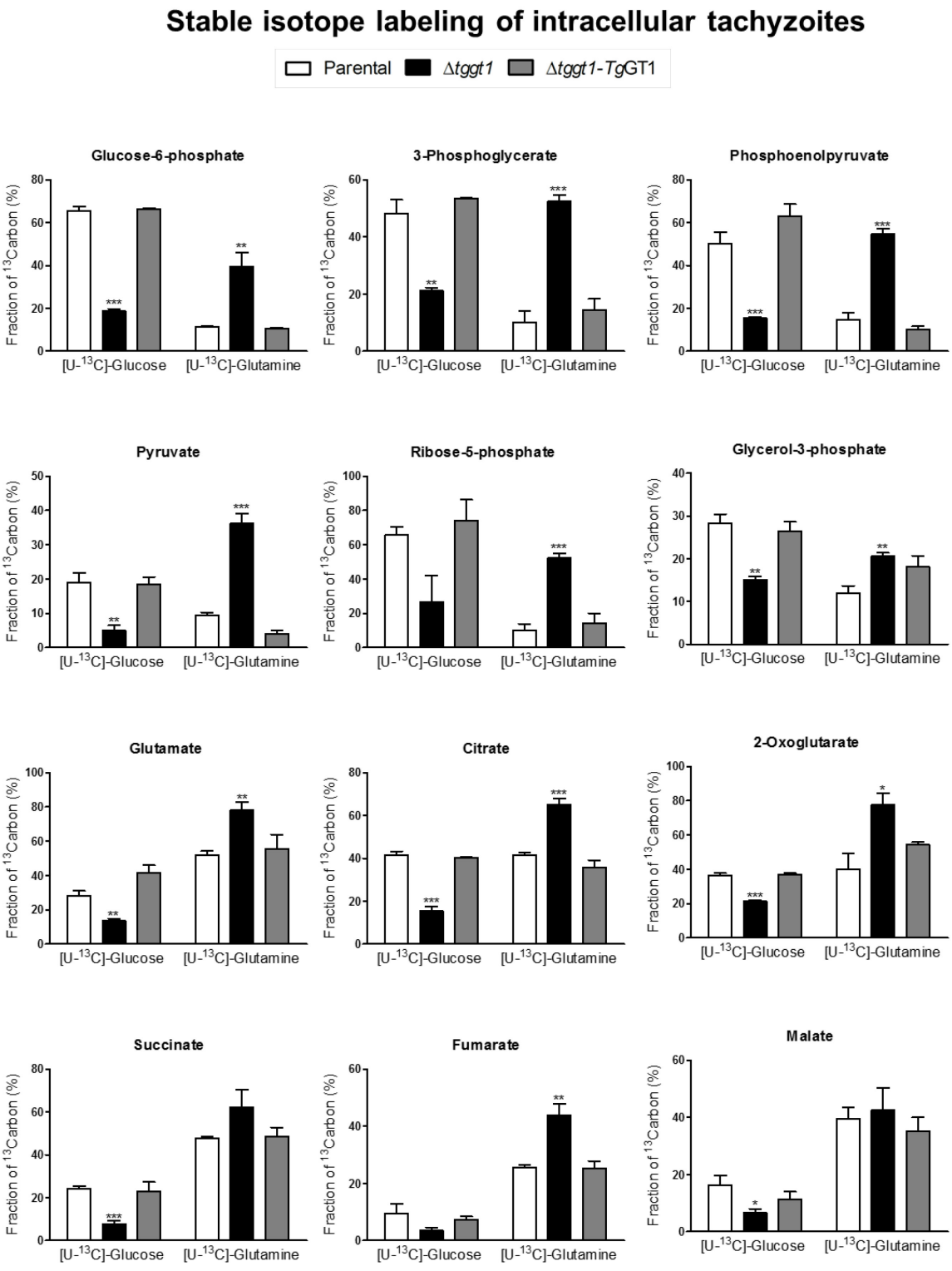


Figure 6. Glutamine-derived carbon flux is induced in intracellular tachyzoites of the Δ*tggt1* strain.

Fractional abundance of ^{13}C atoms in the carbon pool of select metabolites from the indicated parasite strains incubated with 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or with 2 mM [U-^{13}C]-glutamine and 5 mM glucose. Intracellular parasites were labeled with either of the isotopes for 4 h (37°C, 5% CO_2) and subjected to metabolomics analysis by GC-MS, as defined in methods (mean +/- SEM, n=4 assays). Statistical significance was done separately for each group compared to the parental strain using student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

3.1.2. The Δ*tggt1* mutant shows induction of glutamine dependent gluconeogenesis

It has been shown that the parasite can utilize host-derived glutamine, and its consumption is enhanced when glucose is not available (40); therefore a substitutive role of glutamine is anticipated. However, it is not known how the carbon fluxes of the two nutrients are balanced in extracellular and intracellular Δ*tggt1* parasites. To address this question, the mutant as well as parental and complemented strains were labeled with ^{13}C-glutamine in cultures supplemented with glucose followed by GC-MS analysis. All three strains utilized glutamine-derived carbon into the TCA cycle of extracellular (Figure 5, Figure S22, Figure S23) as well as of intracellular (Figure 6, Figure S24, Figure S25) stages under glucose-replete milieus. In contrast to glucose, incorporation of glutamine was more prevalent in the TCA cycle intermediates than glycolysis in the parental strains. Inclusion of glutamine into metabolites of central carbon metabolism was distinctly increased in the Δ*tggt1* mutant. Unlike the reference strains, the Δ*tggt1* mutant showed a prominently higher labeling of all detectable analytes of glycolysis and pentose phosphate pathway with glutamine, indicating the activation of gluconeogenesis (Figure 6, Figure S24, Figure S25). Isotope labeling was reverted when glucose import was reinstated, which indicated a co-regulation of sugar and amino acid metabolism.

Glucose and glutamine together accounted for about 60 to 80% of carbon tracer in TCA cycle metabolites (citrate, 2-oxoglutarate, succinate and malate) and glutamate in all three strains even though their relative proportions varied among individual strains (Figure 5, Figure 6). In other words, glutamine carbon largely compensated for the deficit of sugar in intracellular Δ*tggt1* parasites, and catabolism of the amino acid reverted to the parental level when glucose transport was restored by complementation. Consistent with a high metabolic demand imposed by parasite division, labeling of intracellular parasites was usually more prominent than extracellular stage. Collectively, the results show a constitutive metabolism of glutamine, and suggest a mutual regulation of glycolysis, TCA cycle and gluconeogenesis in *T. gondii*.

3.1.3. Glutamine is required to establish the infection but not for replication of the Δ*tggt1* mutant

To examine the relative importance of glucose and glutamine for the overall growth of *T. gondii*, plaque assays were performed (Figure 7, Figure S26). The Δ*tggt1* mutant displayed an expected 25% growth defect in standard medium when compared to the parental and complemented

parasites (40). An early removal of glutamine from cultures (Figure 7B; 0 h sample, sedimented, *i.e.* natural floating of parasites to the host-cell monolayer) abolished the plaque formation in the mutant (no plaques), while the control strains exhibited normal plaque numbers. Consistently, the number of plaques formed by Δ*tggt1* mutant was nearly ablated when a known analog inhibitor of glutamine catabolism, 6-diazo-5-oxo-L-norleucine (DON, (75)), was applied while setting up the assay (0 h). Conversely, consistent with glutamine-free cultures, the plaque numbers were largely unaffected in the parental and complemented strains treated with DON (compare 0 h samples, Figure 7B). All three strains formed fairly similar number of large plaques when withdrawal of glutamine or treatment with DON was deferred by 4 hours to enable the initial parasite infection of host cells. Interestingly, the parental and complemented strains grown in glutamine-depleted medium showed only a modest 25% decline in plaque sizes when compared to the control cultures, while the Δ*tggt1* mutant displayed a somewhat accentuated 45% growth defect (Figure 7C). Likewise, DON treatment exerted either negligible or moderate (40%) effect on the control and Δ*tggt1* strains, respectively. These data demonstrate that exogenous glutamine is expendable in parasite cultures. They also show a differential dependence of glycolysis-competent and glycolysis-deficient parasites on glutamine catabolism for the lytic cycle.

The aforementioned plaque assays also indicated a possible role of glutamine in host-cell invasion by tachyzoites. Given that plaques are formed after multiple rounds of cell lysis and re-invasion, a modest difference in plaque size upon glutamine withdrawal (4 h) can also be interpreted as no subsequent invasion defect in the Δ*tggt1* strain. In other words, the invasion defect is seen only upon addition of the mutant to the wells in the absence of glutamine (0 h), which may be caused by the loss of parasite viability during the time they naturally float down to host cells. In subsequent cycles, parasites can invade host cells more rapidly, because they have less far to travel. To test the notion, plaque assays were set up, in which parasites were rapidly settled by centrifugal force, thereby reducing the travel time to ≤10 min (0 h, centrifuged; Figure 7B). Indeed, some plaques were observed in the mutant strain cultured in glutamine-free medium; however, their number was still markedly reduced (80%) compared to control strains. Moreover, consistent with other data (Figure 7C), the areas of those few emergent plaques were only moderately affected. These results together suggest a critical requirement of exogenous glutamine for the initial infection event (*i.e.* host-cell invasion) when glucose import is impaired.

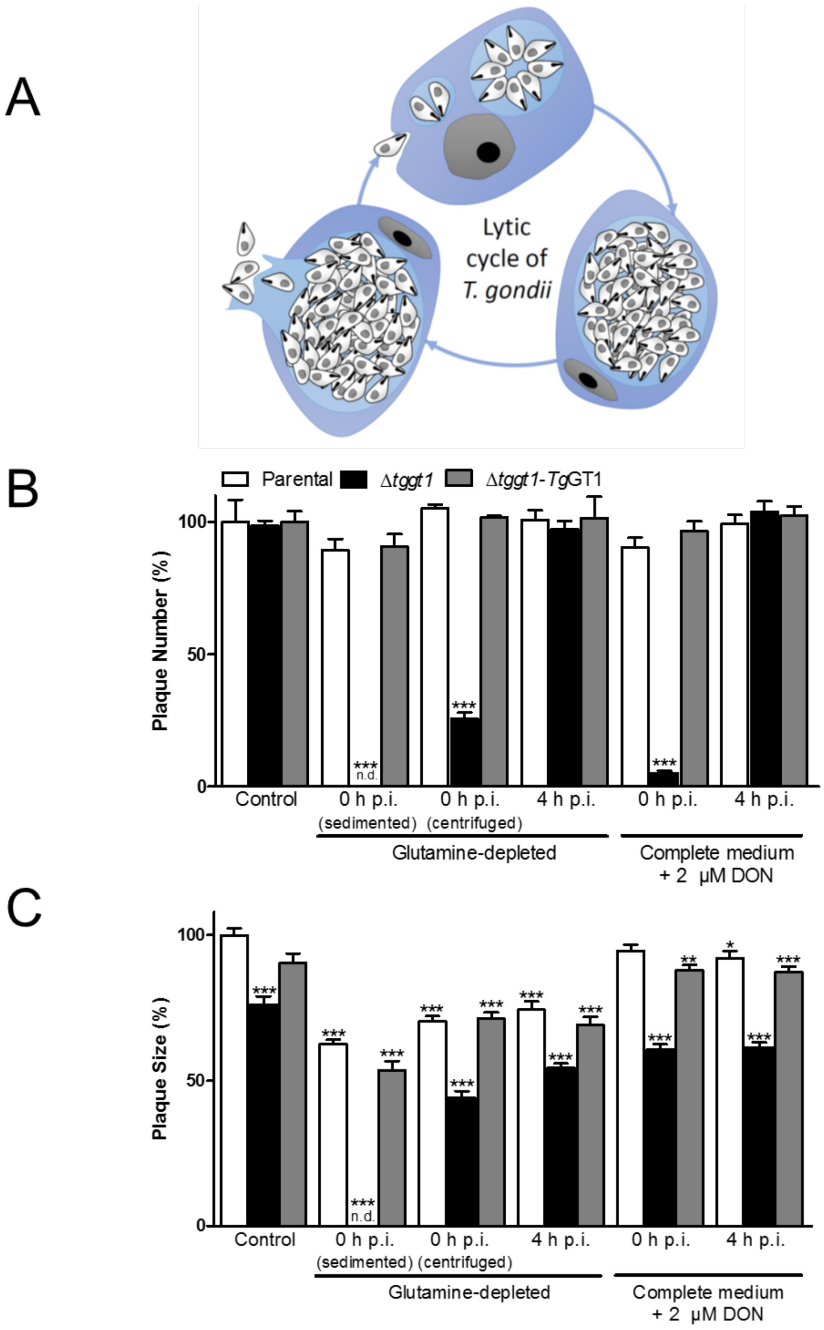


Figure 7. The Δ*tggt1* mutant can replicate without exogenous glutamine, even though it is vital to establish the parasite infection.

(A) Schematized lytic cycle of *T. gondii* tachyzoites showing the consecutive events of invasion, replication and egress. (B-C) Tachyzoites were cultured with or without glutamine (2 mM) in medium containing 10% dialyzed serum, or treated without or with DON (2 µM) in standard culture medium (37°C, 7d, 5% CO_2). Parasites added into the wells were allowed to sediment onto host cells by natural floating (sedimented), or centrifuged at 400*g* for 10 min directly after adding parasites (centrifuged). DON was added at the time of (0 h) or after (4 h) infection of host-cell monolayers. Plaques were stained by crystal violet and analyzed using the ImageJ suite. Plaque numbers (B) and sizes (C) from 3 assays are shown (mean +/- SEM). Statistics (student's t-test) in panel B-C was done with respect to the parental strain grown under control condition (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Abbreviation: n.d., not detectable.

3.1.4. Glycolysis or oxidative phosphorylation alone is sufficient to drive the invasion by *T. gondii*

Next, the relative importance of glucose and glutamine catabolism was tested by invasion assays using human fibroblast host cells (Figure 8A-C). Under normal culture conditions (control), all parasite strains invaded with a similar efficiency, which remained unaltered for the parental and complemented strains when glutamine was omitted. The Δ*tggt1* strain exhibited a nearly complete block of invasion in glutamine-free culture (Figure 8A). To determine a direct involvement of glutamine in host-cell invasion, synchronized invasion assay were performed, *i.e.* let parasite settle in high K^+ medium before switching to low K^+ (high Na^+) medium to induce invasion (68). The mutant displayed a nearly complete loss of invasion when supplied with glutamine-free medium at the time of potassium shift, indicating a need of glutamine for host-cell infection. In contrast, a switching to glutamine-supplemented medium exerted only ≈20% decline, which confirmed the viable and infective nature of most mutant parasites (Figure 8B). Invasion rates were examined in the presence of DON (75) and atovaquone (inhibitor of respiratory chain (76)). Neither of the two inhibitors exerted a significant effect on the parental and complemented strain compared to the control samples; in contrast, both of them abolished invasion of the Δ*tggt1* strain (Figure 8C). The results are consistent with plaque numbers in glutamine-depleted and DON-treated cultures (0 h time-point in Figure 7B). They also resonate well with hitherto known facts that the Δ*tggt1* mutant is impaired in gliding motility (required for invasion) in minimal medium, which could be restored by glutamine but not by pyruvate (40). Consistently, pyruvate (supplied in medium used for invasion assays) seems unable to counteract the collective absence of glucose and glutamine. These results show a strong dependence of tachyzoites on glutamine to facilitate the invasion and motility in the absence of glucose import.

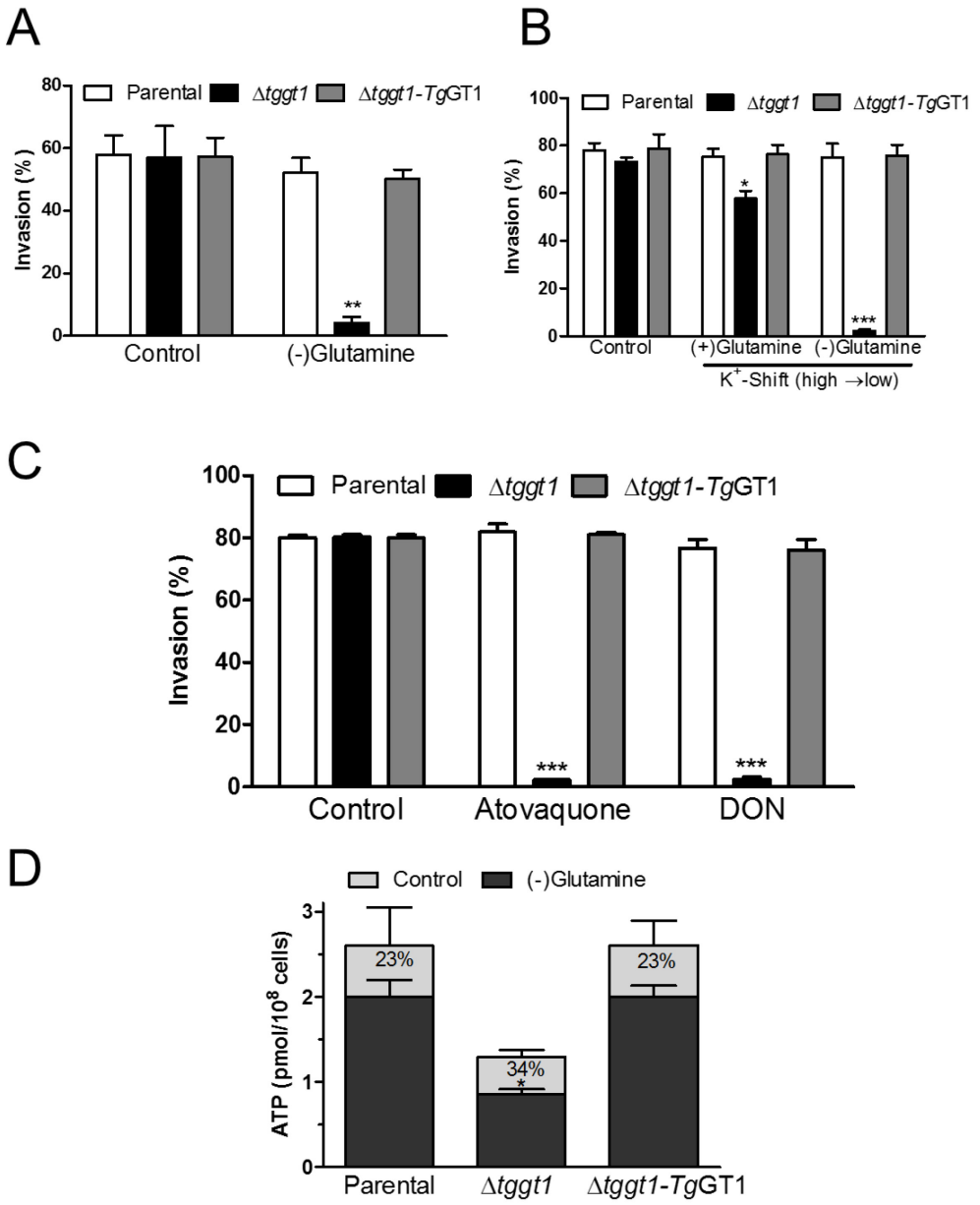


Figure 8. Glucose as well as glutamine alone can supply ample energy for host-cell invasion by *T. gondii*.

(A) Invasion efficiency of the indicated parasite strains in standard culture medium supplemented with dialyzed serum (10%), and glutamine (2 mM, if any). Fresh syringe-released extracellular parasites were used to infect human fibroblast cells (37°C, 5% CO_2, 60 min) followed by staining with α-TgSag1 and α-TgGap45 antibodies. Glutamine-free samples were incubated without the amino acid for 30 min prior to invasion assay. (B) Synchronized host-cell invasion assay. Fresh tachyzoites were suspended in high K+ buffer and added onto host-cell monolayers. Parasites were allowed to settle for 30 min prior to exchanging buffers (high to low K+). Parasites were stained as described in panel A. (C) Effect of known inhibitors of mitochondrial electron transport (atovaquone, 0.1 µM) and of glutamine catabolism (DON, 2 µM) on invasion rates of different parasite strains. Host cells were washed with ice-cold PBS prior to infection, and fresh medium supplemented with individual inhibitors was added at the time of invasion assay. (D) ATP contents of extracellular parasites following incubation (37°C, 5% CO_2, 30 min) in culture medium supplemented with dialyzed FCS, and with or without glutamine (2 mM). Relative declines are shown as percentage. The viability of all parasite strains was nearly 100%, which remained unaltered by glutamine depletion (not shown). Statistical significance (student's t-test) was measured separately for each group by comparing to the parental strain (panel A, B), or to the ATP levels of individual strains under control conditions (panel C) (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

Next ATP levels were measured in fresh extracellular parasites incubated with or without glutamine. The steady-state ATP level in the Δ*tggt1* mutant was nearly half of the parental and complemented strains, which was nonetheless sufficient to sustain the invasion process (Figure 8C-D). A short (30 min) incubation in glutamine-free medium caused a statistically significant 34% decline in ATP content of the mutant compared to a less prominent decline of 23% in the parental and complemented strains. The viability of all parasites in glutamine-free medium was not affected (not shown). The invasion and ATP assays on the parental strains (Figure 8) agree with earlier work reporting that extracellular parasites (RH *hxgprt*$^{-}$) do not require exogenous carbon source for the maintenance of ATP, gliding motility and invasion (61). The fact that ATP content is reduced but not ablated in the glutamine-free Δ*tggt1* tachyzoites can be explained by endogenous glutamine, and/or potential usage of alternative energy sources including gamma-aminobutyric acid (29). A decline in energy below a certain threshold however appears to be inadequate for invasion by the Δ*tggt1* strain (Figure 8). In conclusion, glucose and glutamine can deliver adequate energy for a normal invasion process, and glutamine-dependent ATP synthesis becomes very important when the parasite is unable to import glucose.

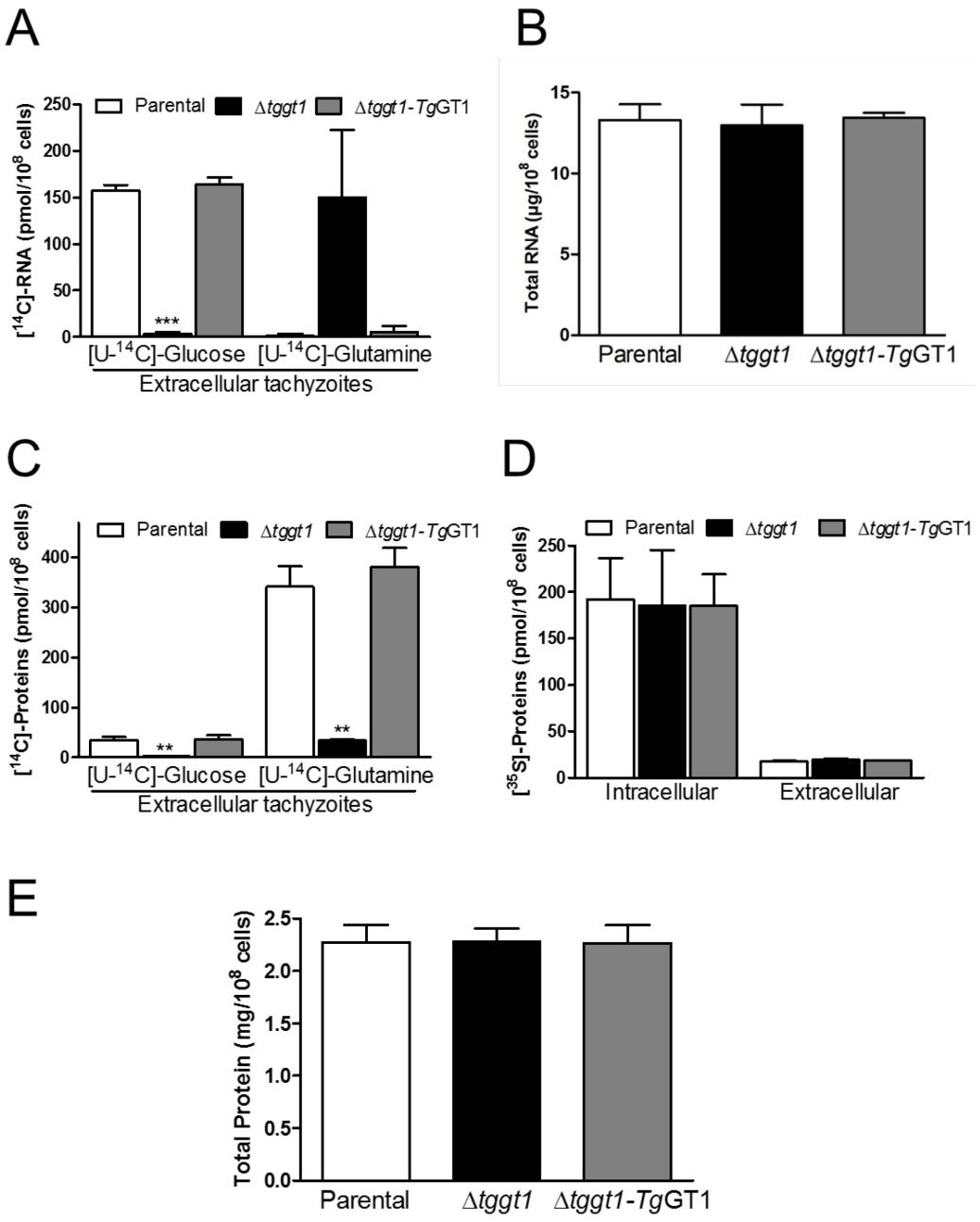


Figure 9. Glucose and glutamine are co-utilized and co-regulated for ribogenesis and protein synthesis.

