SUPPORTING INFORMATION

Fig S1: *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.

Fig S2: *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. Organism Abbreviations: *Ab*, *Aeromonas bivalvium*; *At, Arabidopsis thaliana; Bb*, *Babesia bovis*; *Bi*, *Brucella inopinata*; *Br*, *Brassica rapa*; *Cr*, *Chlamydomonas reinhardtii*; *Cv*, *Chlorella variabilis*; *Ec*, *Escherichia coli*; *Em*, *Enterobacter massiliensis*; *Er*, *Eubacterium ramulus*; *Et*, *Eimeria tenella*; *Ga*, *Gossypium arboretum; Hh*, *Hammondia hammondi*; *Hs*, *Homo sapiens*; *Ia*, *Ignavibacterium album*; *Kp*, *Klebsiella pneumoniae*; *Kr*, *Ktedonobacter racemifer*; *Lb*, *Leptospira biflexa*; *Nc*, *Neospora caninum*; *Pf*, *Plasmodium falciparum*; *Pj*, *Pneumocystis jiroveci*; *Sa*, *Staphylococcus aureus*; *Sb*, *Salmonella bongori*; *Sc*, *Saccharomyces cerevisiae*; *Sl*, *Suillus luteus*; *Tg*, *Toxoplasma gondii*; *Tp*, *Theileria parva*; *Yp*, *Yersinia pestis; Zm, Zostera marina*

Fig S3: *TgPEPCK$_{net}$ 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.

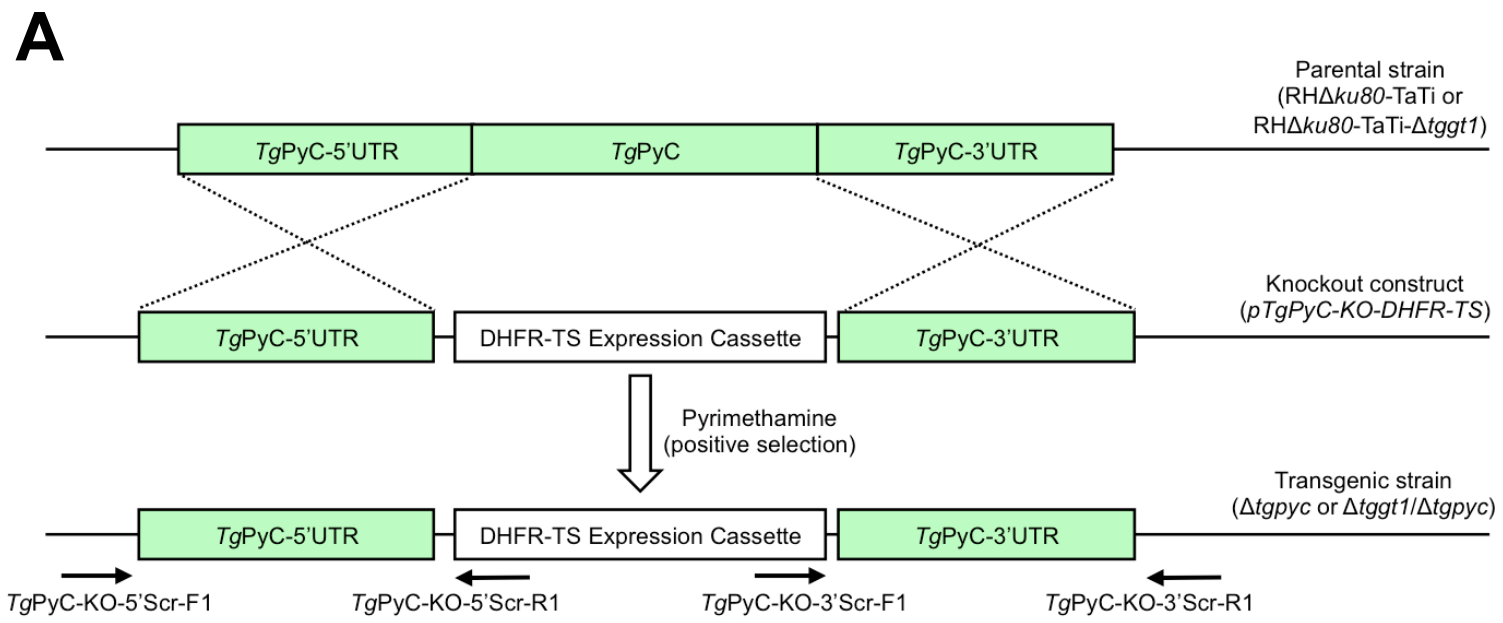
Fig S4: *Metabolites of gluconeogenesis are reduced upon knockdown of TgPEPCK*$_{mt}$ *in the Δtggt1 mutant.* Summed intensities of all isotopomers after isotopic abundance correction of metabolites associated with gluconeogenesis **(A)** and TCA cycle **(B)** are presented as a comparative measurement 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; R5P, ribose 5-phosphate; Glu, glutamate; 2OG, 2-oxoglutarate; Suc, succinate, Fum, fumarate; Mal, malate; Cit, citrate.

Fig S5: *Supplementation with additional serine and glycine can partly restore off-state growth of the Δtggt1/iΔtgpepck*$_{mt}$ *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 (**, $p<0.01$).

Table S1: Oligonucleotides used in this study

Primer name (Restriction site)	Primer sequence (Restriction site underlined)	Purpose (Plasmid name)
	***Tg*PyC**	
*Tg*PyC-ORF-F1 (*Nsi*I)	CTCATCATGCATATGATCATGGCACCTCTGACT	Ectopic overexpression (*pTgGRA2-UPKO*)
*Tg*PyC-ORF-HA-R1 (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTAT GGGTATAGAATGCGGACTAGCAAGTCA	
*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 (*Eco*RI)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTATGG GTATAGAATGCGGACTAGCAAGTCA	
*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 (*Eco*RI)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTATGG GTAGGAAGGGAGACTGTGGGTG	
*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	Tetracycline-regulated expression (*pTETO7SAG1-UPKO*)
*Tg*PEPCK$_{mt}$-ORF-HA-R1 (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTAT GGGTAAAAAACCGGACCAGCAGC	
*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)	CTCATCCCACCGGTCACCTGGGGTCTGATTTAGTGGCG CTAAG	Cloning of crossover sequence for 3'HA tagging (*p3'IT-HXGPRT*)
*Tg*PEPCK$_{mt}$-3'IT-COS-HA-R1 (*Eco*RI)	CTCATCGAATTCCTAAGCGTAATCTGGAACATCGTATGG GTAAAAAACCGGACCAGCAGC	
*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	

Fig S1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Knockout construct
(pTgPyC-KO-DHFR-TS)
TgPyC-5'UTR
DHFR-TS Expression Cassette
TgPyC-3'UTR
Pyrimethamine
(positive selection)
Transgenic strain
(Δtgpyc or Δtggt1/Δtgpyc)
TgPyC-5'UTR
DHFR-TS Expression Cassette
TgPyC-3'UTR
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