(A) Metabolic labeling of RNA with [U-^{14}C]-glucose or [U-^{14}C]-glutamine in fresh extracellular parasites. Fresh syringe-released parasites were cultured with either of the radioisotopes followed by estimation of radiolabeling in total purified RNA (mean +/- SEM, n=3). RNA yields in all radiolabeled samples were similar (not shown). (B) Steady-state RNA content in extracellular tachyzoites, as quantified by UV-absorption spectroscopy. (C) Radioisotope labeling of nascent proteins in extracellular parasites incubated with [U-^{14}C]-glucose or [U-^{14}C]-glutamine. The parasite proteins were extracted to determine the incorporated radioactivity (mean +/- SEM, n=3). (D) Radiolabeling of total proteins in extracellular and intracellular parasites with a commercial mixture of 35S-methionine and ^{35}S-cysteine (mean +/- SEM, n=3). (E) Protein contents in extracellular parasites, as measured by bicinchoninic acid assay. Statistics was performed separately for each group using student's t-test (**, $p<0.01$; ***, $p<0.001$).

3.1.5. Glucose and glutamine together facilitate the biogenesis of biomass in *T. gondii*

To investigate the importance of glucose and glutamine for anabolic metabolism, their usage into major biomass components was determined, *i.e.* nucleotides, proteins and lipids. Extracellular parasites of aforementioned strains were incubated with either ^{14}C-glucose or ^{14}C-glutamine in defined medium with both carbon sources and quantified the incorporation of radioactivity into individual biomass fractions (Figure 9, Figure 10A). A robust incorporation of radioactive label from glucose into RNA of the parental and complemented strains was detected, confirming the operation of pentose phosphate shunt in *T. gondii* (Figure 9A), while incorporation of glutamine was significantly lower. Consistent with its inability to import the sugar (40), utilization of ^{14}C-glucose was negligible in the Δ*tggt1* mutant. Conversely, ^{14}C-glutamine labeling was similar to that of ^{14}C-glucose in the parental and complemented strains. Total RNA levels were comparable in all three strains (Figure 9B), signifying that even upon impairment of glycolysis, the parasite maintained the necessary carbon flux through pentose phosphate pathway, apparently using glutamine-fueled gluconeogenesis.

The radiolabeling of total parasite protein in the parental and complemented strains demonstrated that incorporation of glucose was about six-fold lower than glutamine (Figure 9C). Labeling of nascent proteins by glucose can be explained by the synthesis of nonessential amino acids (*e.g.* glutamate) from sugar (Figure 9, Figure 10), whereas glutamine is possibly incorporated into proteins directly. A fraction of sugar-labeled proteins may also represent glycosylated proteins present in tachyzoites (77). Protein labeling was either completely ablated (in the case of ^{14}C-glucose) or evidently reduced (if ^{14}C-glutamine was used) in the Δ*tggt1* mutant. Although absence of protein labeling with radioactive glucose in Δ*tggt1* strain can be explained by impaired glycolysis, a significant decline in glutamine usage is intriguing. To test whether such a regression was caused by a systemic shutdown of protein synthesis in the mutant due to compromised flow of carbon, tachyzoites were labeled with ^{35}S-methionine and ^{35}S-cysteine (Figure 9D). As anticipated, protein synthesis in intracellular and extracellular parasites corresponded to their metabolically active and quiescent nature, respectively. More importantly, all three strains exhibited a comparable inclusion of radioisotopes into nascent protein pools during extracellular as well as intracellular stages. In accord with a comparable protein synthesis, protein contents of the strains were also equivalent (Figure 9E). The observed decline in glutamine labeling of the Δ*tggt1* mutant therefore appears to be a consequence of metabolic rewiring aimed at accommodating the energy demands of extracellular parasites.

3.1.6. The Δ*tggt1* mutant displays a defective biogenesis of lipids

Next contributions of glucose and glutamine for lipogenesis in extracellular tachyzoites were analyzed. Similar to the RNA and protein pools, a considerable labeling of lipids with radioactive glucose in the parental and complemented strains was observed, which indicated influx of sugar-derived carbon (*e.g.*, G3P, fatty acids) for the membrane biogenesis (Figure 10A). Labeling was nearly absent in the Δ*tggt1* strain, as predicted by glycolytic defect. No obvious inclusion of glutamine into lipids was detected in any of the three strains, which ruled out its possible contribution to lipid synthesis, and signified a lack of counteracting mechanism to sustain lipid biogenesis in the Δ*tggt1* strain. To further examine the observation, three major phospholipids were quantified by chemical phosphorous assays. Indeed, a statistically significant and consistent ≈20% decrease in PtdCho and PtdEtn was observed (Figure 10B, Figure S27). To confirm whether such declines were caused by defective syntheses of indicated lipids in the mutant, metabolic labeling was performed with corresponding head groups (^{14}C-choline, ^{14}C-ethanolamine, ^{14}C-serine), as reported in earlier work (33). Whereas choline and ethanolamine are used to produce PtdCho and PtdEtn, respectively, serine is adeptly utilized to synthesize both PtdSer and PtdEtn (33,34). Indeed, radiolabeling with two of three major precursors of phospholipid synthesis (choline and serine) was decreased in the Δ*tggt1* strain (Figure 10C). The mutant also showed declines in predominant species of triacylglycerol (Figure 10D), as determined by lipidomics analyses. A comparative estimate of fatty acids bound to all membrane lipids present in the Δ*tggt1* strain revealed an apparently specific regression in long-chain fatty acids (26:1, 22:1; Figure 10E). These results together showed an important role of glycolysis in lipogenesis and implied a deficit of glucose-derived acetyl-CoA for fatty acid synthesis in the mutant.

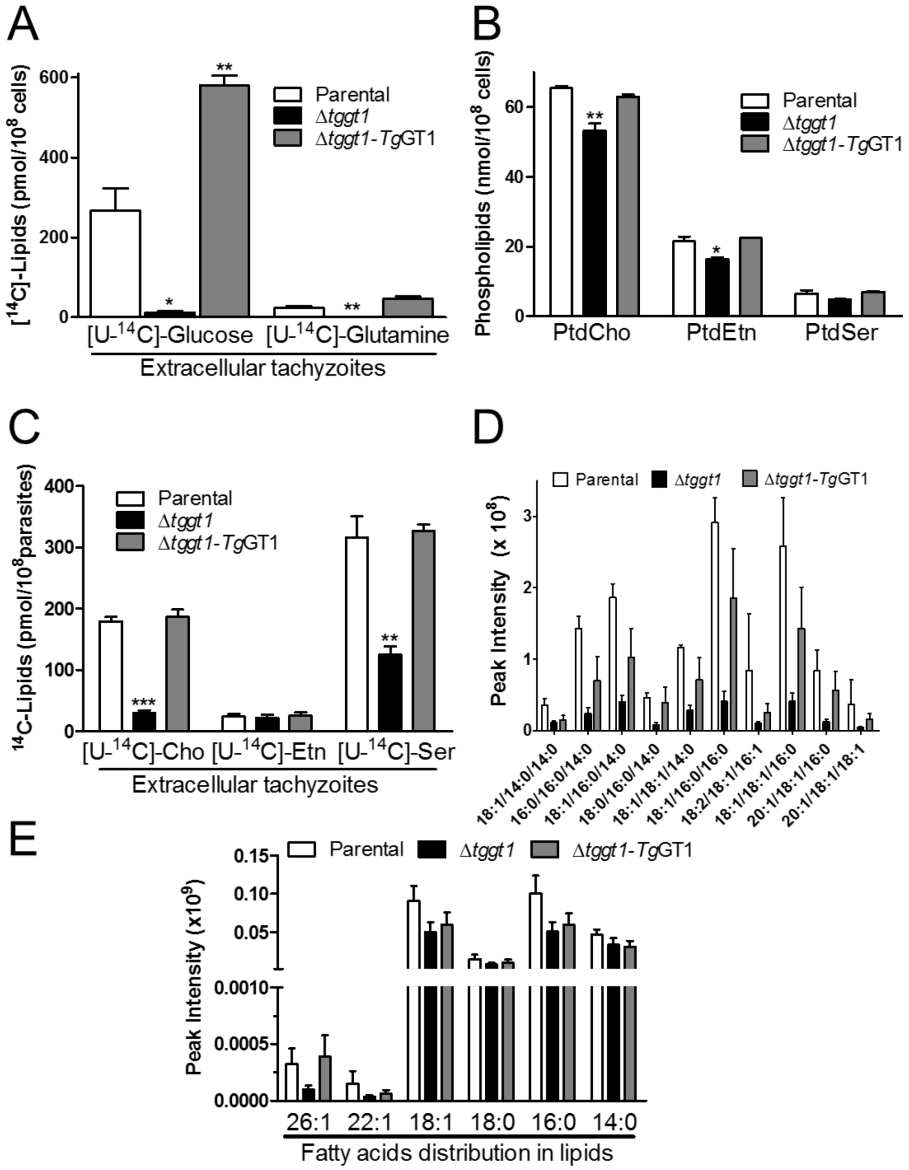


Figure 10. A dysfunctional glycolysis is detrimental to the membrane biogenesis.

(A) Metabolic labeling of nascent lipids using [U-^{14}C]-glucose or [U-^{14}C]-glutamine in extracellular parasites. Total parasite lipids were prepared to determine the radiolabeling by liquid scintillation counting. (B) Comparative amounts of three major lipids in the depicted strains. Phospholipids extracted from extracellular parasites were resolved by thin layer chromatography, detected by iodine-vapor staining, and quantified by chemical-phosphorous assay. Abbreviations: PtdCho, phosphatidylcholine, PtdEtn, phosphatidylethanolamine, PtdSer, phosphatidylserine. (C) Radiolabeling of parasite lipids with choline, ethanolamine or serine. Extracellular tachyzoites were incubated with either of the [U-^{14}C]-labeled head groups and radiotracer incorporated into total lipids was measured. The data plotted in panel A-C show mean +/- SEM from three assays. (D) Relative contents of the major triacylglycerol species in the three parasite strains, as monitored by lipidomics analysis. Lipids isolated from fresh extracellular parasites were analyzed by UPLC-MS (mean +/- SEM, n=5). (E) Estimated amounts of acyl chains conjugated to total lipids in specified strains. Intensities on the y-axis denote cumulative sum of all peaks corresponding to a given acyl chain irrespective of the bound lipid (mean +/- SEM, n=5). Lipids bound to the specific acyl chains were quantified from the areas of chromatographic peaks. Statistical significance was measured separately for each group with respect to the parental strain using student's t-test (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). Note that the *Δtggt1* mutant in panel D-E did not show any significant difference for individual lipid species; however, a collective reduction across all lipid species is very significant (2way ANOVA) (Panel D, $p < 0.0001$; Panel E, $p = 0.0060$).

3.1.7. Acetate supplementation can amend the phenotypic defects in the Δ*tggt1* mutant

It has been shown previously that tachyzoites can incorporate acetate, particularly into long-chain fatty acids produced by elongase (and/or FAS1?) pathways (30,31). Accordingly, the bioinformatics search identified a putative acetyl-CoA synthetase (*Tg*ACS) in the parasite database (www.ToxoDB.org). A 3'-genomic tagging of *Tg*ACS protein with a C-terminal HA epitope was performed (Figure 11A). Expression of *Tg*ACS-HA in the eventual transgenic strain was regulated by its native promoter and *TgGra2*-3'UTR. Immunoblot analysis revealing a single band of expected size (80 kDa) confirmed the protein integrity and endogenous expression of *Tg*ACS in tachyzoites (Figure 11B). Immunofluorescence assays showed a predominantly cytosolic expression of the epitope-tagged protein in intracellular tachyzoites, which co-localized with a known cytosolic marker *Tg*Hsp90 ((64), Figure 11C). Next the influence of exogenous acetate on the plaque and replication phenotypes of all strains was compared (Figure 11D-E). Consistent with reduced plaque size in regular (acetate-free) medium, the Δ*tggt1* strain replicated slower, as discerned by a higher fraction of smaller vacuoles compared to the control strains. Both phenotypic defects were entirely restored in the Δ*tggt1* mutant cultured with acetate (Figure 11D-E). Labeling of the parasites with radioactive acetate demonstrated a significant incorporation into lipids of all three strains, with intracellular parasites showing an expectedly higher inclusion of isotope (Figure 11F). In agreement with its recovered growth and replication, the Δ*tggt1* mutant assimilated as much isotope as the parental and complemented strains. These results show that a dysfunction of glycolysis impairs lipogenesis, which compromises the lytic cycle of *T. gondii* in standard cultures lacking acetate. The data also show a flexible use of available nutrients by the parasite.

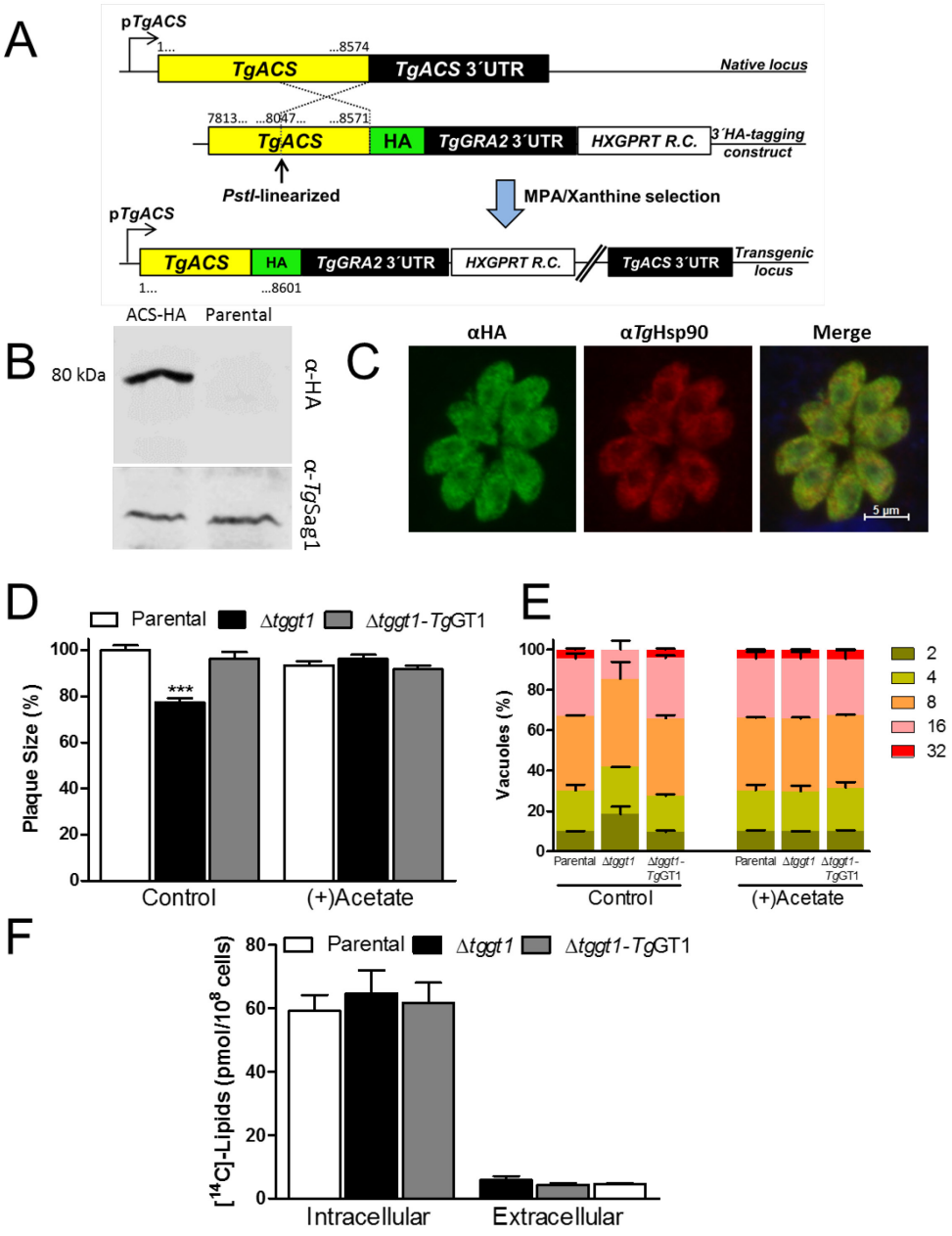


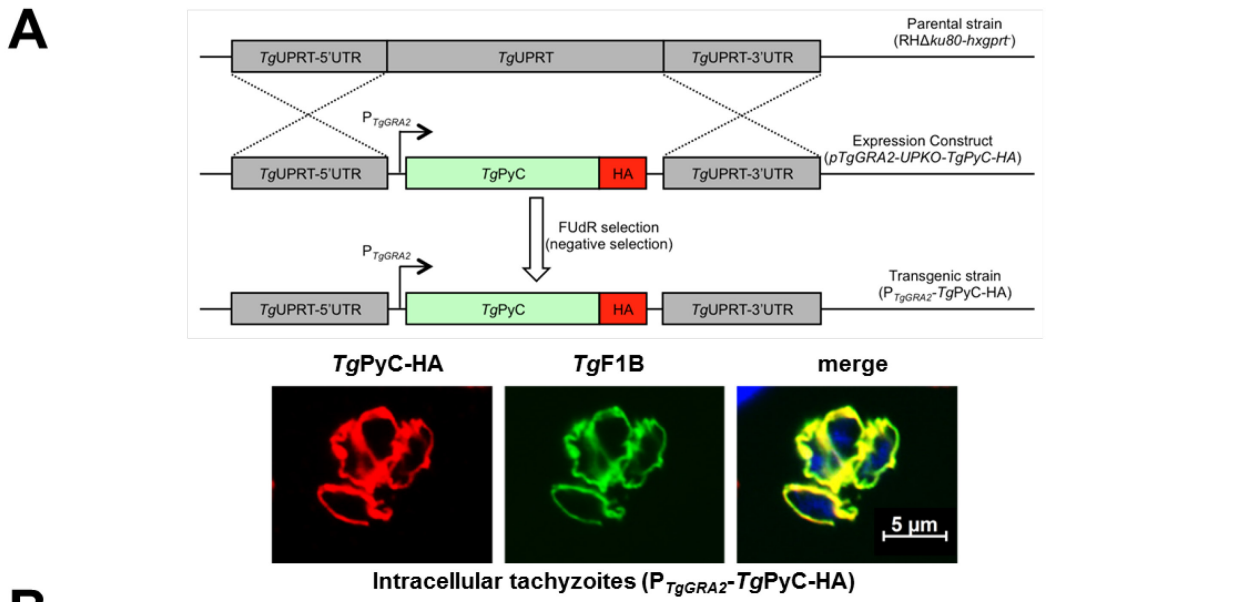
Figure 11. Growth impairment in the Δ*tggt1* mutant is restored by acetate supplementation.

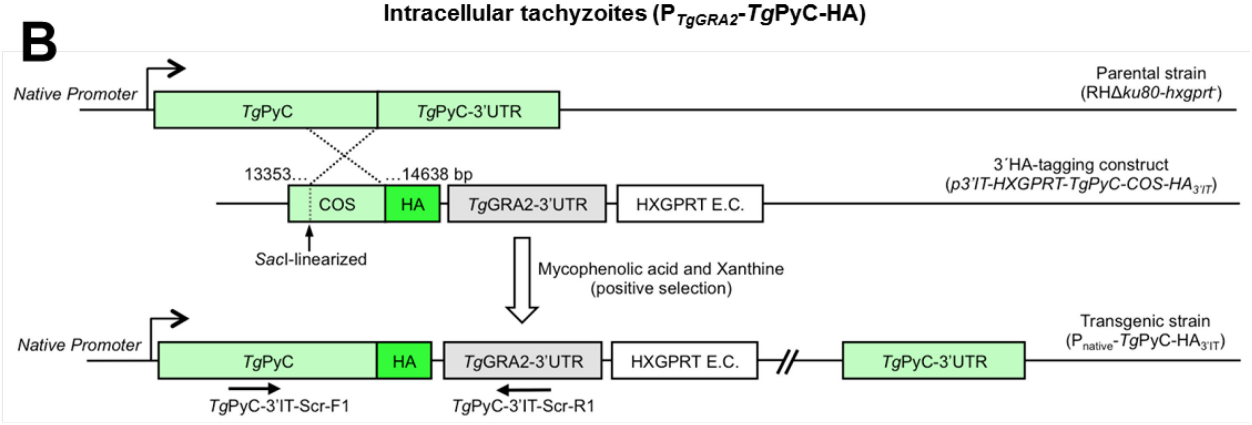
(A) Schematic illustration of epitope-tagging for expressing *Tg*ACS-HA under the control of its native promoter and *Tg*Gra2-3'UTR. The construct for 3'-HA tagging of the *Tg*ACS gene was transfected and drug-selected in the RHΔku80-hxgprt- strain. (B) Immunoblot of transgenic (expressing *Tg*ACS-HA) and parental strains using α-HA and α-TgSag1 (loading control) antibodies. (C) Immunofluorescent localization of *Tg*ACS-HA protein in intracellular tachyzoites. Parasitized cells (24 h infection) were immunostained with α-HA and α-*Tg*Hsp90 (cytosolic marker) antibodies. (D) Plaque growth of the designated parasite strains cultured in standard cell culture medium with or without 2 mM acetate (7d, 37°C, 5% CO_2). Plaques were stained with crystal violet prior to ImageJ analysis (mean +/- SEM, n=3). Statistical significance (student's t-test) was measured separately for each group with respect to the parental strain (***, $p<0.001$). (E) Replication rates of the three parasite strains cultured in the absence or presence of 2 mM acetate. Parasitized fibroblasts (24 h infection) were immunostained with α-*Tg*Gap45, and the parasitophorous vacuoles were scored for the parasite numbers (mean +/- SEM, n=3). (F) Radiolabeling of parasite lipids using [U-^{14}C]-acetate. Extracellular or intracellular tachyzoites were incubated with the radioisotope for 4 h (37°C, 5% CO_2) followed by scintillation counting of lipid fractions isolated from purified parasites (mean +/- SEM, n=3).

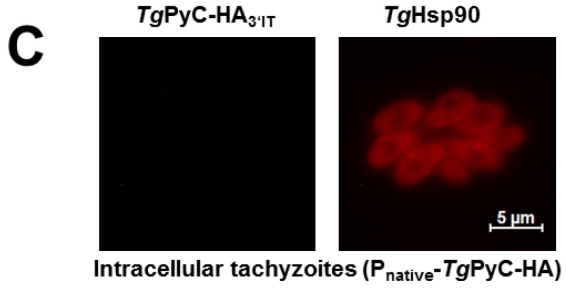
3.2. Glutamine fuelled gluconeogenesis becomes essential in glucose limiting conditions

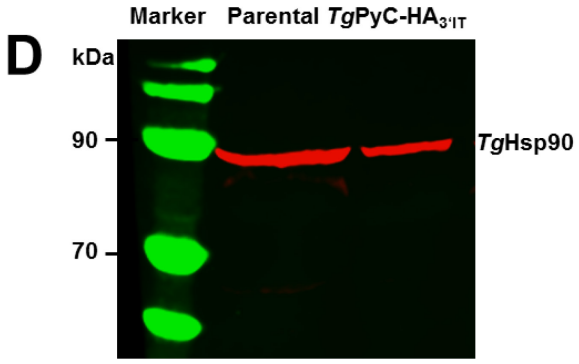
3.2.1. Pyruvate carboxylase is a mitochondrial protein but it is not expressed in tachyzoites

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 (78). A bioinformatics search using mammalian isoforms identified one putative PyC in the parasite genome (TGGT1_284190; www.ToxoDB.org). The complete ORF of *Tg*PyC was cloned, encoding for a protein of 1391 amino acids, which contains a conserved pyruvate carboxylase domain. Analysis of the sequence using MitoProtII suite predicted a mitochondrial localization. In accordance, ectopic expression of C-terminally tagged *Tg*PyC (*Tg*PyC-HA) in intracellular tachyzoites revealed that the protein localized in the mitochondrion, as confirmed by immunofluorescent staining with a known organelle marker, *Tg*F1B ATPase (79) (Figure 12A). Subsequently, the corresponding gene was epitope-tagged to examine the native expression (Figure 12B). Unexpectedly, neither by immunofluorescence nor by immunoblot analyses expression of *Tg*PyC-HA in tachyzoites was detected (Figure 12C-D).


A
Parental strain
(RHΔku80-hxgprt-)
TgUPRT-5'UTR
TgUPRT
TgUPRT-3'UTR
P TgGRA2
TgPyC
HA
Expression Construct
(pTgGRA2-UPKO-TgPyC-HA)
FUdR selection
(negative selection)
Transgenic strain
(P TgGRA2-TgPyC-HA)
TgPyC-HA
TgF1B
merge
5 µm
Intracellular tachyzoites (P TgGRA2-TgPyC-HA)


B
Native Promoter
TgPyC
TgPyC-3'UTR
Parental strain
(RHΔku80-hxgprt-)
13353...
...14638 bp
COS
HA
TgGRA2-3'UTR
HXGPRT E.C.
3´HA-tagging construct
(p3'IT-HXGPRT-TgPyC-COS-HA3'IT)
SacI-linearized
Mycophenolic acid and Xanthine
(positive selection)
Transgenic strain
(P native-TgPyC-HA3'IT)
TgPyC-3'IT-Scr-F1
TgPyC-3'IT-Scr-R1


C
TgPyC-HA3'IT
TgHsp90
5 µm
Intracellular tachyzoites (P native-TgPyC-HA)


D
Marker
Parental TgPyC-HA3'IT
kDa
90
70
TgHsp90

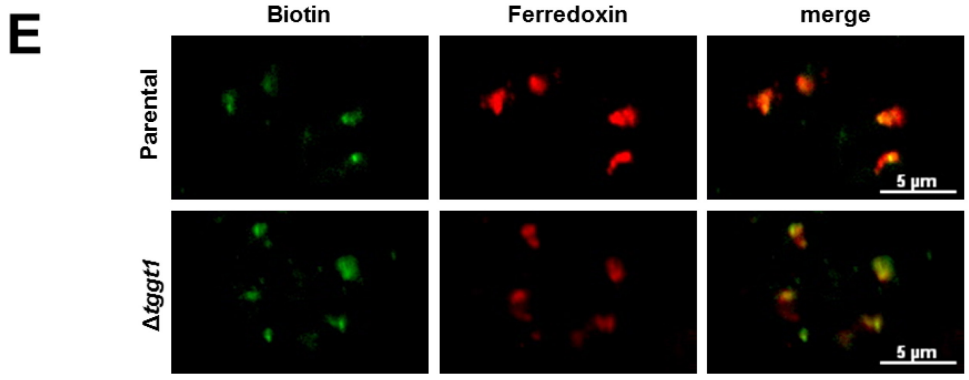

E
Biotin
Ferredoxin
merge
Parental
Δtggt1
5 µm
5 µm

Figure 12. 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 *Tg*PyC tagged with a hemagglutinin epitope (HA) and regulated by the *TgGRA2* elements was targeted at the *TgUPRT* locus *via* double homologous recombination in the RHΔ*ku80-hxgprt*$^-$ strain. Stable transgenic parasites were selected by FUdR for the loss of UPRT function and subjected to immunostaining with α-HA and α-*Tg*F1B antibodies. (B) Genomic tagging of the endogenous *Tg*PyC 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 *Sac*I enzyme and transfected in the RHΔ*ku80-hxgprt*$^-$strain. Parasites expressing *Tg*PyC-HA$_{3'IT}$ under the control of native promoter and *TgGRA2*-3'UTR were drug-selected, screened by genomic PCR using recombination-specific primers (*Tg*PyC-3'IT-Scr-F1/R1) and sequencing of the transcript. (C-D) Detection of *Tg*PyC-HA$_{3'IT}$ by immunofluorescence and western blot analysis. Intracellular parasites (24 h infection) were immunostained with α-HA and α-*Tg*Hsp90 antibodies to monitor the endogenous expression and location of *Tg*PyC-HA$_{3'IT}$. For immunoblot, extracellular tachyzoites (10^7) were subjected to SDS-PAGE, blotting and staining with α-HA and α-*Tg*Hsp90 (loading control) antibodies. (E) Detection of *Tg*PyC in the parental (RHΔ*ku80-hxgprt*$^-$) and Δ*tgpyc* strains using α-biotin and α-*Tg*Fd 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.

To further examine the expression of *Tg*PyC under normal glucose-replete condition and in a glucose-uptake impaired mutant, anti-biotin antibody staining of the prosthetic group was utilized (Figure 12E). Yet again, it failed to detect immunostaining of the parasite mitochondrion, suggesting the absence of expression. In contrast, it was feasible to detect another biotin-dependent enzyme acetyl-CoA carboxylase that colocalized with ferredoxin, a marker of the apicoplast in tachyzoites (80). 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 or deficient glycolysis.

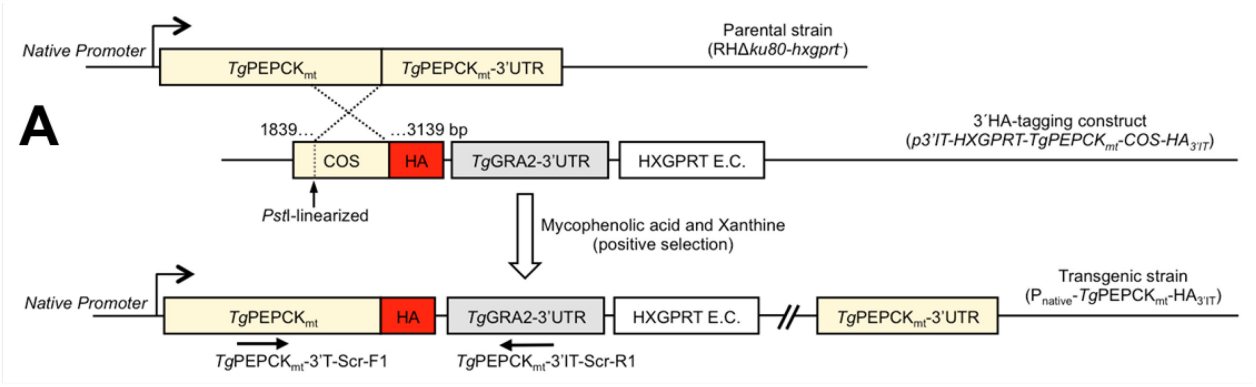
3.2.2. Pyruvate carboxylase is dispensable for the lytic cycle of tachyzoites

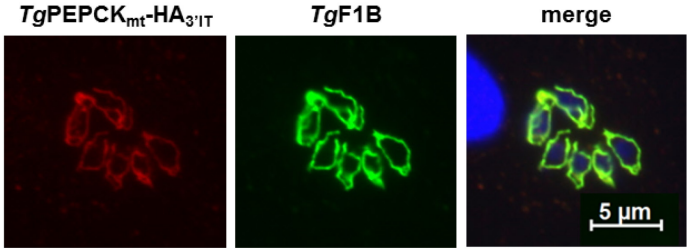
In proliferating mammalian cells, one of the established functions of pyruvate carboxylase is to drive anaplerosis under normal condition (78). A second equally important function is to power gluconeogenesis under glucose starvation. To investigate anaplerotic and gluconeogenic significance of *Tg*PyC in parasites, knockout mutants in glycolysis-competent and glycolysis-deficient strains were engineered, respectively. The *TgPyC* gene was replaced by homologous recombination-mediated insertion of the DHFR-TS expression cassette using pyrimethamine selection (Figure S29A). The absence of *Tg*PyC transcript in representative clones of Δ*tgpyc* and Δ*tggt1*/Δ*tgpyc* strains confirmed the genetic deletion of pyruvate carboxylase (Figure S29B). The successful making of the two mutants shows that *Tg*PyC is not required for the survival of tachyzoites. The mutants were assessed for the overall growth fitness in plaque assays (Figure S29C). None of the knockout strains of *Tg*PyC exhibited a noteworthy defect. The sizes as well as numbers of plaque formed by the Δ*tgpyc* and Δ*tggt1*/Δ*tgpyc* mutants were comparable to respective progenitor strains. These results resonate well with insignificant expression of pyruvate carboxylase in the tachyzoite stage and establish its physiological dispensability irrespective of glucose catabolism.

3.2.3. The genome of *T. gondii* harbors two distinctive orthologs of PEPCK

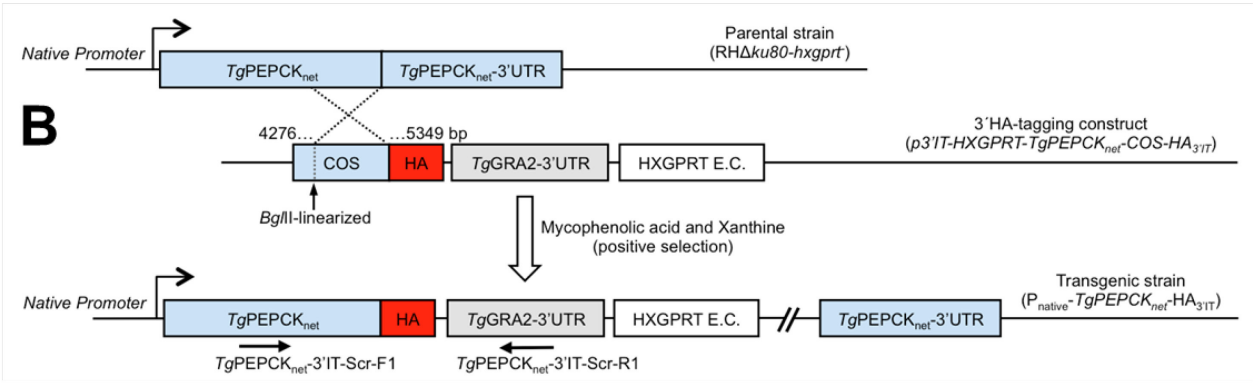
A surprisingly nonessential nature of *Tg*PyC prompted us to examine metabolic relevance of PEPCK for the asexual reproduction of tachyzoites in glucose-replete and glucose-deprived states. Two PEPCK paralogs were identified, *Tg*PEPCK1 (TGGT1_289650) and *Tg*PEPCK2 (TGGT1_289930) in the database (www.ToxoDB.org), which encode for 677 and 614 amino acids, respectively (Figure S30A). Sequence alignment with *bona fide* orthologs confirmed the presence of a complete catalytic domain containing substrate, metal and nucleotide binding residues. MitoProtII analysis predicted a high probability (>95%) of *Tg*PEPCK1 being located in the parasite mitochondrion *via* a mitochondrial targeting peptide (Figure S30B). Phylogenetic clustering demonstrated that *Tg*PEPCK1 forms a distinct clade with other PEPCKs from the phylum apicomplexa and closely related to the ATP-dependent orthologs from fungi and plants (Figure S30C). *Tg*PEPCK2 is markedly distinct from *Tg*PEPCK1 and shows substitutions of otherwise-conserved residues in the nucleotide and substrate-binding sites. Quite notably, orthologs of *Tg*PEPCK2 appear to be absent in most organisms; they could only be identified in selected coccidian parasites (*Neospora caninum*, *Hammondia hammondi*).

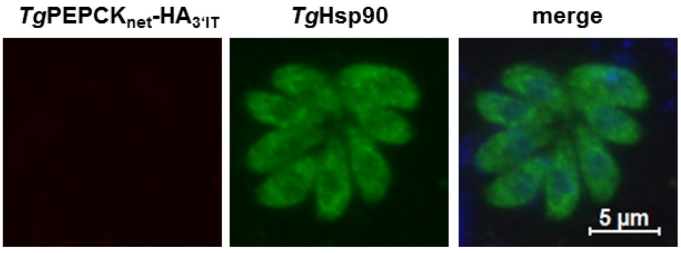
Subsequently, the endogenous expression and subcellular localization of the two PEPCK isoforms in tachyzoites were determined. To this end, crossover-mediated tagging of both genes was performed with a 3'HA epitope (Figure 13A-B). Immunofluorescence staining established that C-terminally HA-tagged *Tg*PEPCK1 (*Tg*PEPCK1-HA$_{3'IT}$) was distributed in the mitochondrion, which was confirmed by its costaining with the organelle marker *Tg*F1B ATPase (Figure 13A). By contrast, *Tg*PEPCK2-HA$_{3'IT}$ was not detectable in tachyzoites despite a successful epitope-tagging of its gene (Figure 13B). Consistently, it was possible to amplify transcript of *Tg*PEPCK1 but not of *Tg*PEPCK2, further corroborating the expression of only former isoform (Figure S31C). Immunoblot analysis confirmed the constitutive expression of *Tg*PEPCK1-HA$_{3'IT}$ (~76 kDa), while *Tg*PEPCK2-HA$_{3'IT}$ could not be detected (Figure 13C). Following these results, *Tg*PEPCK1 was renamed to *Tg*PEPCK$_{mt}$ (mitochondrial), and *Tg*PEPCK2 to *Tg*PEPCK$_{net}$ (not expressed in tachyzoites). According to transcriptomics data (81), *Tg*PEPCK$_{net}$ is significantly induced during development of the parasite in the felid host (enteroepithelial stages; Figure 13D). Therefore, it appears as though *Tg*PEPCK$_{mt}$ and *Tg*PEPCK$_{net}$ serve different developmental stages during the natural lifecycle of *T. gondii*.



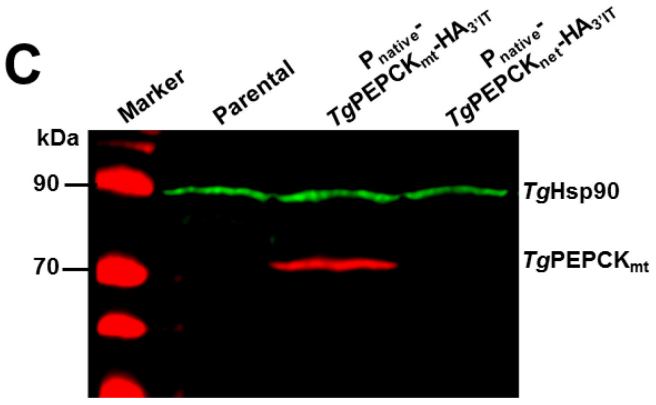


Intracellular tachyzoites (P_{native}-*Tg*PEPCK$_{mt}$-HA$_{3'IT}$)





Intracellular tachyzoites (P_{native}-*Tg*PEPCK$_{net}$-HA$_{3'IT}$)



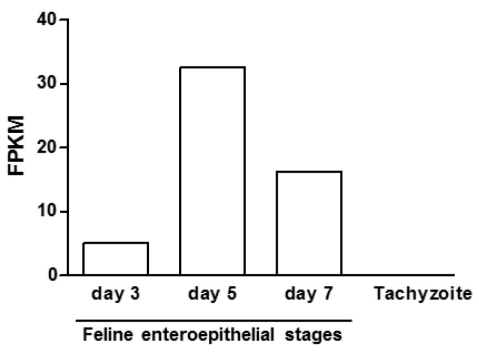


Figure 13. *Tg*PEPCK$_{mt}$ localizes in the mitochondrion, whereas *Tg*PEPCK$_{net}$ is not expressed in the tachyzoite stage of *T. gondii.*

(A) 3'-insertional tagging (3'IT) of the *TgPEPCK$_{mt}$* gene with a HA tag in tachyzoites and detection of *Tg*PEPCK$_{mt}$-HA$_{3'IT}$ protein by immunofluorescence. The *Pst*I-digested plasmid construct with a crossover sequence (COS) targeting the 3'-end of the *TgPEPCK$_{mt}$* gene was transfected into the RHΔ*ku80-hxgprt*$^-$ strain. Tachyzoites encoding *Tg*PEPCK$_{mt}$-HA$_{3'IT}$ under the control of its native promoter and *TgGRA2*-3'UTR were selected with indicated drugs, screened by genomic PCR using *Tg*PEPCK$_{mt}$-3'IT-Scr-F1/R1 primers, and verified by sequencing. Intracellular parasites (24 h infection) were immunostained with α-HA and α-*Tg*F1B antibodies to determine the subcellular location of *Tg*PEPCK$_{mt}$-HA$_{3'IT}$. (B) Genomic tagging of the *TgPEPCK$_{net}$* gene and fluorescent detection of *Tg*PEPCK$_{net}$-HA$_{3'IT}$ in tachyzoites. Stable transgenic parasites were generated and immunostained with α-HA and α-*Tg*Hsp90 antibodies, as stated in p*anel A*. (C) Immunoblot showing the natural expression of *Tg*PEPCK$_{mt}$-HA$_{3'IT}$, *Tg*PEPCK$_{net}$-HA$_{3'IT}$ and *Tg*Hsp90 in transgenic tachyzoites from *panel A-B*. Extracellular parasites (10^7) of the indicated strains were subjected to protein isolation followed by SDS-PAGE and immunostaining using α-HA and α-*Tg*Hsp90 (loading control) antibodies. Parental strain served as a negative control for α-HA staining. (D) Comparative levels of *Tg*PEPCK$_{net}$ 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.

3.2.4. Only *Tg*PEPCK$_{mt}$ is required for an optimal lytic cycle under glucose-replete condition

The biological importance of both PEPCKs was examined in the glycolysis-competent as well as in glycolysis-deficient mutants. First, mutants of *Tg*PEPCK$_{net}$ in the standard (RHΔ*ku80*-TaTi) and its derivative Δ*tggt1* strains were generated by exchanging the gene with a DHFR-TS expression cassette *via* double homologous recombination (Figure S31A). The events of 5' and 3' crossover were verified by screening of pyrimethamine-resistant parasite clones. In agreement with aforementioned results (Figure 13C), neither the parental nor the Δ*tggt1* strain displayed the expression of *Tg*PEPCK$_{net}$ transcript (Figure S31B). Consequently, the resulting Δ*tgpepck*$_{net}$ and Δ*tggt1*/Δ*tgpepck*$_{net}$ mutants also lacked the mRNA of *Tg*PEPCK$_{net}$. No significant impairment in the plaque area or plaque number of the single and double mutants was evident when compared to respective progenitor strains (Figure S31C). It can therefore be concluded that *Tg*PEPCK$_{net}$ is dispensable for the lytic cycle of tachyzoites.

Using a similar approach, the physiological requirement of *Tg*PEPCK$_{mt}$ was examined for the parasite growth in the presence or absence of glucose catabolism (Figure 14A). Indeed, it was feasible to knock out successfully the *Tg*PEPCK$_{mt}$ gene locus by introducing a DHFR-TS expression cassette, albeit only in the standard parental (RHΔ*ku80*-TaTi) strain. Pyrimethamine-resistant parasite clones were screened by genomic PCR, which displayed amplification of 5' and 3' recombination-specific bands in selected clones (Figure 14B). Sequencing of amplicons from a clone representing the Δ*tgpepck$_{mt}$* mutant confirmed the deletion of the *TgPEPCK$_{mt}$* locus. As expected, the Δ*tgpepck$_{mt}$* mutant lacked the transcript

expression, further endorsing the gene knockout (Figure 14C). The mutant formed smaller plaques (30% growth defect) when compared to the parental strain, while plaque numbers were not changed (Figure 14D). To ascertain the requirement of *Tg*PEPCK$_{mt}$ in glucose-limiting conditions, it was attempted to delete the gene in the Δ*tggt1* strain. However, multiple efforts to make a Δ*tggt1*/Δ*tgpepck$_{mt}$* 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-derived condition.

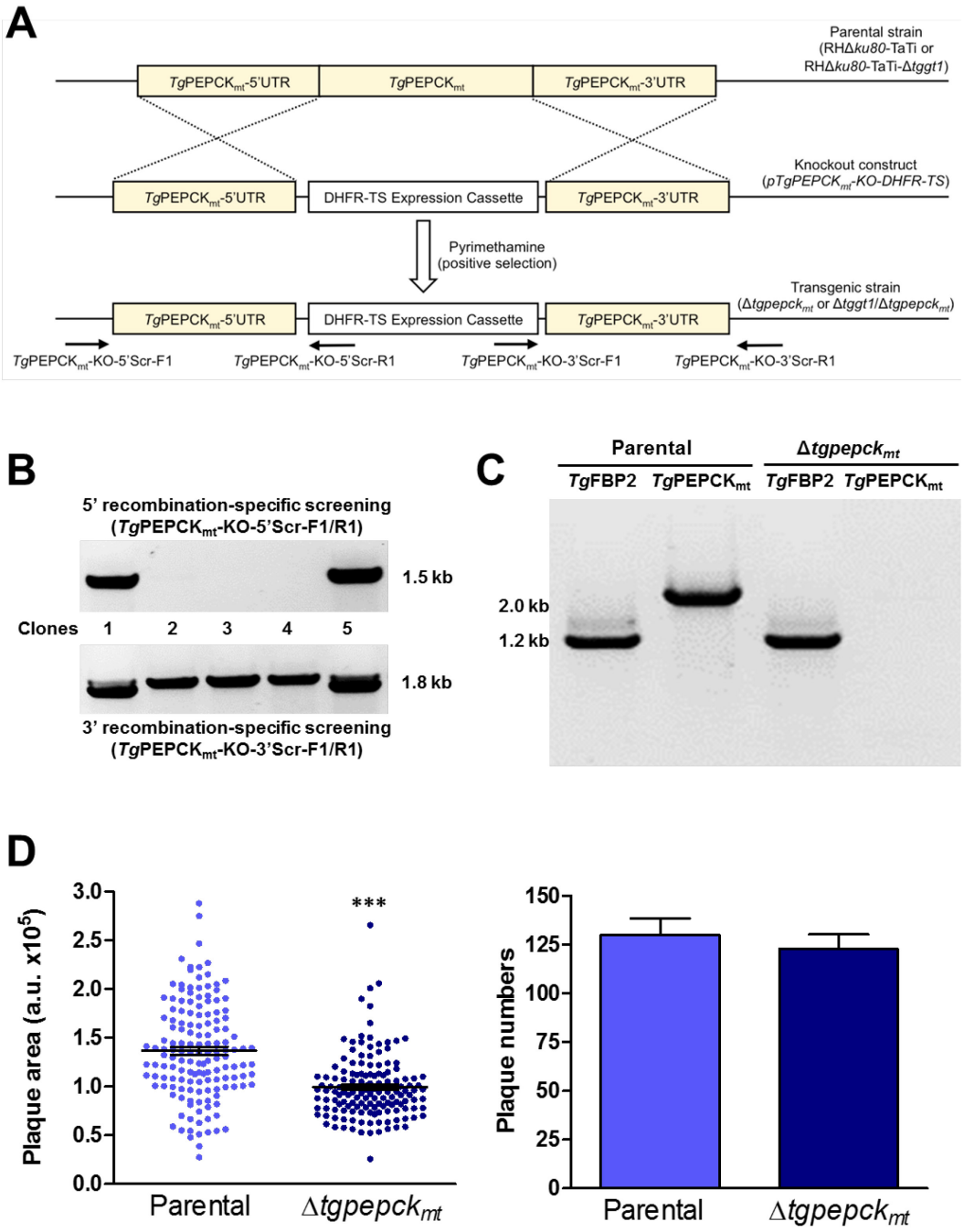


Figure 14. *Tg*PEPCK$_{mt}$ promotes the lytic cycle of glycolysis-competent tachyzoites.

(A) Scheme depicting double homologous recombination-mediated knockout of the *TgPEPCK$_{mt}$* 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 (*Tg*PEPCK$_{mt}$-KO-5'Scr-F1/R1 or *Tg*PEPCK$_{mt}$-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$_{mt}$* knockout in recombination-positive clones was confirmed by sequencing of PCR amplicons. (C) Validation of the Δ*tgpepck$_{mt}$* mutant by transcript analysis. A representative clonal mutant was examined for the presence or absence of *Tg*PEPCK$_{mt}$ and *Tg*FBP2 (control) transcripts using ORF-specific primer sets. Parental strain was included as a positive control. (D) Plaque assays revealing comparative growth of the Δ*tgpepck$_{mt}$* 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 (***, $p<0.001$).

3.2.5. *Tg*PEPCK$_{mt}$ is critical for glucose-independent growth of tachyzoites

An apparent lethality of the Δ*tggt1*/Δ*tgpepck$_{mt}$* mutant prompted us to engineer an inducible knockdown of *Tg*PEPCK$_{mt}$ in the Δ*tggt1* strain (Δ*tggt1*/iΔ*tgpepck$_{mt}$*). To achieve this, first an ATc-regulated and C-terminally HA-tagged ORF of *Tg*PEPCK$_{mt}$ was introduced at the *Tg*UPRT locus by FUdR selection and subsequently knocked out the gene by a DHFR-TS expression cassette using pyrimethamine selection (Figure 15A). As envisioned, the *Tg*PEPCK$_{mt}$ transcript was significantly repressed (not detectable) upon treatment of the conditional mutant with ATc for 72 h (Figure 15B). Consistently, immunofluorescence and immunoblot assays confirmed a marked inhibition of protein expression in the Δ*tggt1*/iΔ*tgpepck$_{mt}$* strain within a day of ATc treatment, which was abolished upon prolonged exposure to the drug (see 48-72 h in Figure 15C-D).

A chemically repressible conditional mutant of *Tg*PEPCK$_{mt}$ in the Δ*tggt1* strain enabled us to evaluate its importance for glucose-independent plaque growth of tachyzoites (Figure 16A-B). As expected, the precursor Δ*tggt1* mutant was not impacted by treatment with ATc, whereas the double mutant was severely impaired upon repression of *Tg*PEPCK$_{mt}$. The condition mutant formed miniscule-size plaques even though their numbers were comparable with the Δ*tggt1* strain. The measurements of growth rates by serial passage (Figure 16C) demonstrated a continued unaltered reproduction of the Δ*tggt1* mutant irrespective of ATc. Likewise, the double mutant behaved normally without ATc, but slowed progressively during successive passages and ceased to grow within a week of drug exposure in cultures, establishing an essential role of the mitochondrial PEPCK in glycolysis-deficient tachyzoites.

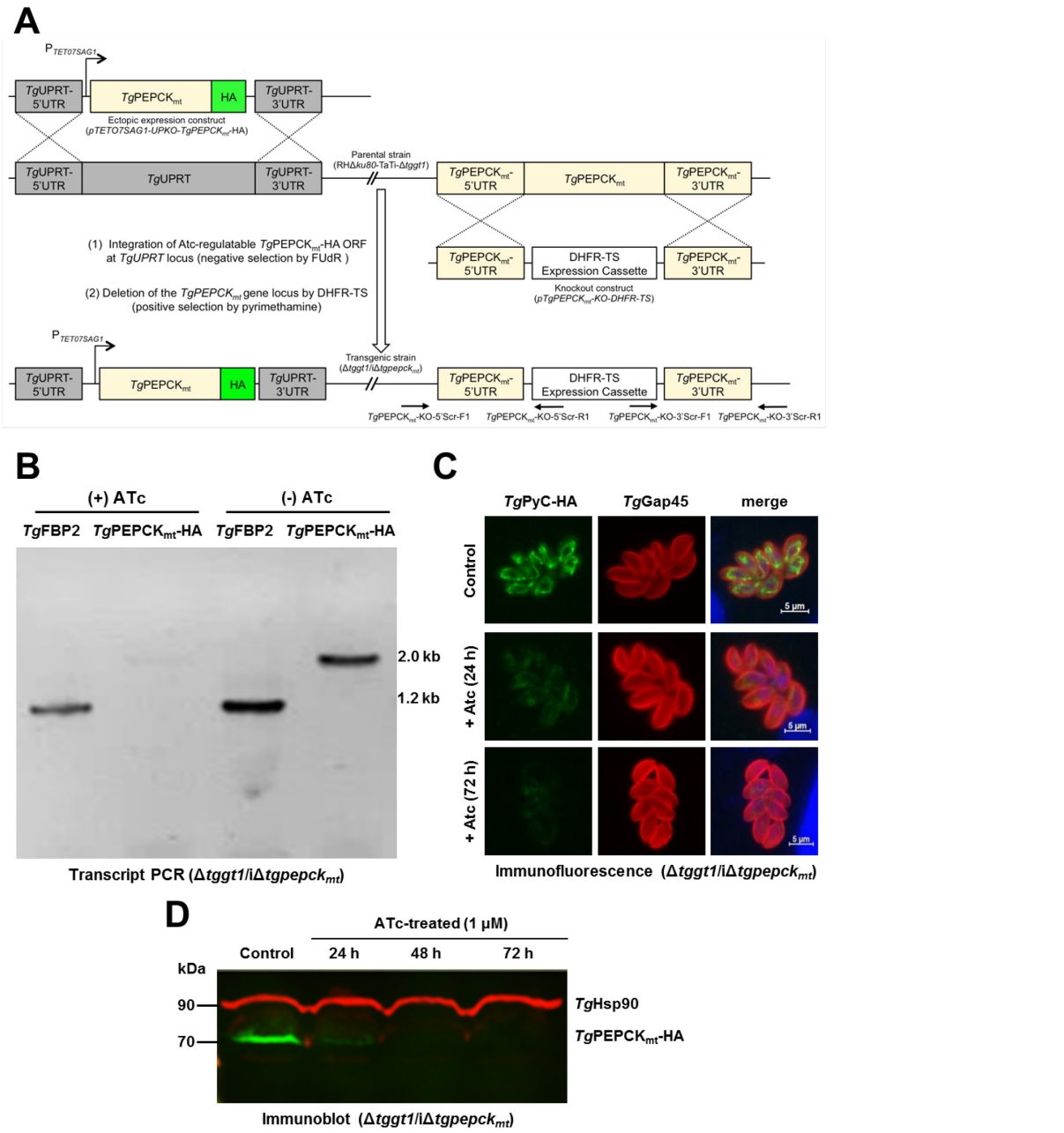


Figure 15. Conditional mutagenesis enables a tetracycline-regulated knockdown of *Tg*PEPCK$_{mt}$ in glycolysis-impaired tachyzoites.

(A) Scheme for generating a tetracycline-inducible mutant of *Tg*PEPCK$_{mt}$ (iΔ*tgpepck$_{mt}$*) in a GT1-knockout strain with impaired glycolysis (RHΔ*ku80*-TaTi-Δ*tggt1*). In the first step, an ATc-repressible ORF of *Tg*PEPCK$_{mt}$-HA was integrated at the *TgUPRT* locus by FUdR selection and then *TgPEPCK$_{mt}$* gene was replaced by DHFR-TS. The eventual Δ*tggt1*/iΔ*tgpepck$_{mt}$* mutant was identified by genomic PCR using 5′ and 3′-crossover-specific primers (*Tg*PEPCK$_{mt}$-KO-5′Scr-F1/R1 and *Tg*PEPCK$_{mt}$-KO-3′Scr-F1/R1). (B) PCR confirming the regulation of *Tg*PEPCK$_{mt}$ transcript by ATc in the Δ*tggt1*/iΔ*tgpepck$_{mt}$* mutant. Total parasite RNA was used to amplify *Tg*PEPCK$_{mt}$-HA and *Tg*FBP2 (control for RNA integrity) using ORF-specific primers. (C) Immunostaining of the Δ*tggt1*/iΔ*tgpepck$_{mt}$* strain showing ATc-regulation of *Tg*PEPCK$_{mt}$-HA protein. The untreated control and drug-treated parasites (24 h infection) were stained using α-HA and α-*Tg*Gap45 (a marker of inner membrane complex) antibodies. (D) Immunoblot depicting the ATc-mediated repression of *Tg*PEPCK$_{mt}$-HA in the conditional mutant. Parasites (10^7) were subjected to immunoblot analyses using α-HA and *Tg*Hsp90 (loading control) antibodies.

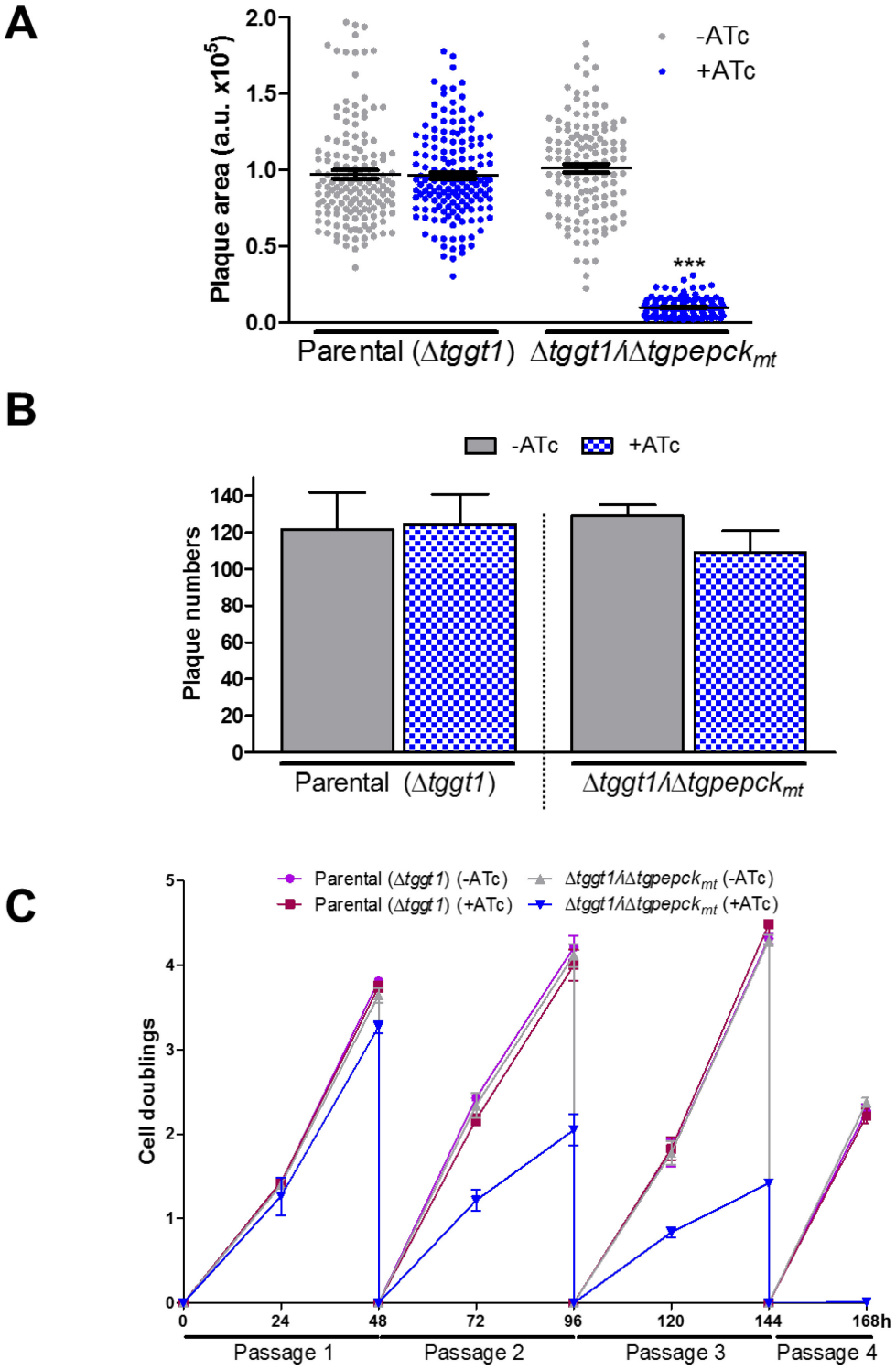


Figure 16. *Tg*PEPCK$_{mt}$ is essential for the lytic cycle of tachyzoites with impaired glycolysis.

(A-B) Growth phenotype of the ATc-regulatable Δ*tggt1*/iΔ*tgpepck*$_{mt}$ strain. The *Tg*PEPCK$_{mt}$ mutant and parental (RHΔ*ku80*-TaTi-Δ*tggt1*) 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).

3.2.6. *Tg*PEPCK$_{mt}$ regulates glutamine-derived gluconeogenic flux in tachyzoites

To discern the underlying basis of observed phenotype in the Δ*tggt1*/iΔ*tgpepck$_{mt}$* mutant, stable isotopes labeling assays were performed. The mutant was cultured with or without ATc for 2 passages and then labeled in standard medium (+/- ATc), in which glutamine was exchanged by [U-^{13}C]-glutamine. The viability of the parasite inoculum was >95% for both conditions. Isotopomer analysis of the double mutant showed a notable flux of glutamine-derived carbon into metabolites associated with TCA cycle and gluconeogenesis in the absence of ATc (Figure S32).

The carbon flux from glutamine into select metabolites linked to gluconeogenesis (PEP, 3PG, G3P, R5P, Pyr) declined in ATc-treated samples that was reflected in their abundance (Figure S32, Figure S33). A somewhat modest but evident decrease in labeling of certain TCA cycle intermediates was observed (Cit, 2OG, Suc, Fum); though, the abundance of these metabolites did not mirror isotope inclusion. A combined assessment of all glutamine-labeled metabolites confirmed a perturbed gluconeogenesis and TCA cycle when expression of *Tg*PEPCK$_{mt}$ was turned off (Figure 17A). The impairment of gluconeogenesis was more obvious than impairment of the citric acid cycle. Last but not least, uniformly (+6) labeled citrate in the *on state* was detected, which was reduced in the *off state*, confirming the occurrence of a complete TCA cycle operated by glutamine only (Figure S32). The observations underline *Tg*PEPCK$_{mt}$ as a metabolic shunt connecting the mitochondrial and cytosolic flux of glutamine-derived carbon.

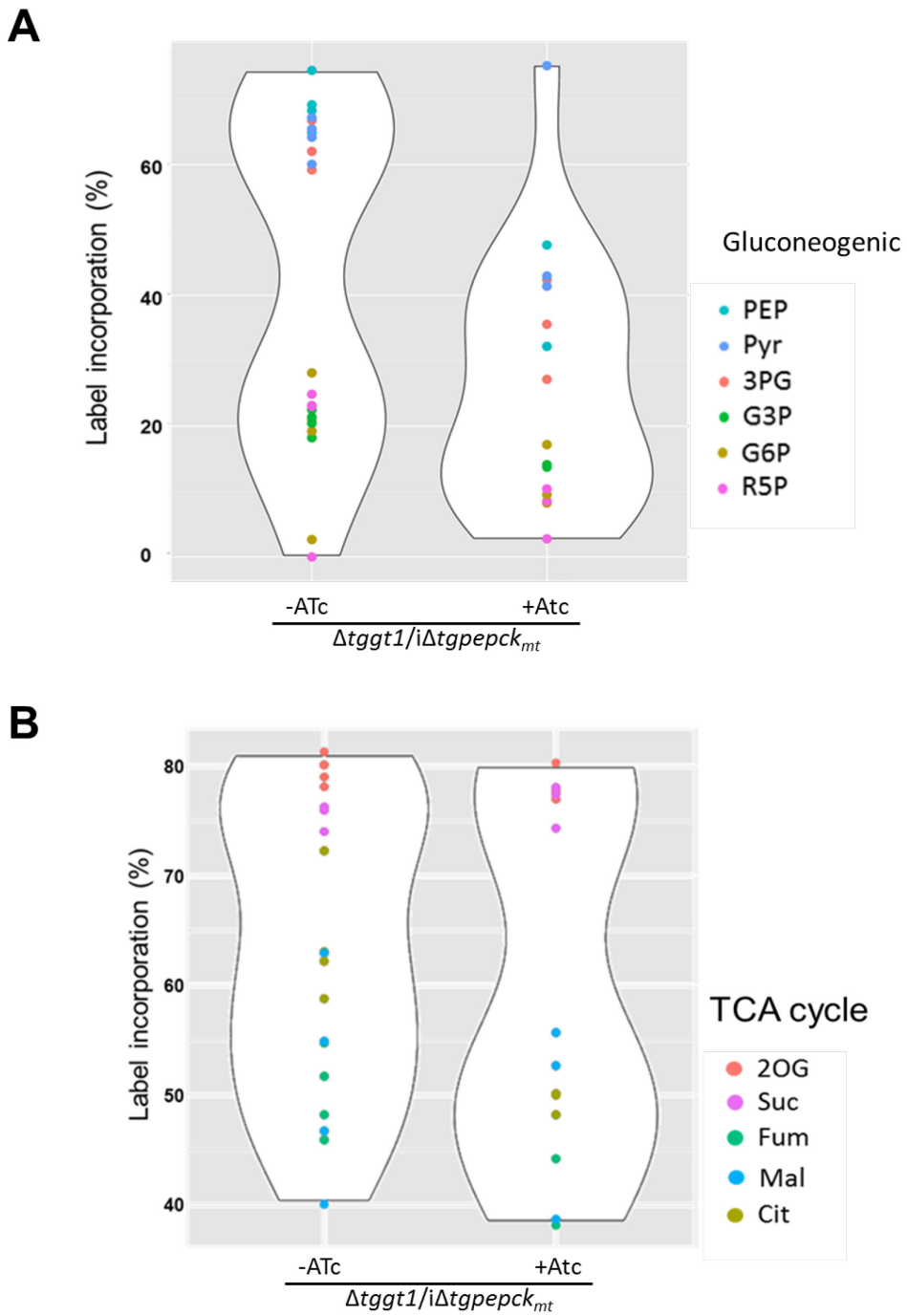


Figure 17. *Tg*PEPCK$_{mt}$ regulates the flux of glutamine-carbon through gluconeogenesis and TCA cycle.

(A-B) Violin plots showing the impairment of glutamine flux into metabolites involved in or directly associated with gluconeogenesis and TCA cycle in the Δ*tggt1*/iΔ*tgpepck*$_{mt}$ mutant. Isotope inclusion was evaluated by metabolic labeling of intracellular tachyzoites with [U-^{13}C]-glutamine in standard culture medium (4 h, 37°C, 5% CO_2). Polar metabolites were isolated from isotope-labeled parasites and subjected to GC-MS analysis. 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.

4. Discussion

4.1. Metabolic cooperation of glucose and glutamine in *Toxoplasma gondii*

Toxoplasma gondii is an obligate parasite, which can infect and replicate in nearly all nucleated cells of an exceptionally wide range of host organisms. Asexual growth of this parasite requires successive rounds of lytic cycles. This work shows that glucose and glutamine together furnish a major fraction of biomass and energy in a co-regulated manner, and such a cooperative metabolism is essential to realize a successful lytic cycle of *T. gondii* in human cells. The facts that the parasite can endure a dearth of either nutrient and it continues to replicate even without these two carbon sources reflect an unparalleled metabolic plasticity in *T. gondii* given its evolutionary adaptation to obligate intracellular parasitism. Furthermore, the parasite is competent in utilizing acetate when available, which can override the glycolytic impairment. Not only do these features ensure the survival of extracellular and intracellular states, they may underlie the parasite growth in variable nutritional milieus encountered in distinct host-cell types. Last but not least, metabolism of intracellular parasites is much more prominent than in the extracellular stage, which resonates well with their high and low proliferative states.

Extracellular parasites strictly depend on glucose and/or glutamine to invade host cells, likely because the extracellular milieu lacks any other substitutive nutrients, which can operate glycolysis and/or TCA cycle adequately enough to facilitate the invasion process. Intriguingly, despite a substantially reduced ATP level in the absence of glucose, the parasite invasion is not influenced. However, an inhibition of oxidative phosphorylation or a withdrawal of glutamine from cultures completely abolished the invasion by the Δ*tggt1* strain, even though ATP levels were reduced further only by a modest 34%. These interesting results imply that either a threshold energy pool is required by extracellular parasites and/or another factor (e.g. redox state) plays a key role in sustaining the process of invasion. Unlike ATP, the protein and RNA contents of the Δ*tggt1* mutant were surprisingly normal, which implied a flexible usage of compensatory nutrients during its replication. Intracellular parasites exhibited a much greater resilience apparently by exploiting host-derived glycolytic intermediates, amino acids and fatty acids. In accord, intracellular Δ*tggt1* mutant showed a residual labeling of 3-phosphoglycerate and of the TCA cycle with ^{13}C-glucose, which can perhaps be attributed to host glycolysis, known to be induced in parasitized human cells (27). Likewise, the residual tracer in the TCA

cycle may come from glucose-derived non-essential amino acids (glutamate, aspartate *etc.*) and other unknown metabolites produced in the host cell.

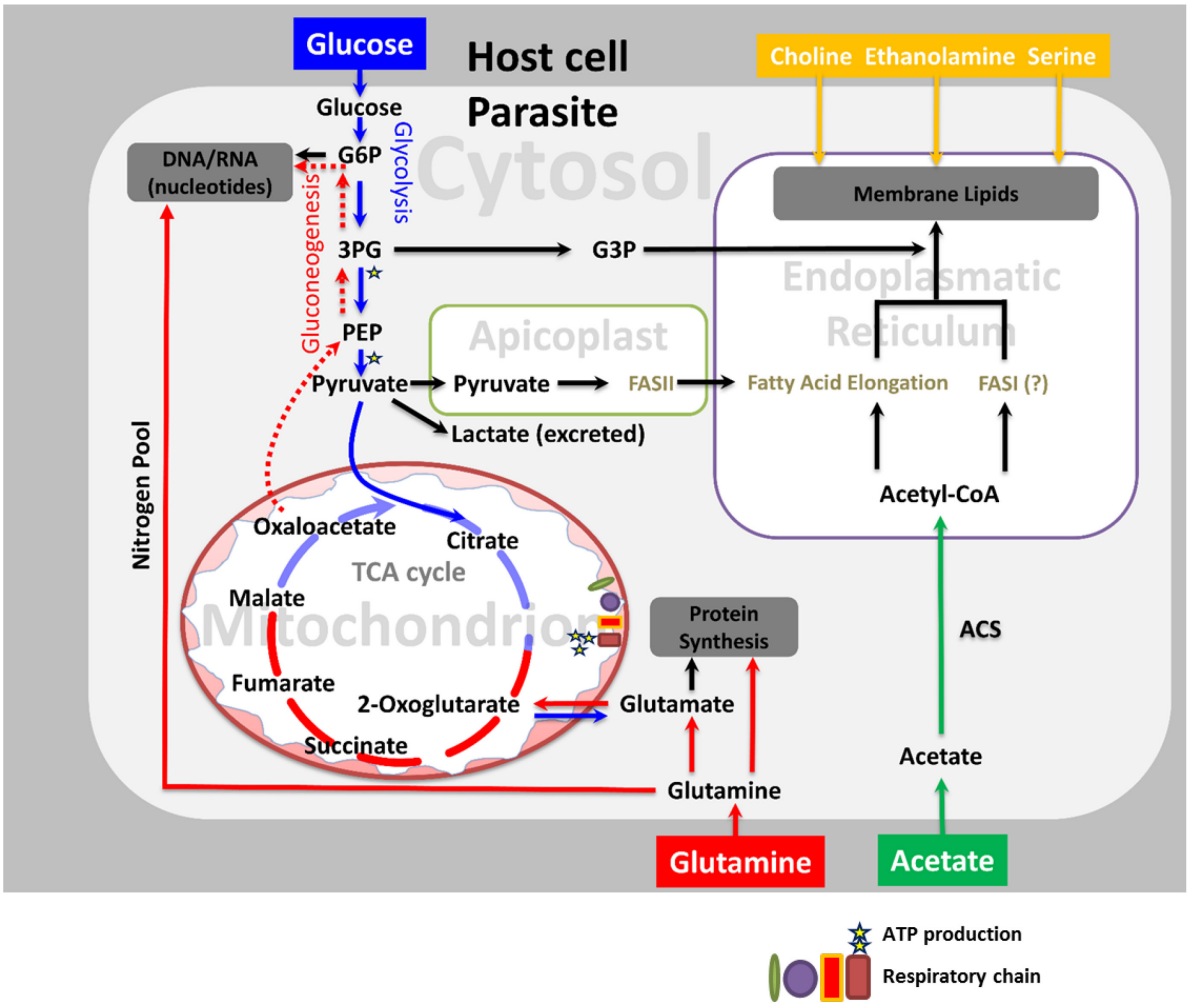


Figure 18. Carbon metabolism of *T. gondii* converges with tumor cells.

Proposed model of central carbon metabolism is constructed based on this work, published literature and annotations of select enzymes expressed in the tachyzoite stage (www.ToxoDB.org). Only those metabolites detectable or relevant to this work are shown for simplicity. Glucose and glutamine are co-utilized to satisfy the demands of biomass (proteins, nucleotides, lipids), energy, and reducing equivalents (not depicted). Nucleotides synthesis requires ribose 5-phosphate produced by diversion of glycolytic metabolites to the pentose phosphate shunt. Lipid biogenesis utilizes acetyl-CoA and glycerol-3-phosphate, which are primarily derived from glucose under normal condition. Likewise, protein synthesis needs glucose-derived amino acids. When replicating intracellular, glutamine catabolism enables an efficient biosynthetic use of glucose by replenishing the TCA cycle drained by biogenesis of macromolecules. Glutamine also confers the much-needed pool of nitrogen for nucleotide and protein syntheses. Extracellular parasites can use either of the two nutrients to produce sufficient energy for the host-cell invasion. Glutamine-derived carbon flux (TCA cycle and gluconeogenesis) sustains the parasite survival with a minimal growth defect, when glycolysis is compromised. The parasite can also deploy acetate as a carbon source when available in culture. Carbon metabolism is reprogrammed according to proliferating (intracellular) and non-proliferating (extracellular) demands and in response to the available nutrients. Abbreviations: ACS, Acetyl-CoA synthetase; GA3P, glyceraldehyde 3-phosphate; G3P, glycerol-3-phosphate; PEP, phosphoenolpyruvate.

Unlike biogenesis of nucleotides and proteins, glutamine does not seem to sustain lipogenesis, which results in an evident and wide-spectrum deficit of lipids with long acyl chains due to dysfunction of the elongation and/or FAS1 system upon glycolytic impairment. Lipids harboring medium chains were not affected to the same extent, which is consistent with their proposed import from host in the absence of a functional FAS2 pathway in the parasite apicoplast (30,82). It seems as though the pools of acetyl-CoA for the FAS1, FAS2 and elongase pathways are distinctly regulated. While acetyl-CoA for FAS2 pathway is derived primarily *via* glycolysis (30), other two pathways may have multiple sources, such as glycolysis, ATP-citrate lyase (TGME49_223840, www.ToxoDB.org) and/or acetyl-CoA synthetase. It is known that acetate does not label fatty acids generated by FAS2 pathway in the apicoplast (82); instead it is adeptly utilized by FAS1 and/or elongase (30), which is consistent with the expression of an acetyl-CoA synthetase, defect in synthesis of long acyl chains in the Δ*tggt1* mutant and complementation by acetate. Although yet to be studied in *T. gondii*, the cytoplasmic ATP-citrate lyase (citrate shuttle) is required for glucose-dependent fatty acid synthesis in cancer cells under normal conditions (83) and is linked to histone acetylation (84). Under hypoxia, reductive metabolism of glutamine can support lipogenesis and cell proliferation (85). Inability of glutamine to recompense for lipogenesis in the Δ*tggt1* mutant under tested (normoxic) condition could be due to rewiring of TCA cycle to prioritize essential pathways, *e.g.* the citrate shuttle to sustain redox balance in the apicoplast (29), ATP synthesis and ribogenesis. Such an efficient, regulated and discretionary reprograming of central carbon metabolism in response to nutritional cues guarantees the parasite's survival with a minimal trade-off in its growth fitness.

4.2. Importance of gluconeogenesis in *Toxoplasma gondii*

This work shows that *Tg*PEPCK$_{mt}$ functions as a nodal enzyme, which coordinates glycolysis, gluconeogenesis and TCA cycle, and allows tachyzoites to metabolize both carbon sources for energy and anabolic activities. In sugar-starved cells, *Tg*PEPCK$_{mt}$ enables the production of glutamine-derived PEP, which in turn is used to generate several biosynthetic precursors. Glutamine furnishes gluconeogenic intermediates and ensures a canonical TCA cycle upon glycolytic defect. Consistent with the absence of glucose 6-phosphatase gene, tachyzoites do not produce sugar from ^{13}C-glutamine, signifying that parasites engage only in early steps of gluconeogenesis and fully consume gluconeogenic intermediates to support biosynthetic activities required for glucose-independent survival. Such a metabolic phenotype essentially

converges with cancer cells, which also do not express glucose 6-phosphatase and depend on PEPCK-mediated flux of glutamine-carbon through reverse glycolysis for synthesizing lipids, nucleotides and amino acids instead of producing glucose (86,87).

Tachyzoites can use glutamine as an anaplerotic substrate to produce 2-oxoglutarate and subsequent biosynthetic precursors. The process enables the TCA cycle to function as a fuel source for energy and a biosynthetic hub. Glutamine-derived anaplerosis can more or less fully compensate for glucose-derived anaplerosis to ensure the functioning of a canonical TCA cycle. The function of citric cycle in glucose-deprived cells 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 cancer cells (86,87). Even in glucose-replete milieu, 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 basis PyC uses 1 ATP per oxaloacetate molecule, whereas glutaminolysis does not need ATP; instead, glutamine catabolism yields reducing equivalents for oxidative phosphorylation and enzymatic reactions (88). Although a continued operation of the TCA cycle and oxidative phosphorylation by glutamine may produce adequate energy and NAD(P)H to drive invasion and sustain enzymatic catalysis, it cannot fully account for the replication of glucose-starved tachyzoites because glycolytic metabolites are still needed to generate the macromolecules essential for cell proliferation.

Surprisingly, *Plasmodium falciparum* harbours an additional phosphoenolpyruvate carboxylase (PEPC), which catalyses the reverse reaction of PEPCK and exhibits an essential anaplerotic function and maintains cytosolic and mitochondrial redox balance in RBC stages of *P. falciparum* (89) and might substitute for lack of PyC. In contrast, *T. gondii* genome lacks PEPC, while PEPCK might be able to catalyse conversion of phosphoenolpyruvate to oxaloacetate under glucose replete conditions.

This work shows that glucose-derived carbon is incorporated into R5P and nucleic acids. Synthesis of R5P from glutamine is induced in the absence of glucose import and declined upon repression of *Tg*PEPCK$_{mt}$, indicating an important role of glutamine in supplying carbon for the synthesis of nucleotides under conditions of glucose deprivation. Besides, two carbon atoms in purines originate from glycine, and 2 one-carbon units are derived from *N*-^{10}-formyl-tetrahydrofolate, which requires serine (88). Synthesis of both, serine and glycine, requires 3-phosphoglycerate, a glycolytic intermediate, which has to be generated from glutamine in the Δ*tggt1* strain. Indeed, supplement of exogenous serine and glycine partially restored *off-state* growth of the Δ*tggt1*/iΔ*tgpepck$_{mt}$* mutant (Figure S34). The fact that the lytic cycle could not be

entirely rescued indicates additional biosynthetic routes fed by glutamine, which could not be satisfied by other nutrients present in the parasite culture. For instance, glutamine-derived G3P may serve as a precursor for glycerophospholipid and nucleotide syntheses (88). The levels of G3P and several such metabolites involved in or associated with gluconeogenesis were reduced in the conditional mutant, when *Tg*PEPCK$_{mt}$ was shut down, leading to impaired biomass production and eventual demise of tachyzoites.

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 Δ*tggt1*/iΔ*tgpepck$_{mt}$* strain. Previous results indicated that Δ*tggt1* mutant is unable to utilize exogenous pyruvate, and lactate synthesis depends on the extent of glycolysis (40). 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 (90), but there is no genetic or biochemical evidence of this pathway in *T. gondii*. Other anaplerotic substrates (capable of making oxaloacetate) include branched-chain amino acids (leucine, valine, isoleucine) that can enter the cycle through metabolism of propionyl-CoA, the relevance of this pathway in tachyzoites is unclear however (91). These data together underpin the physiological importance of glutamine over other nutrients during the lytic cycle, which is further intensified by the fact that it is the second most plentiful nutrient in mammalian tissues after glucose (92).

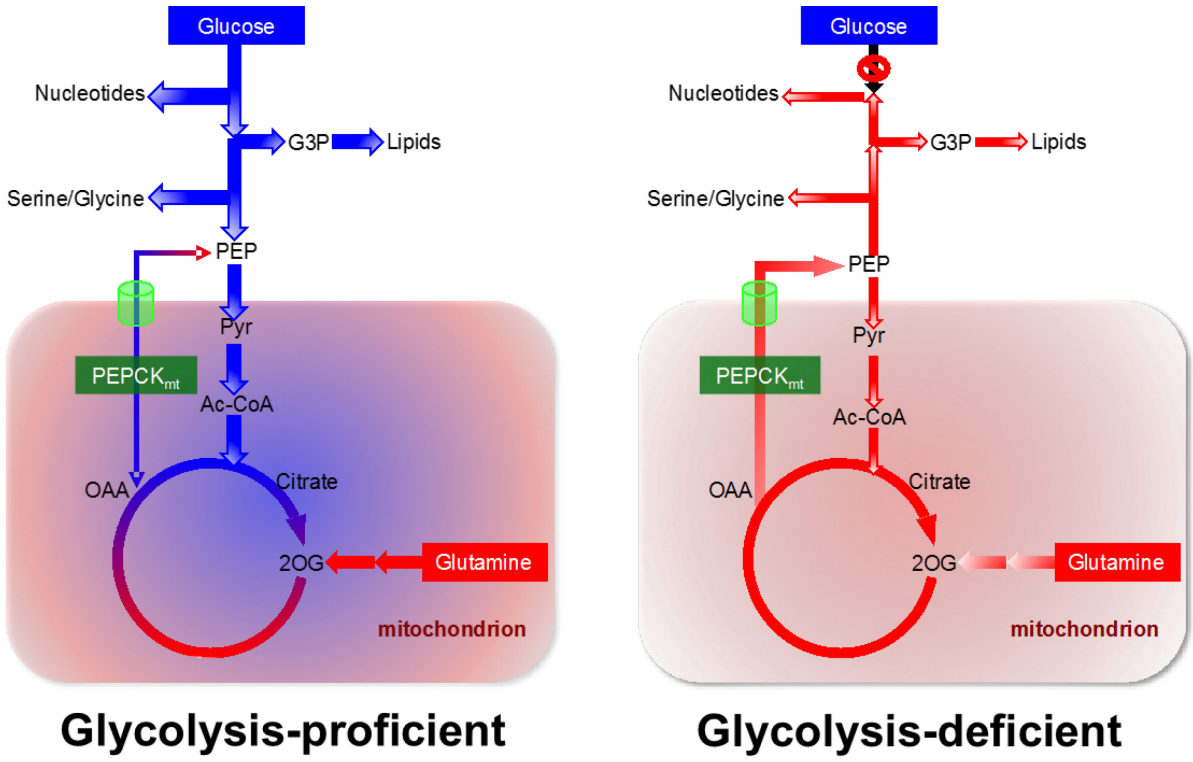


Figure 19. A prototype model featuring the function of *Tg*PEPCK$_{mt}$ as a metabolic shunt in central carbon metabolism of tachyzoites.

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 *Tg*PEPCK$_{mt}$, since *Tg*PyC and *Tg*PEPCK$_{net}$ 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.

A role of PEPCK in promoting the entry of carbon from glutamine into biosynthetic precursors *via* PEP converges with cancer cells. Montal *et al.* (87) and Vincent *et al.* (93) 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 non-glucose substrates and diversion of intermediates into anabolic pathways therefore appears to be a common strategy exploited by tachyzoites and tumor cells to counteract a dearth of glucose. Both PEPCK-M mRNA and protein are induced upon withdrawal of glucose in tumor cells (94). The enzymatic activity of PEPCK-M depends on the mitochondrial GTP (95) as well as on deacetylation (96). Unexpectedly, *Tg*PEPCK$_{mt}$ is constitutively expressed and appears to be an ATP-dependent enzyme, which suggests an alternative regulation of gluconeogenesis by acetylation-deacetylation in tachyzoites. Indeed, acetylation of *Tg*PEPCK$_{mt}$ does occur (97) and genetic depletion of acetyl-CoA is associated with activation of gluconeogenesis in tachyzoites (59). Therefore it was postulated that a decrease in the mitochondrial acetyl-CoA in the Δ*tggt1* mutant causes deacetylation of *Tg*PEPCK$_{mt}$, which subsequently turns on gluconeogenesis.

In conclusion, this work shows that the mitochondrial isoform of PEPCK embodies a key nodal point linking catabolism of glucose, glutamine with anabolic pathways in tachyzoites of *T. gondii* (Figure 19). 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 alternative nutrients provides parasites with a selective advantage to face the bioenergetic demand of a fluctuating nutritional milieu.

4.3. Metabolic convergence between parasites and cancer cells

Over the last decade, a consistent picture of carbon metabolism has emerged from studies on diverse types of proliferating cells (cancer cells, stem cells, lymphocytes), whose metabolic needs are different from differentiated (quiescent) cells. Proliferating cells must continually produce the major constituents of biomass (nucleic acids, proteins and membranes) (88,98). Most quiescent cells, however, are quite relieved of such liabilities and reduce their carbon flux with an aim to promote cell maintenance and survival. Metabolic demands of differentiated cells are primarily met by glucose, most of which (>90%) enters the TCA cycle as pyruvate, and only a small fraction (<10%) is converted to lactate. Most dividing cells instead require a rapid glycolysis along with lactate production and glutamine catabolism (99). Such a carbon flux seems rather inefficient for making ATP and the waste of 3 carbons as lactate at first glance; however, it confers much-needed benefit to dividing cells by routing glycolytic and TCA cycle metabolites for the synthesis of macromolecules. Moreover, two recent studies show that several types of cancer cells fuel biogenesis by avidly consuming acetate (100,101). Parasites

appear to show a comparable metabolic phenotype in cultures. This work demonstrate that co-usage, quintessence (tangible metabolic benefits) and cooperation of glucose, glutamine and acetate in *T. gondii* resembles the physiology of tumor cells. Such a metabolic convergence may eventually be exploited to develop common therapeutics targeting the parasite and cancer metabolism.

5. Outlook and Conclusions

This thesis establishes a deeper understanding of the central carbon metabolism of the obligate intracellular parasite *Toxoplasma gondii*. It reveals the mechanism of glutamine utilization by the parasite and determines its role as the major gluconeogenic substrate. This work highlights the metabolic flexibility of *Toxoplasma gondii* to adapt quickly to changes in the nutritional environment.

While the cooperativity of glucose and glutamine have been described quite extensively, the regulation of the carbon sources and the interaction with the host cell remains mainly elusive. For example, the role of *Tg*PEPCK$_{mt}$ under glucose replete conditions is poorly understood. The gluconeogenic enzymes *Tg*FBP2 and *Tg*PEPCK$_{mt}$ are constitutively expressed and while there has been a futile cycle model proposed for *Tg*FBP2, the role of *Tg*PEPCK$_{mt}$ remains to be determined. Metabolomics analysis of intracellular parasites has become a powerful standard technique, circumventing the lack of functional enzymatic data on many enzymes in these parasites (102). The stable isotope labelling with glucose and glutamine are appropriate tools to investigate the function of *Tg*PEPCK$_{mt}$ and investigate a potential anaplerotic function of the enzyme or a gluconeogenic flux under glucose replete conditions. The presence of +3 isotopomers in malate when labelling parental strains with glucose suggests this anaplerotic role, while residual labelling of glycolytic intermediates with glutamine in the parental strain underlines a potential futile cycle (Figure S25).

Furthermore, the knockout strains presented in this study can be ideally used to examine the importance of the different pathways of the central carbon metabolism *in vivo*. Especially as it has been shown that glucose is not be the only contributor to the central carbon metabolism and glutamine levels are fairly high in different tissues.

Another interesting aspect is the role of metabolic regulation of stage differentiation. As it has been shown that tachyzoites alter gene expression in host cells, the metabolism could be one of the crucial regulation steps in differentiation. Therefore knockouts of the examined enzymes should be conducted in type II strain and examined for altering in stage conversion.

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7. Supplemented figures

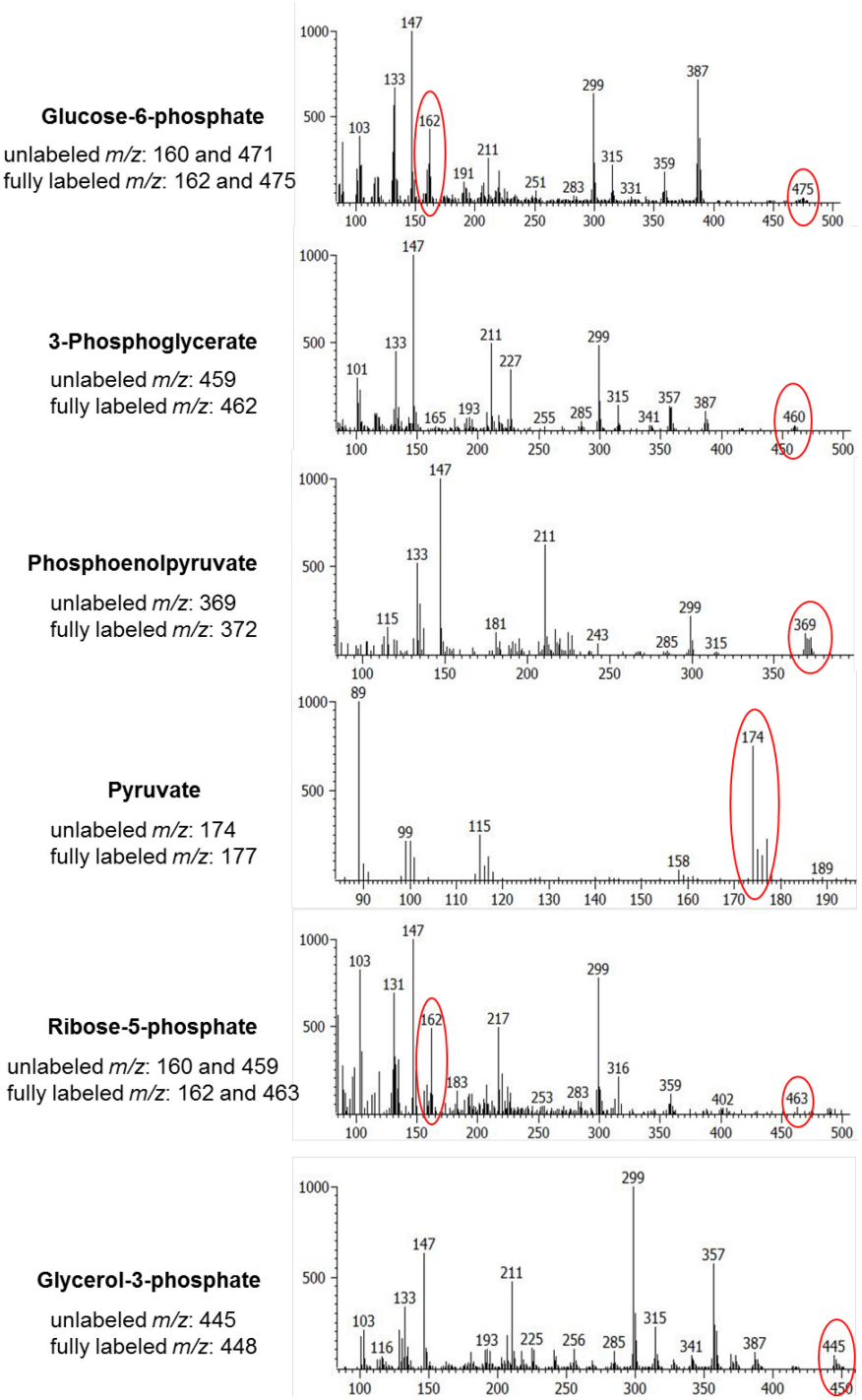


Figure S20. EI-MS spectra of metabolites isolated from purified intracellular tachyzoites.

HFF cells, infected with the parental strain were labeled with 5 mM [U-^{13}C]-glucose and 2 mM glutamine (37°C, 4 h, 5% CO_2) followed by parasite purification, metabolite extraction and GC-MS analysis. The peaks corresponding to the non-fragmented derivatized analyte are encircled in red. X-axis and Y-axis denote the *m/z* values and intensities, respectively.

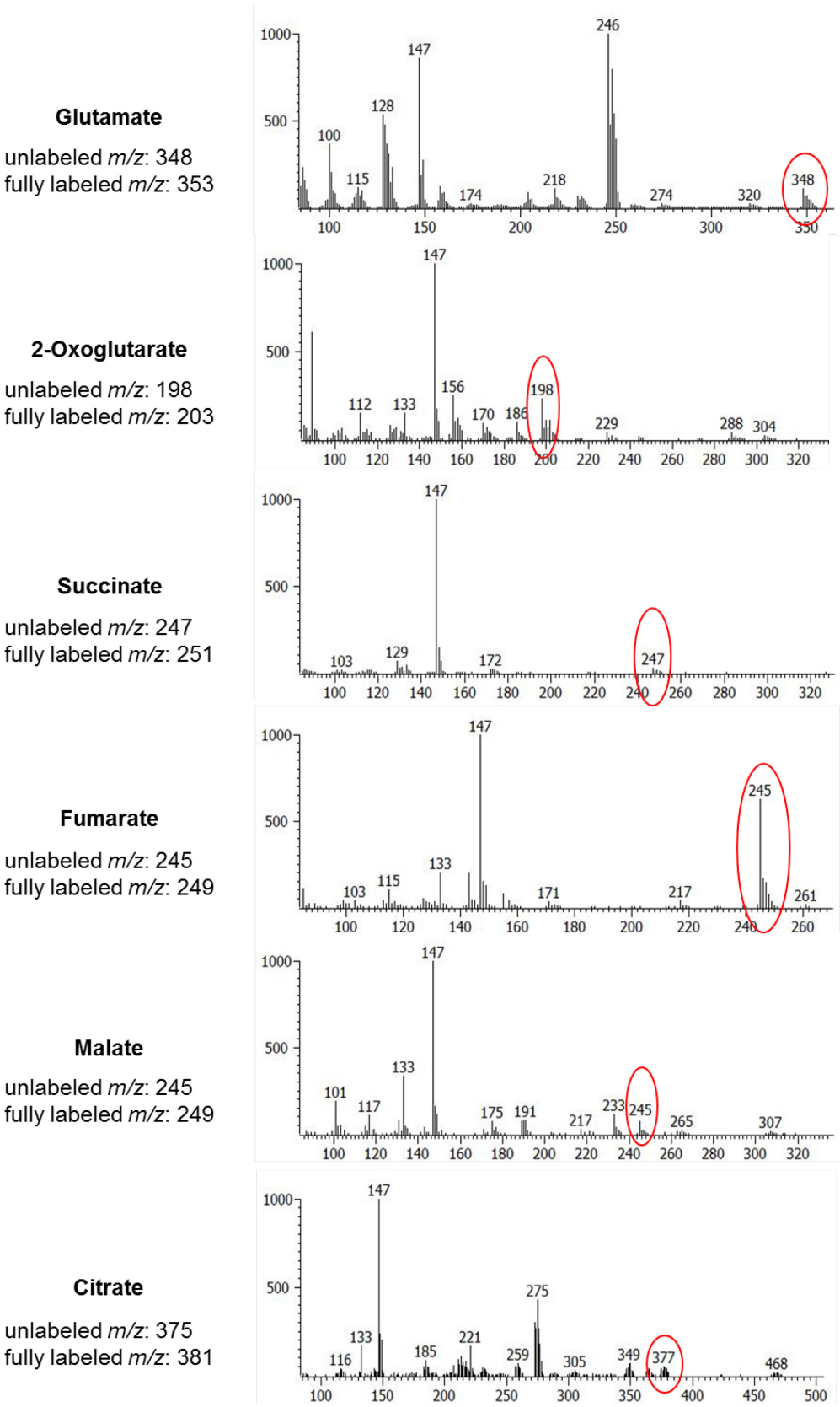


Figure S21. EI-MS spectra of metabolites isolated from purified intracellular tachyzoites.

HFF cells, infected with the parental strain were labeled with 5 mM [*U-*^{13}*C*]-glucose and 2 mM glutamine (37°C, 4 h, 5% CO_2) followed by parasite purification, metabolite extraction and GC-MS analysis. The peaks corresponding to the non-fragmented derivatized analyte are encircled in red. X-axis and Y-axis denote the *m/z* values and intensities, respectively.

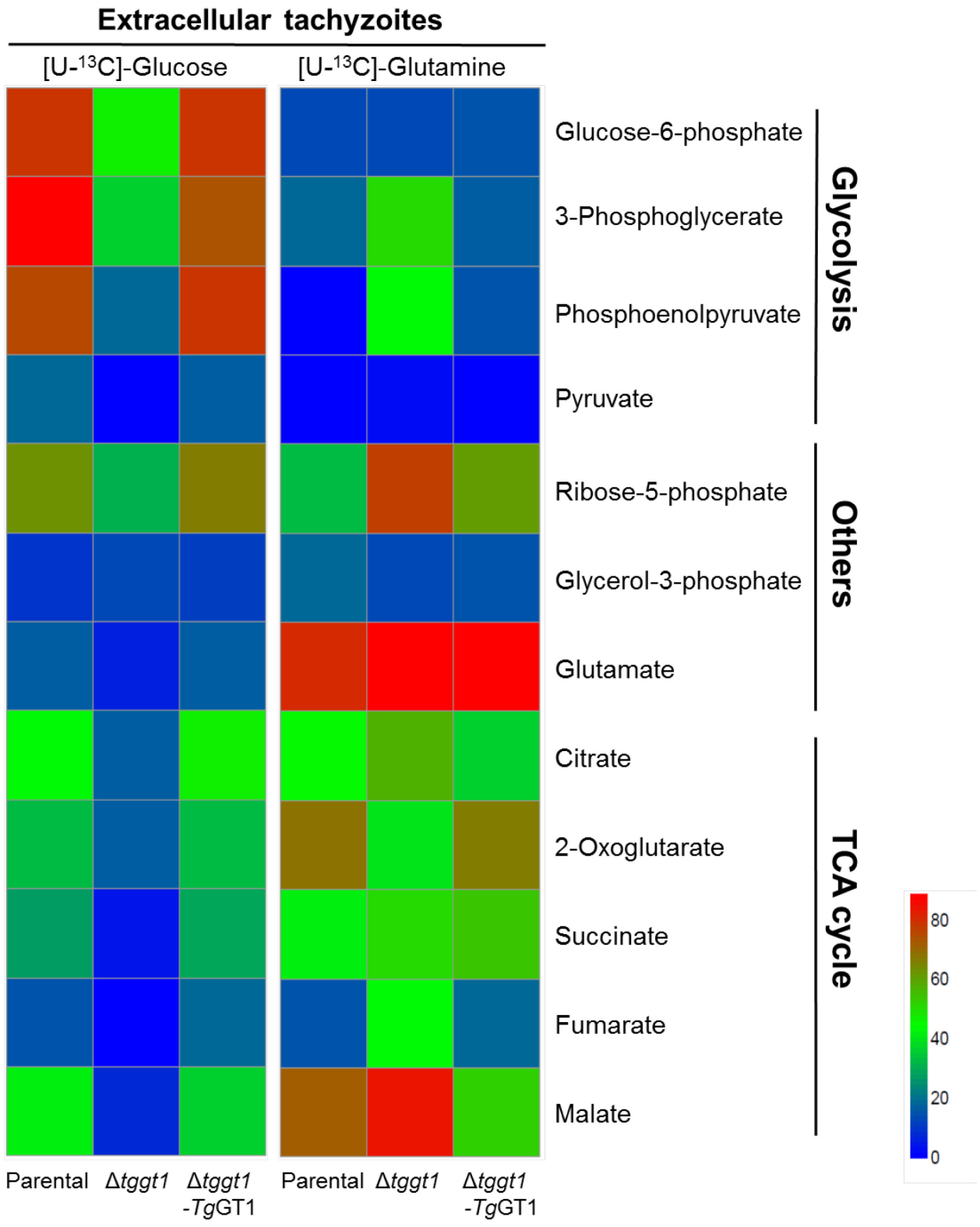


Figure S22. Heat maps showing glucose and glutamine labeling of extracellular tachyzoites.

Extracellular parasites of the specified strains were labeled either with 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or with 2 mM [U-^{13}C]-glutamine and 5 mM glucose (37°C, 4 h, 5% CO_2). The mean percentages of the ^{13}C pool in each metabolite from four assays are shown on a color-gradient scale (0%, blue; 50%, green; 100%, red).

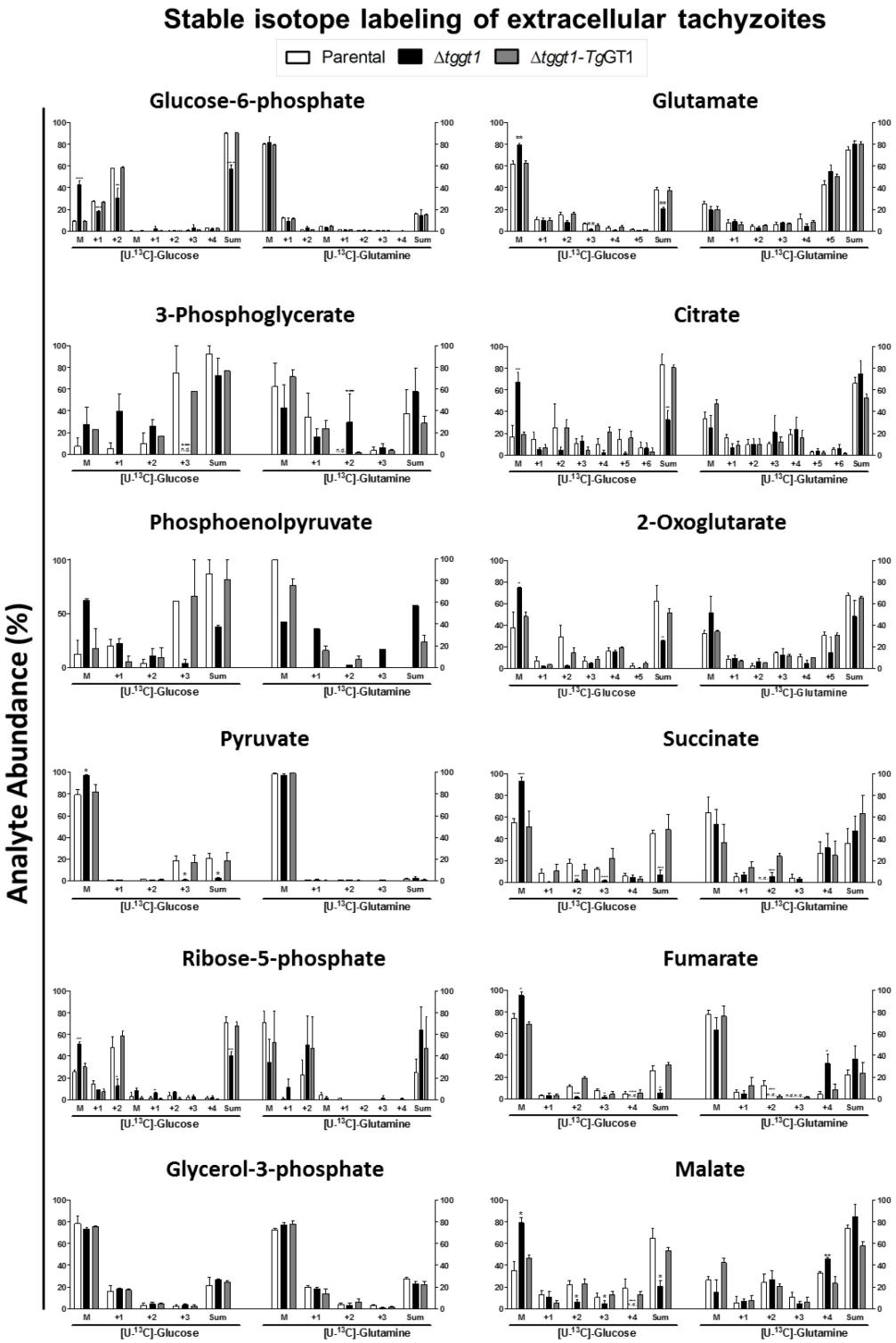


Figure S23. Fractional abundance of the isotopomers detected after stable isotope labeling of extracellular tachyzoites.

Extracellular parasites were incubated either with 5 mM [U-^{13}C]-glucose and 2 mM glutamine, or with 2 mM [U-^{13}C]-glutamine and 5 mM glucose (37°C, 4 h, 5% CO_2). '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 glucose-6-phosphate and ribose-5-phosphate. Statistical significance was measured separately for each group compared to the parental strain using student's *t*-test (n = 4 assays, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$). *Abbreviation*: n.d., not detectable

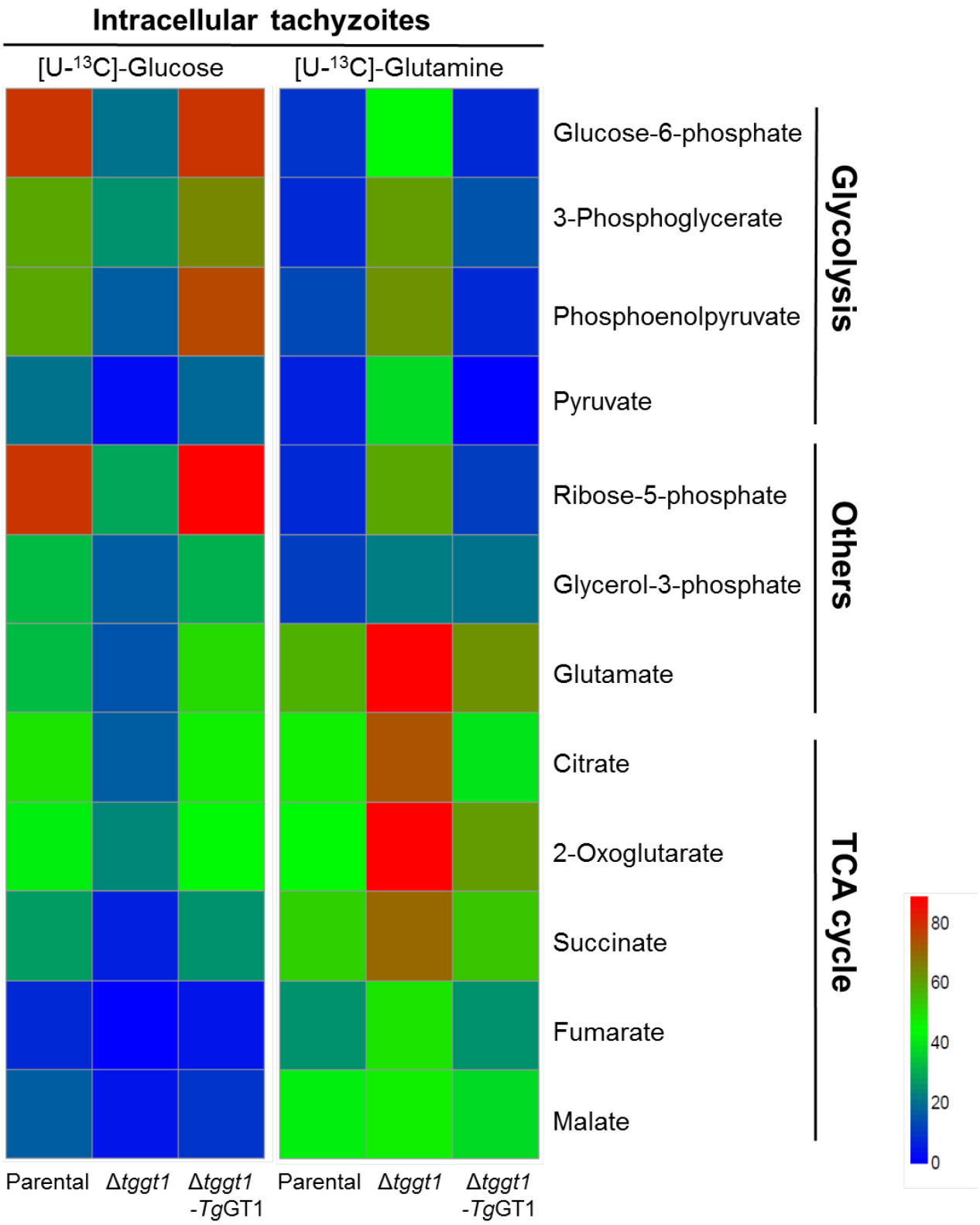


Figure S24. Heat maps showing glucose and glutamine labeling of intracellular tachyzoites.

Intracellular parasites of the indicated strains were labeled either with 5 mM [*U-13C*]-glucose and 2 mM glutamine, or with 2 mM [U-13C]-glutamine and 5 mM glucose (37°C, 4 h, 5% CO_2). The mean percentages of the ^{13}C pool in each metabolite from four experiments are shown on a color-gradient scale (0%, blue; 50%, green; 100%, red).

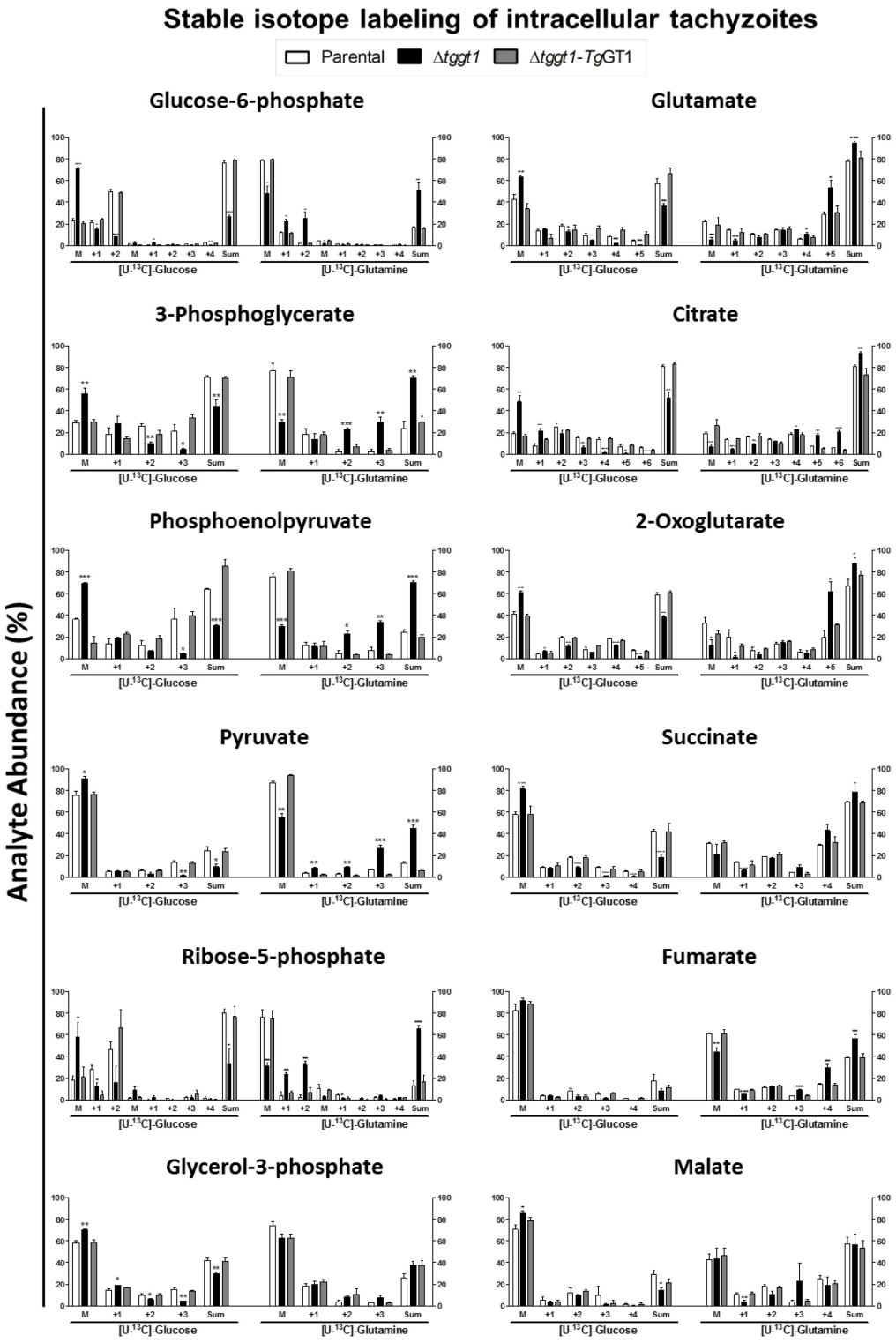


Figure S25. Fractional abundance of the isotopomers detected after stable isotope labeling of intracellular tachyzoites.

Intracellular parasites were incubated either with 5 mM [*U-13C*]-glucose and 2 mM glutamine, or with 2 mM [*U-13C*]-glutamine and 5 mM glucose (37°C, 4 h, 5% CO_2). '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 glucose-6-phosphate and ribose-5-phosphate. Statistical significance was measured separately for each group compared to the parental strain using student's *t*-test (n = 4 assays, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

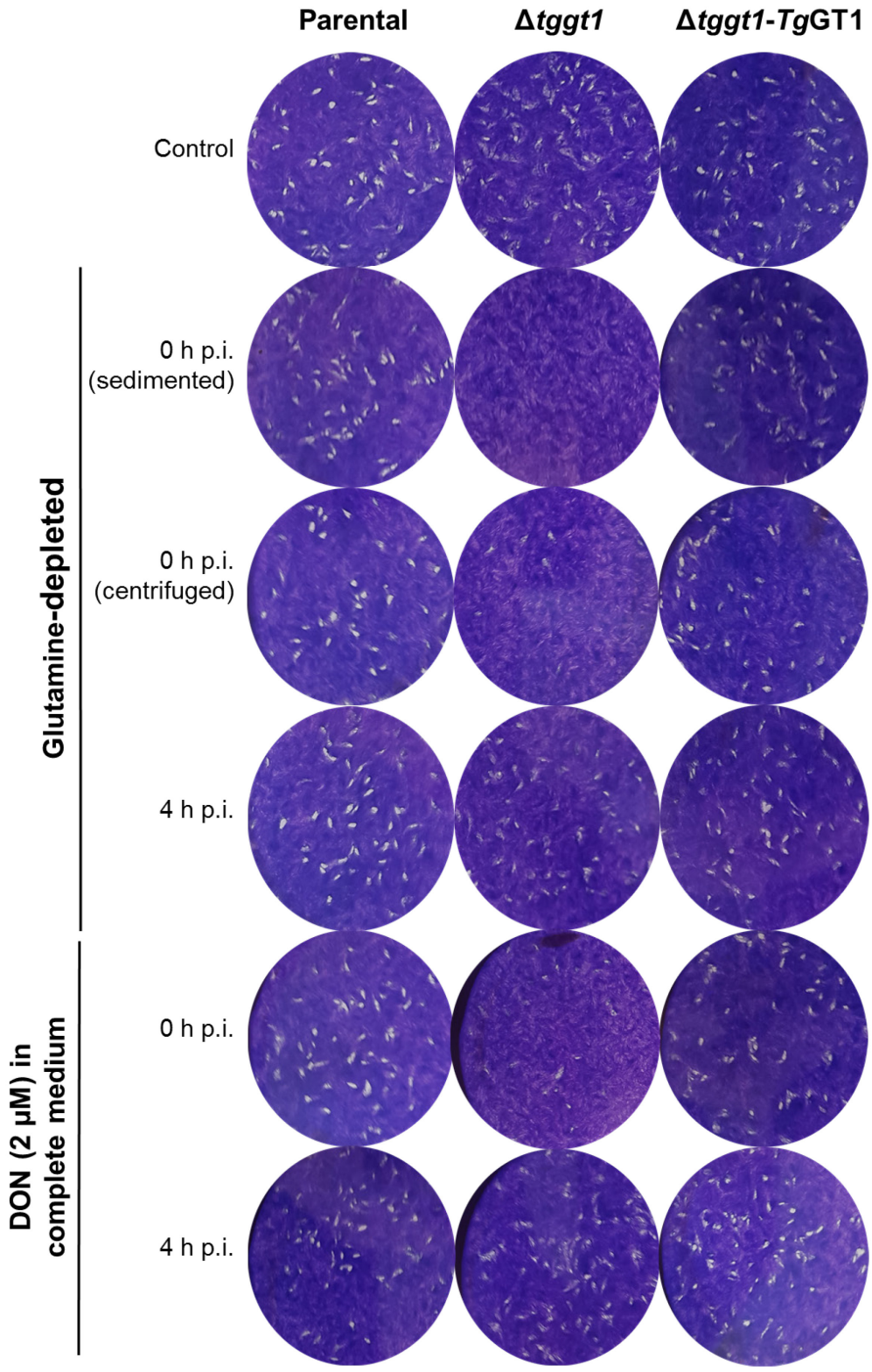


Figure S26. Glutamine is critical for the lytic cycle of tachyzoites defective in glucose import.

Representative plaque images show the overall growth fitness of the indicated strains under various conditions. Assays were performed as describe in *methods*. Parasites were cultured with or without glutamine (2 mM) in standard culture medium containing 10% dialyzed serum (37°C, 7d, 5% CO_2). DON (2 µM) was added at the time of (0 h) or after (4 h) infection of the host fibroblast monolayers. '*sedimented*' parasites were allowed to float down naturally, whereas '*Centrifuged*' parasites were centrifuged immediately after adding the parasites onto host-cell monolayers (400*g*, 10 min).

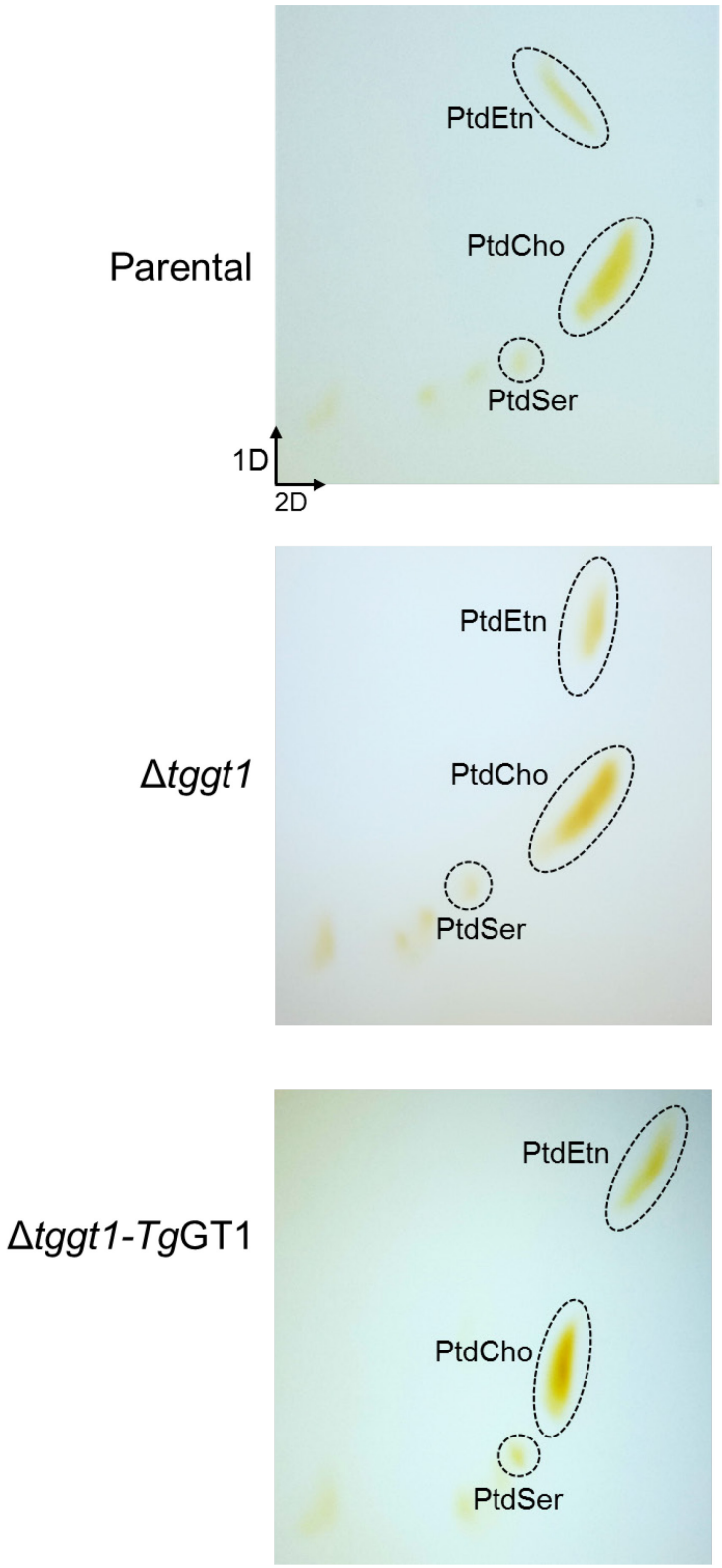


Figure S27. Two-dimensional thin layer chromatography depicting three major phospholipids of *T. gondii* tachyzoites.

Total lipids (10^8 parasites) were extracted by methanol-chloroform and resolved on silica 60 plates in chloroform/methanol/ammonium hydroxide (84.5:45.5:6.5) followed by chloroform/acetic acid/methanol/water (80:12:9:2). Phospholipids were visualized by iodine staining and identified based on their co-migration with commercial lipid standards. Lipid amount in individual bands were determined by chemical phosphorous assay.

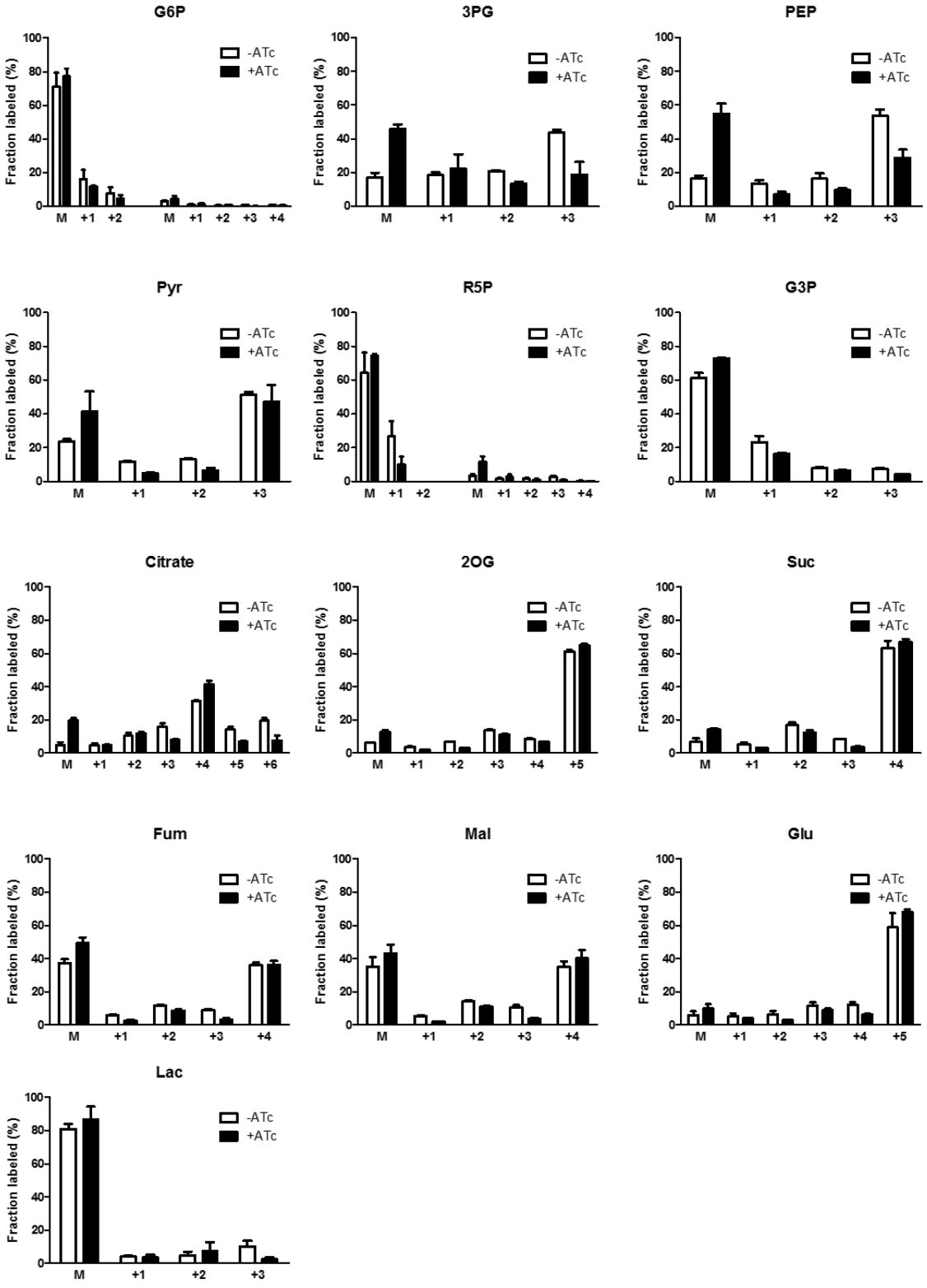


Figure S28. Fractional abundance of the isotopomers detected after stable isotope labeling of intracellular Δ*tggt1*/iΔ*tgpepck*$_{mt}$.

Intracellular parasites were incubated with 2 mM [U-^{13}C]-glutamine and 5 mM glucose (37°C, 4 h, 5% CO_2). 'M' represents unlabeled fractions. Only fragmented analytes were detectable for glucose-6-phosphate and ribose-5-phosphate. Statistical significance was measured separately for each group compared to the parental strain using student's *t*-test (n = 4 assays, *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

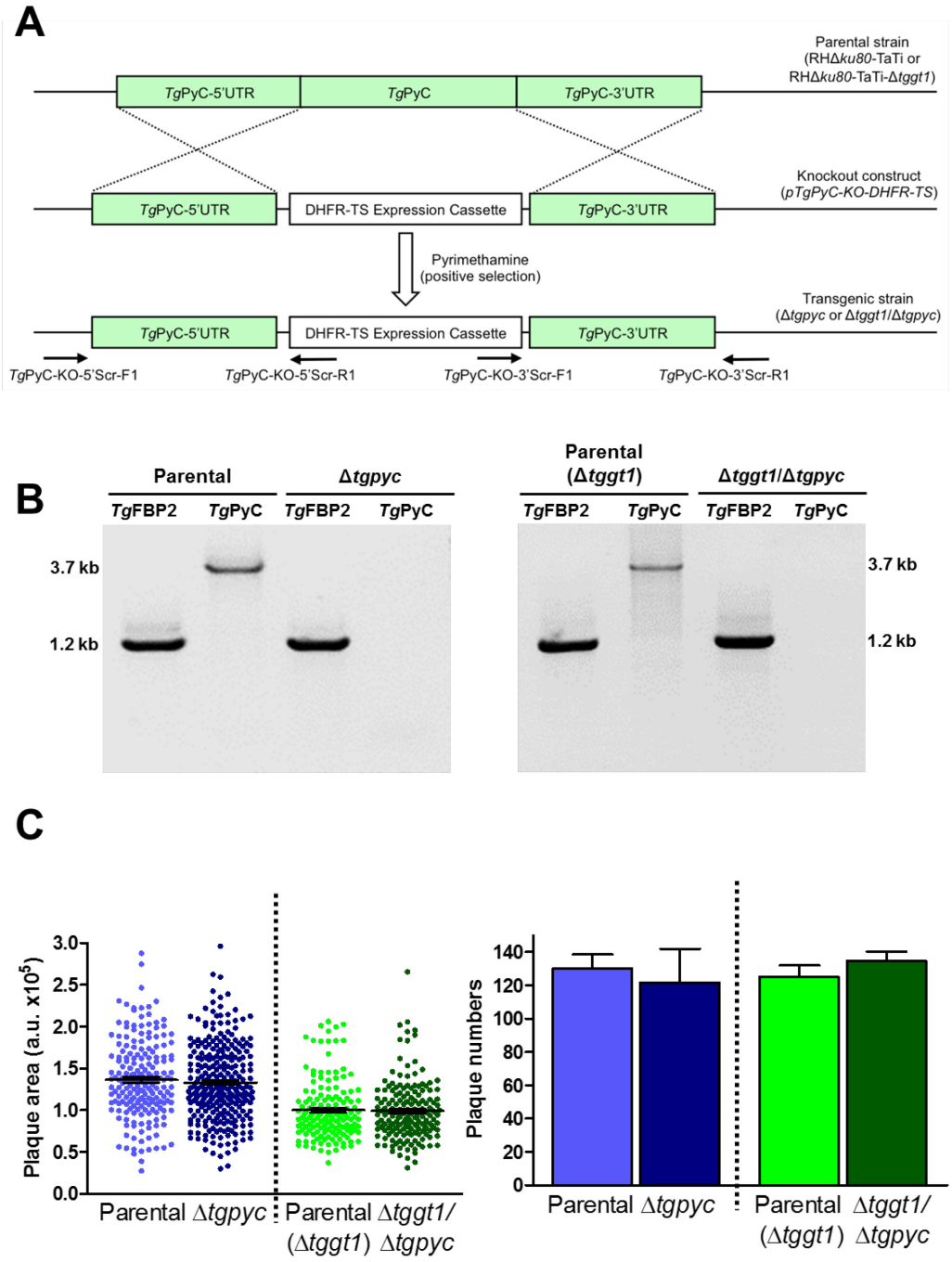


Figure S29. Pyruvate carboxylase is nonessential for tachyzoites regardless of glycolytic function.

(A) Scheme depicting deletion of the *TgPyC* gene by DHFR-TS *via* homologous crossover in tachyzoites. Indicated plasmid construct was transfected into either the RHΔ*ku80*-TaTi or in RHΔ*ku80*-TaTi-Δ*tggt1* strain followed by drug-selection and screening of the parasite clones using indicated primer pairs (*Tg*PyC-KO-5'Scr-F1/R1; *Tg*PyC-KO-3'Scr-F1/R1). The positive Δ*tgpyc* and Δ*tggt1*/Δ*tgpyc* mutants were confirmed by sequencing of recombination-specific PCR amplicons. (B) Detection of *Tg*PyC transcript in the Δ*tgpyc* and Δ*tggt1*/Δ*tgpyc* mutants. A representative clone of each mutant was tested for the presence or absence of *Tg*PyC and *Tg*FBP2 using ORF-specific primers. *Tg*FBP2 serves as a control to ascertain the integrity of RNA. The relevant parental strains (RHΔ*ku80*-TaTi or RHΔ*ku80*-TaTi-Δ*tggt1*) were included as positive controls. (C) Growth of the Δ*tgpyc* and Δ*tggt1*/Δ*tgpyc* mutants compared to their corresponding parental strains, as determined by plaque assay. Plaque area (arbitrary units) and plaque numbers represent the mean ± SE of 3 assays. Note that growth defect in the Δ*tggt1* and its derivative Δ*tggt1*/Δ*tgpyc* strains are due to loss of the *Tg*GT1 gene.

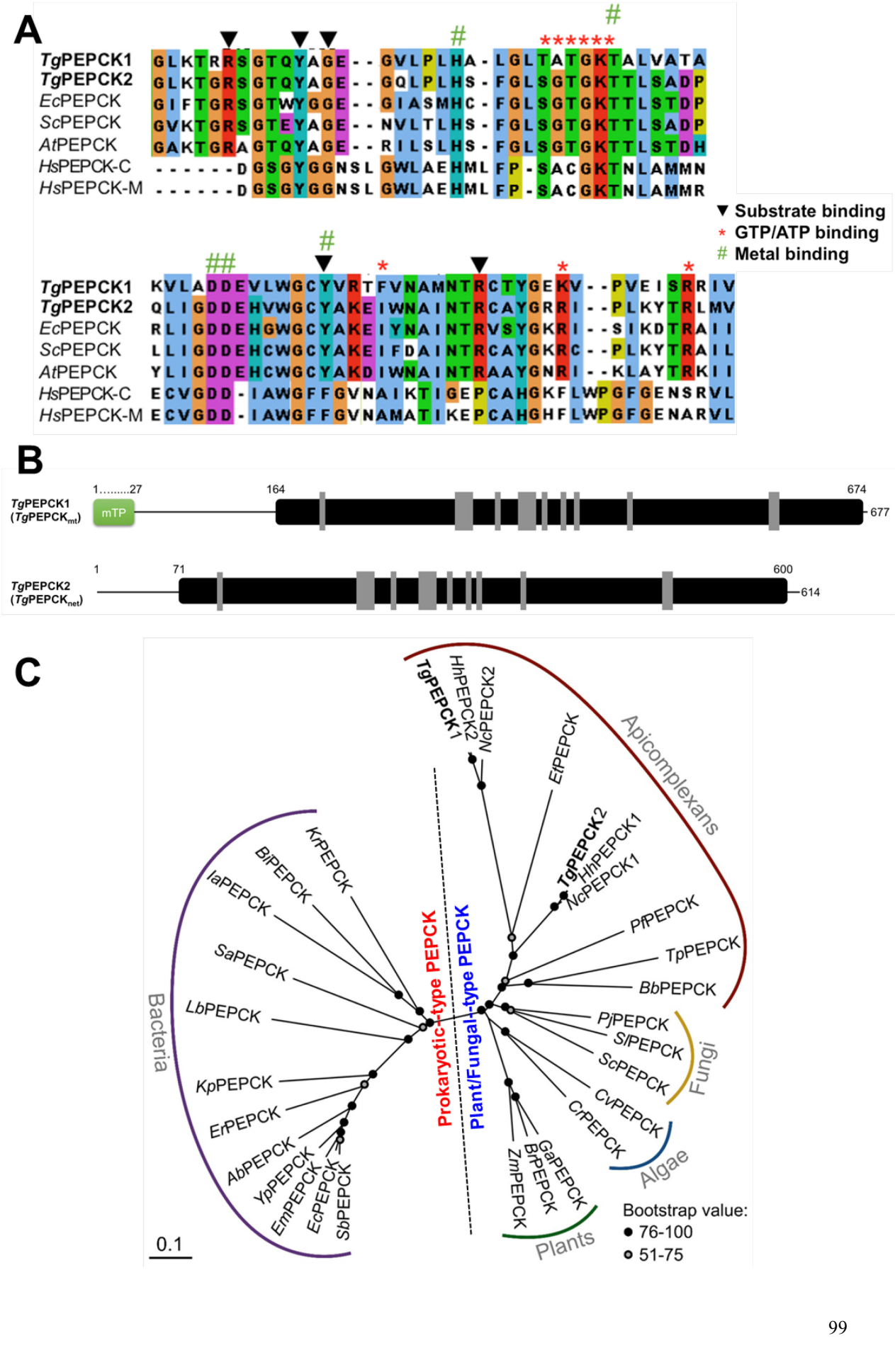

A
TgPEPCK1
TgPEPCK2
EcPEPCK
ScPEPCK
AtPEPCK
HsPEPCK-C
HsPEPCK-M
▼ Substrate binding
* GTP/ATP binding
# Metal binding
B
TgPEPCK1 (TgPEPCKmt)
mTP
TgPEPCK2 (TgPEPCKnet)
C
Apicomplexans
Bacteria
Fungi
Algae
Plants
Prokaryotic-type PEPCK
Plant/Fungal-type PEPCK
Bootstrap value:
76-100
51-75
0.1

Figure S30. *Toxoplasma* harbors two distinct phosphoenolpyruvate carboxykinases.

(A) Multiple sequence alignment of the PEPCK domains from *T. gondii* with their orthologs from selected organisms. Alignment was made using BLAST and MUSCLE programs. Conserved residues are highlighted in colors. Note the sequence variation between ATP-dependent (*Ec*PEPCK, *Sc*PEPCK, *At*PEPCK, *Tg*PEPCK1, *Tg*PEPCK2) and GTP-dependent homologs (*Hs*PEPCK-C, *Hs*PEPCK-M). (B) Primary structure of *Tg*PEPCK1 and *Tg*PEPCK2 protein. The predicted catalytic domain is shown as a black box with active sites annotated in light gray color. The mitochondrial target peptide (mTP) is shown in green. (C) Phylogenetic analysis of PEPCKs from distinct organisms. PEPCK sequences were clustered using CLC Sequence Viewer 7.7 and visualized by FigTree v1.4.2. The single most parsimonious tree of ATP-dependent PEPCK orthologs is shown. Circles on the branches denote bootstrap values for parsimony. *Tg*PEPCK1 and *Tg*PEPCK2 differ considerably from mammalian homologs, which are GTP-dependent and thus could not be parsimoniously grouped with ATP-dependent PEPCKs from bacteria, plants, fungi and parasites. Sequences for performing analysis were obtained from NCBI and parasite databases. NCBI accession: *Tg*PEPCK1 (*Tg*PEPCK$_{mt}$), KX785384; *Tg*PEPCK2 (*Tg*PEPCK$_{net}$), KX785385; *Ec*PEPCK, P22259; *Sc*PEPCK, P10963; *At*PEPCK, Q9T074; *Hs*PEPCK-M, Q16822; *Hs*PEPCK-C, P35558. *Nc, Neospora caninum ; Pf, Plasmodium falciparum; Bb, Babesia bovis; Sc, Saccharomyces cerevisiae; Pj, Pneumocystis jirovecii; Sl, Suillus luteus; Br, Brassica rapa; Ga, Gossypium arboretum; Cr, Chlamydomonas reinhardtii; Cv, Chlorella variabilis; Tp, Theileria parva; Zm, Zostera marina; Hh, Hammondia hammondi; Et, Eimeria tenella; Kr, Ktedonobacter racemifer; Bi, Brucella inopinata; Ia, Ignavibacterium album; Kp, Klebsiella pneumoniae; Ec, Escherichia coli; Lb, Leptospira biflexa; Er, Eubacterium ramulus; Sb, Salmonella bongori; Sa, Staphylococcus aureus; Yp, Yersinia pestis; Ab, Aeromonas bivalvium; Em, Enterobacter massiliensis.*

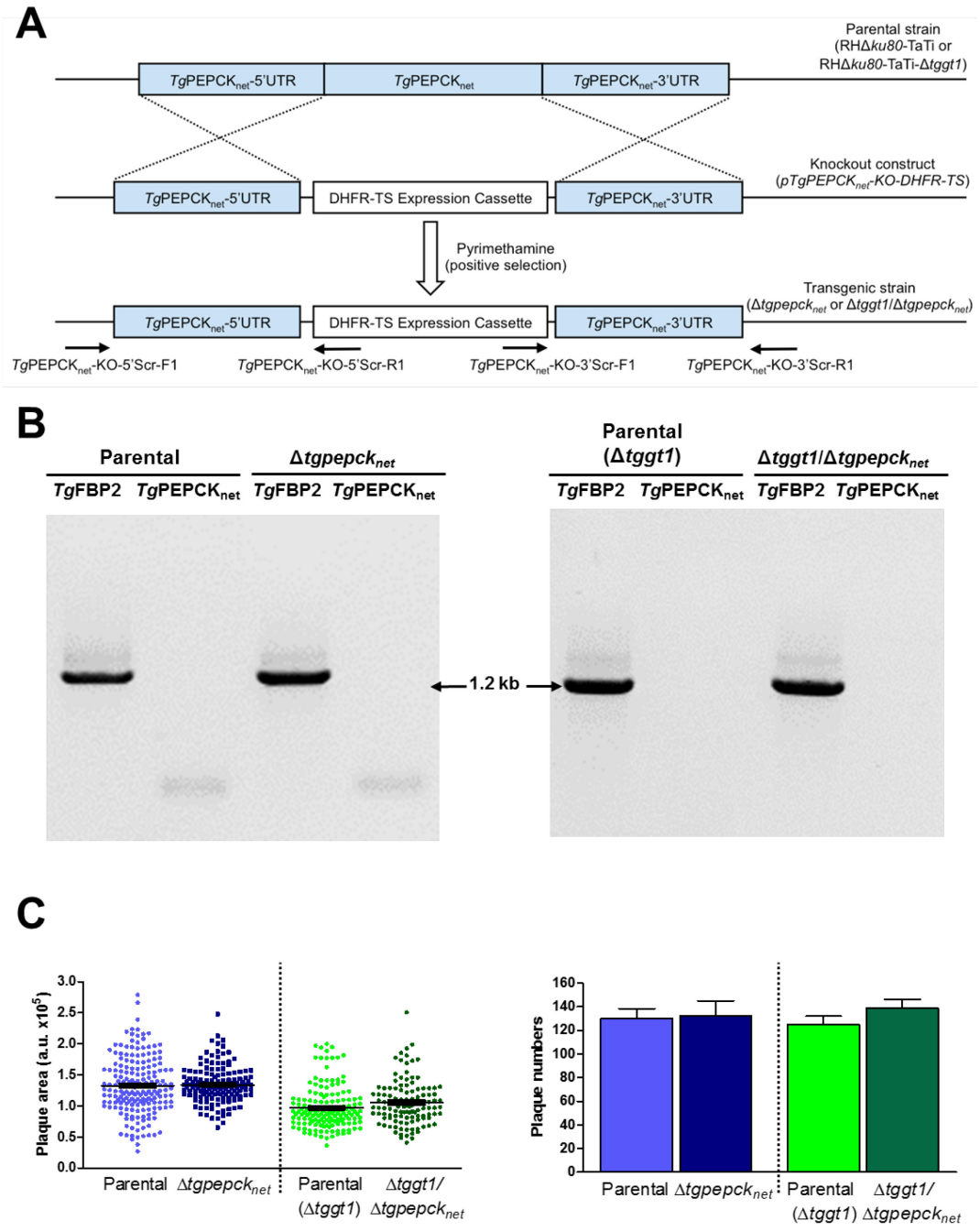


Figure S31. *Tg*PEPCK$_{net}$ is expendable in glycolysis-proficient and glycolysis-deficient parasites.

(A) Genetic deletion of *TgPEPCK$_{net}$* by DHFR-TS in tachyzoites. A gene knockout construct allowing double homologous recombination at the *TgPEPCK$_{net}$* locus was transfected into designated parental strains (RHΔ*ku80*-TaTi or RHΔ*ku80*-TaTi-Δ*tggt1*). The clonal transgenic tachyzoites were obtained by drug selection and screened for 5' and 3' recombination events using pertinent primers (*Tg*PEPCK$_{net}$-KO-5'Scr-F1/R1 or *Tg*PEPCK$_{net}$-KO-3'Scr-F1/R1). The positive clones (Δ*tgpepck$_{net}$* and Δ*tggt1*/Δ*tgpepck$_{net}$* strains) were confirmed by sequencing of recombination-specific amplicons. (B) Detection of *Tg*PEPCK$_{net}$ transcript in the Δ*tgpepck$_{net}$* and Δ*tggt1*/Δ*tgpepck$_{net}$* strains. Each strain was tested for the expression of *Tg*PEPCK$_{net}$ and *Tg*FBP2 (control for RNA integrity) using ORF-specific primers. (C) Plaque assays showing relative growth of the Δ*tgpepck$_{net}$* and Δ*tggt1*/Δ*tgpepck$_{net}$* mutants with respect to analogous ancestral strains. Plaques formed by individual strains were evaluated for their area (arbitrary units) and numbers (mean ± SE, n=3 assays). Note a somewhat slower growth (30% defect) of the two strains lacking *Tg*GT1 expression.

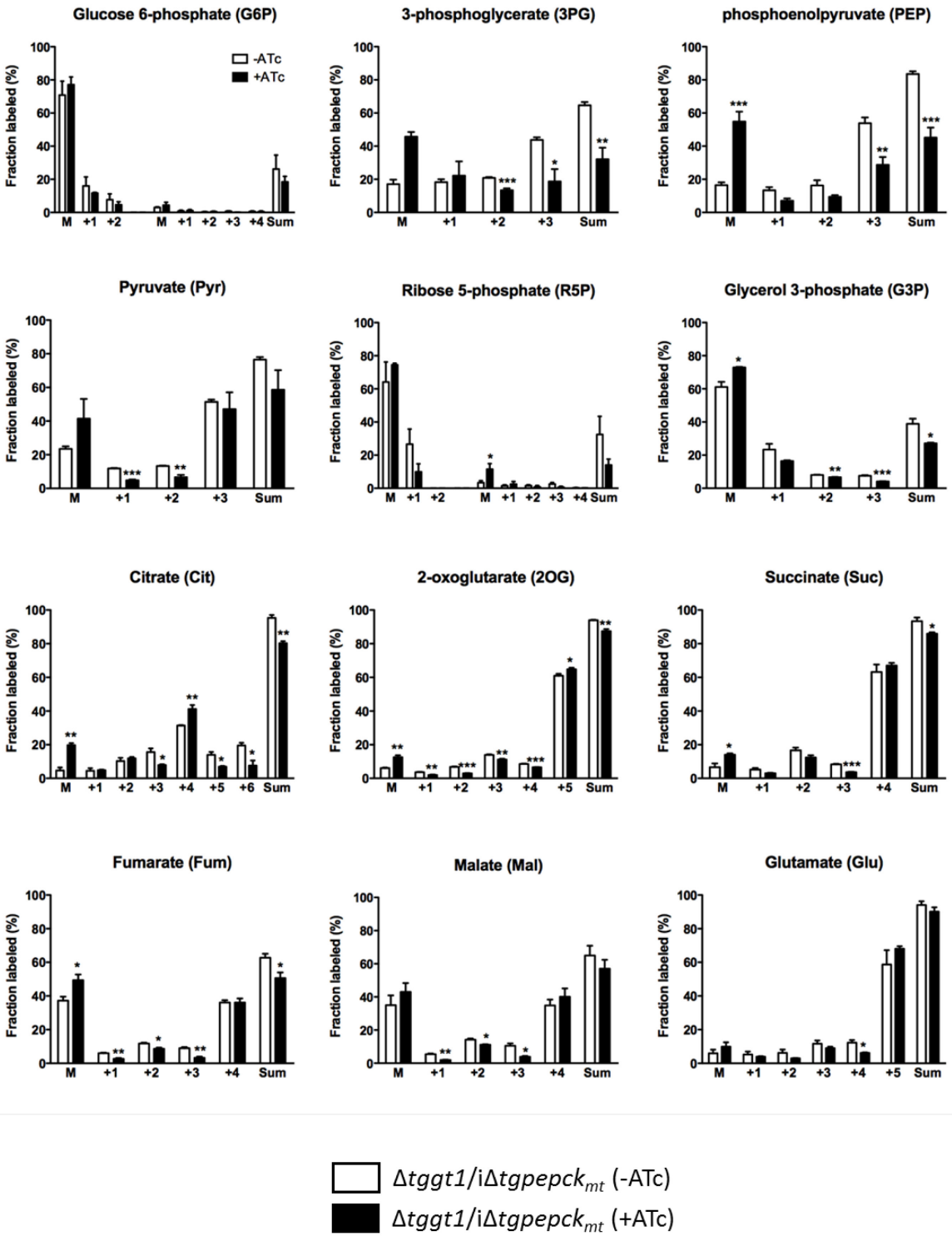


Figure S32. Fractional abundance of the select isotopomers in the Δ*tggt1/iΔtgpepck*$_{mt}$ tachyzoites labeled with [*U-*^{13}*C*]-glutamine.

Intracellular parasites were labeled as described in *methods*. '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. Statistical significance was determined separately for each group (+/-ATc) using student's *t*-test (n = 4 assays; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$).

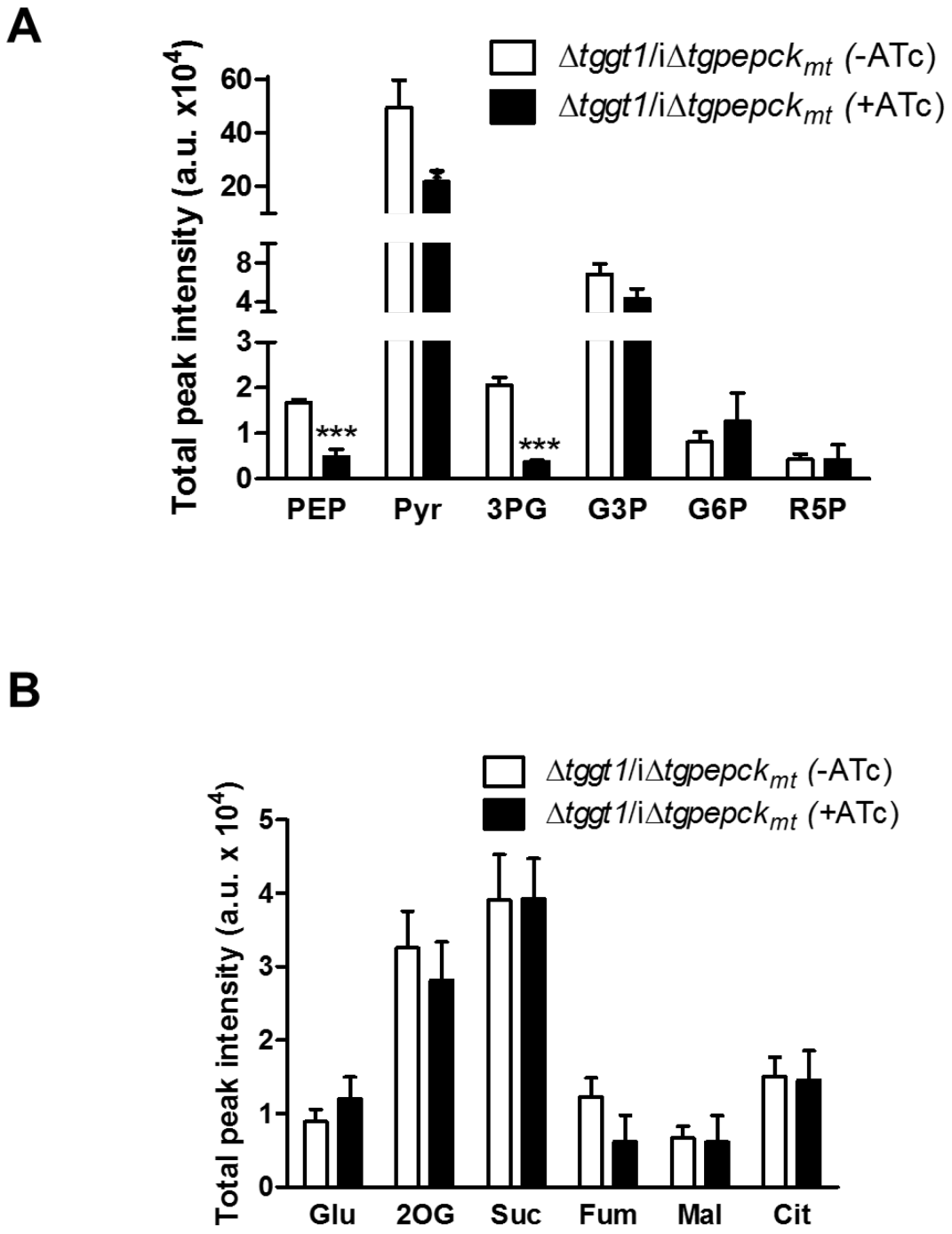


Figure S33. Metabolites of gluconeogenesis are reduced upon knockdown of *Tg*PEPCK$_{mt}$ in the Δ*tggt1* mutant.

Summed intensities of all isotopomers after isotopic abundance correction of metabolites associated with gluconeogenesis (A) and TCA cycle (B) are presented as a comparative measurement of abundance. Tachyzoites of the Δ*tggt1*/iΔ*tgpepck$_{mt}$* strain were subjected to metabolomics analyses as stated in *methods*. 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.

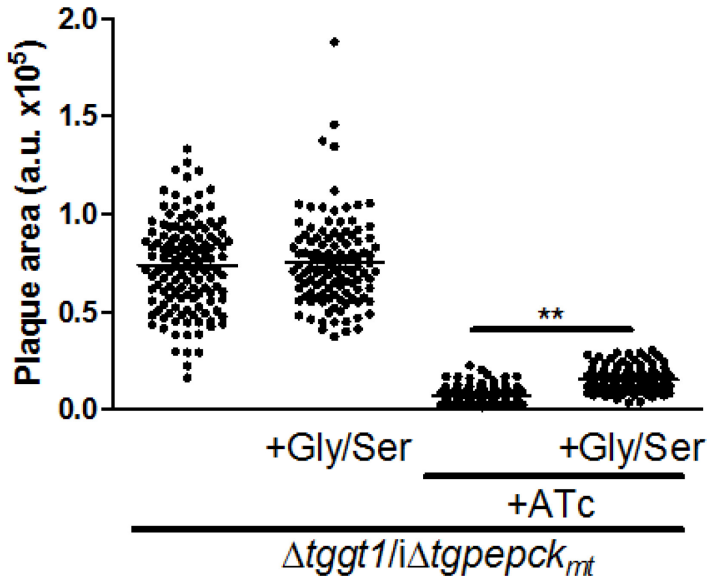
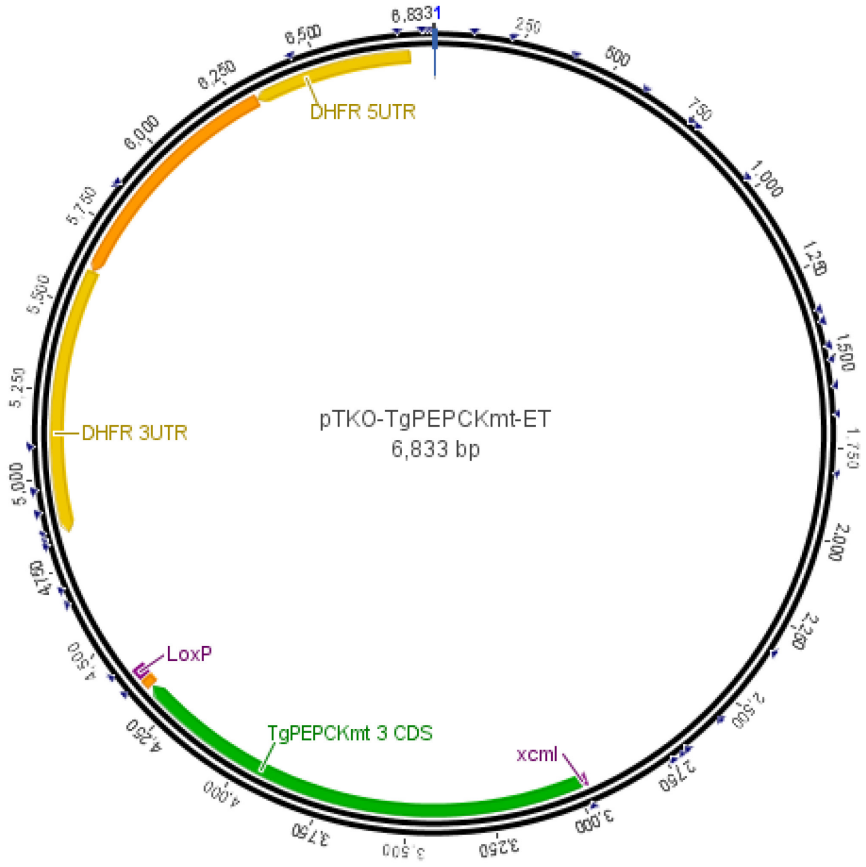


Figure S34. Supplementation with additional serine and glycine can partly restore off-state growth of the Δ*tggt1/i*Δ*tgpepck*$_{mt}$ mutant.

Plaque assays were performed in standard culture medium containing either the normal amounts of serine and glycine (100 µM each) or supplemented with additional amounts of both amino acids (2 mM each). Tachyzoites treated with ATc were precultured for 2 passages with the drug. Shown are the mean plaque area (arbitrary units) with SEM from 3 independent assays. No effect of nutrient supplementation was observed when *Tg*PEPCK$_{mt}$ is expressed (-ATc). Significance was measured separately for each group (+ or - Ser/Gly) using student's *t*-test (***, $p<0.01$).



The pTKO-*Tg*PEPCK$_{mt}$-ET vector was generated for endogenous HA-tagging of *Tg*PEPCK$_{mt}$.

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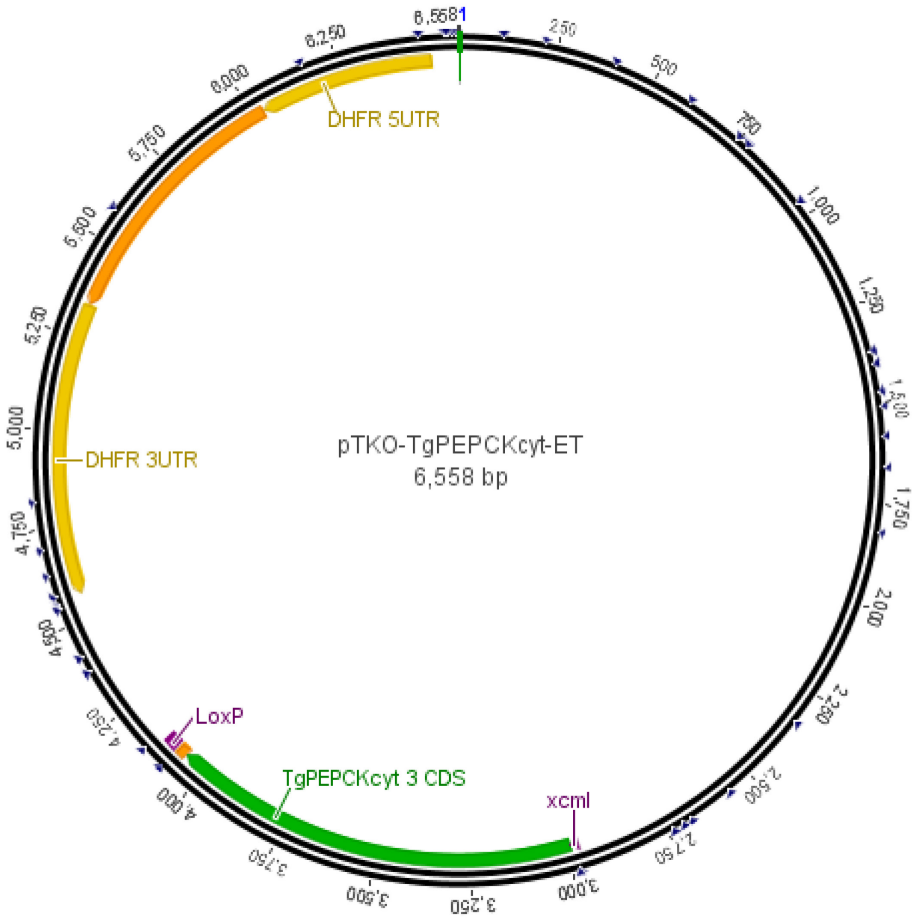

The pTKO-*Tg*PEPCK$_{net}$-ET vector was generated for endogenous HA-tagging of *Tg*PEPCK$_{net}$.

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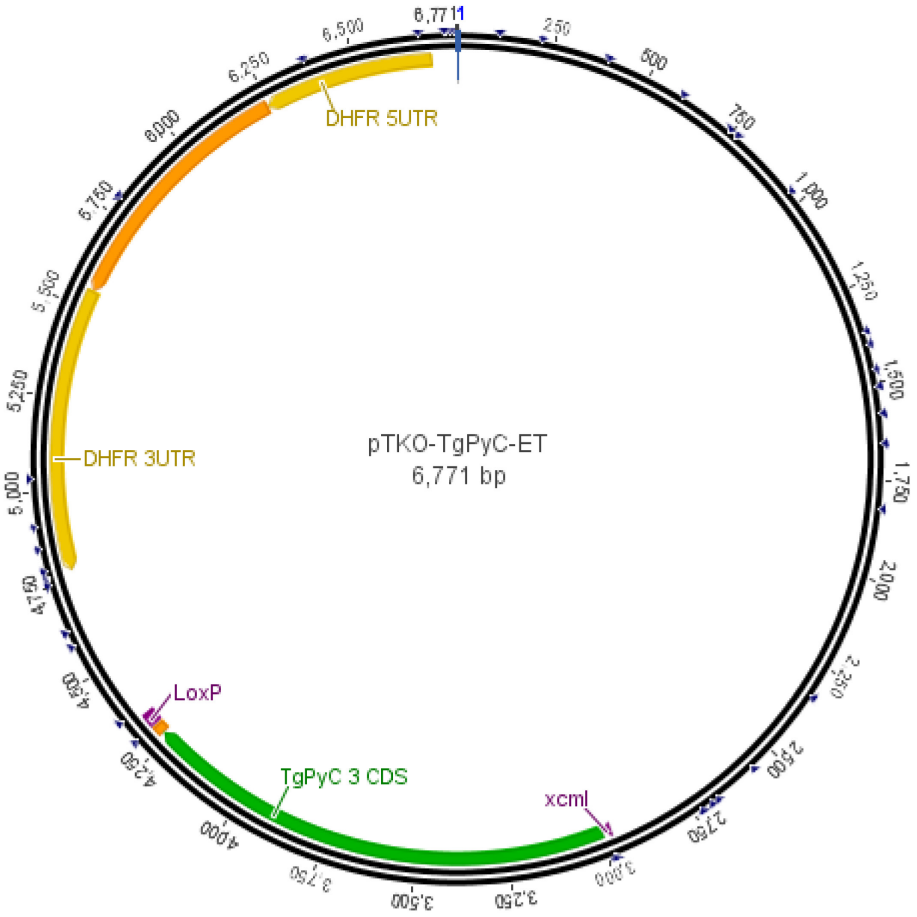

The pTKO-*Tg*PyC-ET vector was generated for endogenous HA-tagging of *Tg*PyC.

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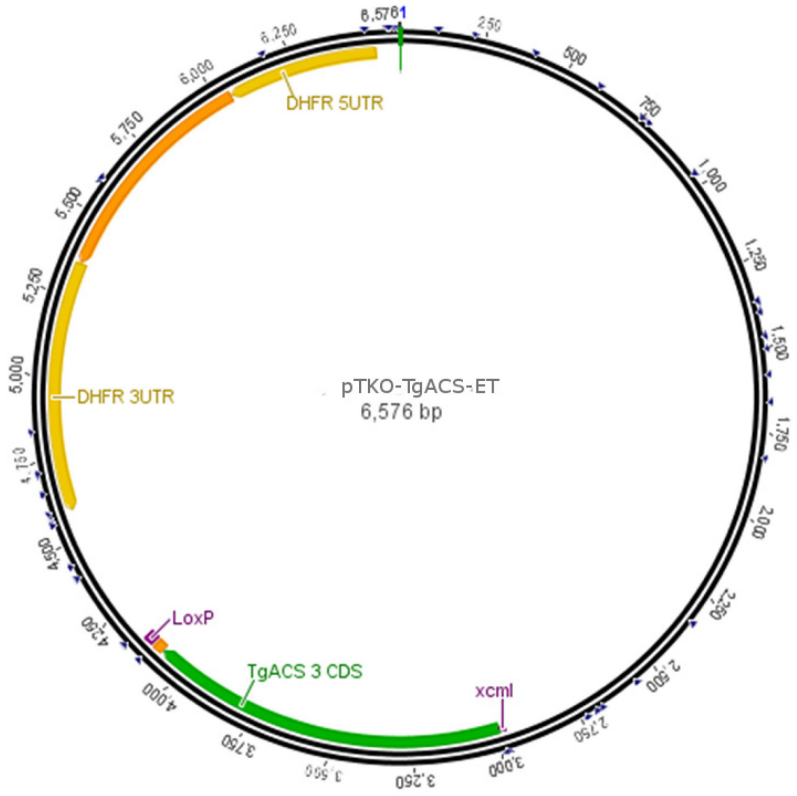

The pTKO-*Tg*ACS-ET vector was generated for endogenous HA-tagging of *Tg*ACS.

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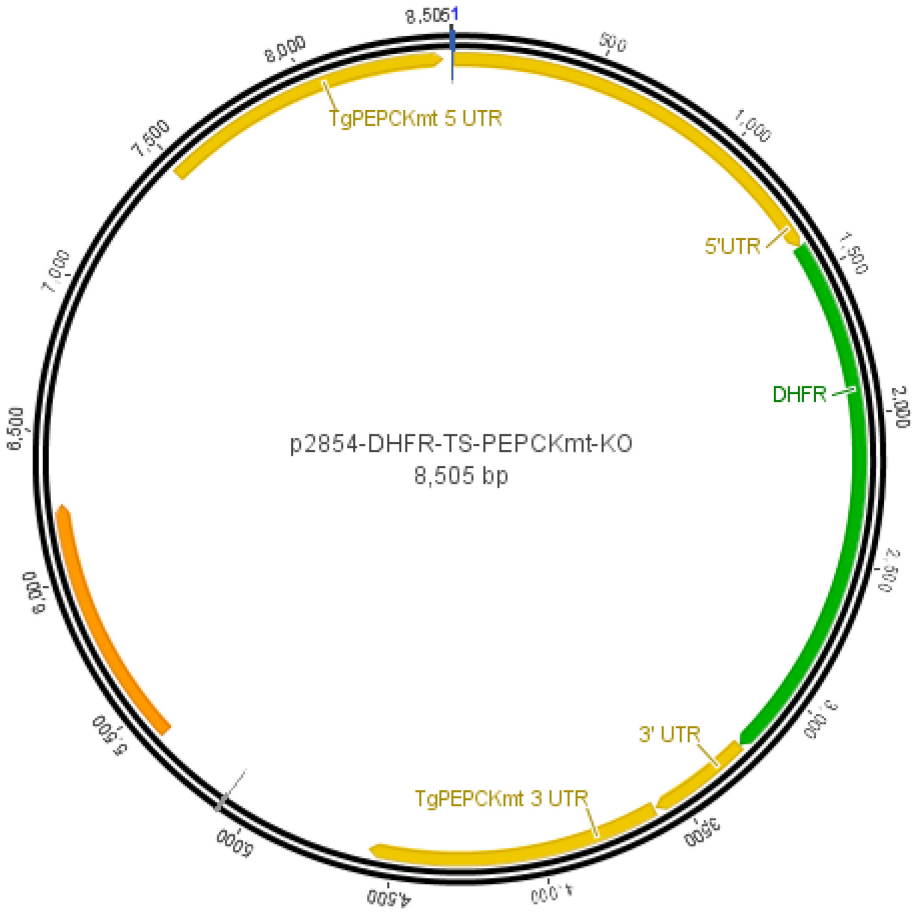

The p2854-DHFR-TS-PEPCK$_{mt}$-KO vector was generated for knockout of *Tg*PEPCK$_{mt}$.

>p2854-DHFR-TS-PEPCK$_{mt}$-KO

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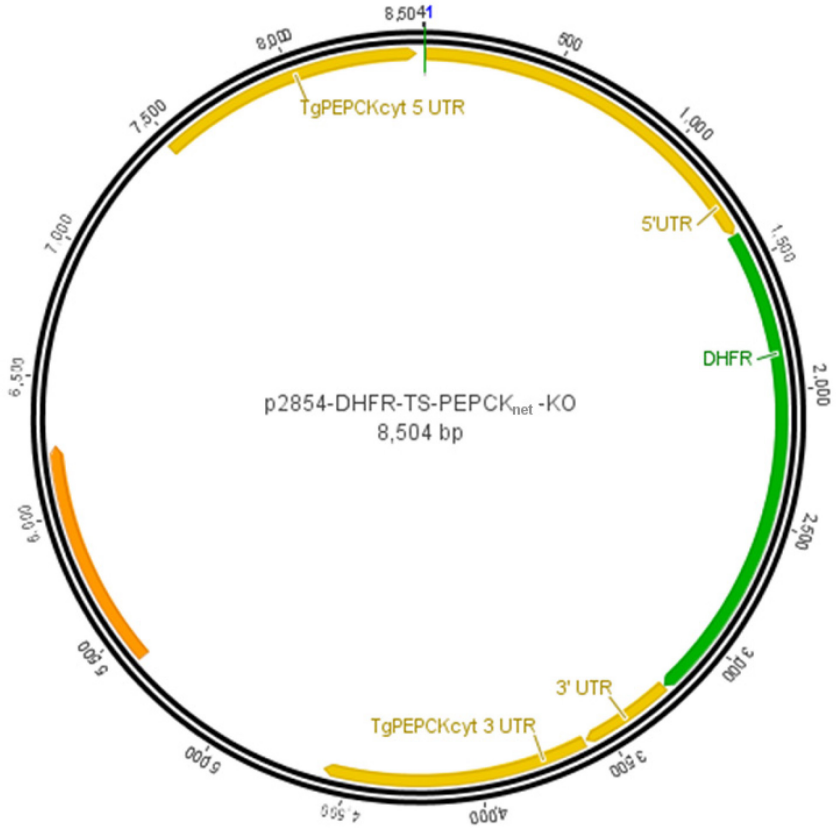

The p2854-DHFR-TS-PEPCK$_{net}$-KO vector was generated for knockout of *Tg*PEPCK$_{net}$.

>p2854-DHFR-TS-TgPEPCK$_{net}$-KO
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TGGGACACGCGAGGGATCATATCATCAATCCGTAGCATGTTCCCGAAGGGCCCCCCCTCGAGGTCGA
CGGTATCGAT

VI. List of publications

Nitzsche, R., Zagoriy, V., Lucius, R., and Gupta, N. (2015) Metabolic cooperation of glucose and glutamine is essential for the lytic cycle of obligate intracellular parasite *Toxoplasma gondii*. ***The Journal of biological chemistry***

Blume, M., **Nitzsche, R.**, Sternberg, U., Gerlic, M., Masters, S. L., Gupta, N., and McConville, M. J. (2015) A *Toxoplasma gondii* Gluconeogenic Enzyme Contributes to Robust Central Carbon Metabolism and Is Essential for Replication and Virulence. ***Cell host & microbe*** **18**, 210-220

Nitzsche, R., Zagoriy, V., Lucius, R., Dunai, I., and Gupta, N. (2016) A mitochondrial phosphoenolpyruvate carboxykinase refers glucose-independant growth of *Toxoplasma gondii*. ***The Journal of biological chemistry*** **(submitted).**

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VIII. Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation

„Genetic Dissection of the Central Carbon Metabolism in the Intracellular Parasite *Toxoplasma gondii*“

selbständig angefertigt und keine anderen Mittel als die angegebenen Hilfsmittel verwendet zu haben.

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Ort, Datum Unterschrift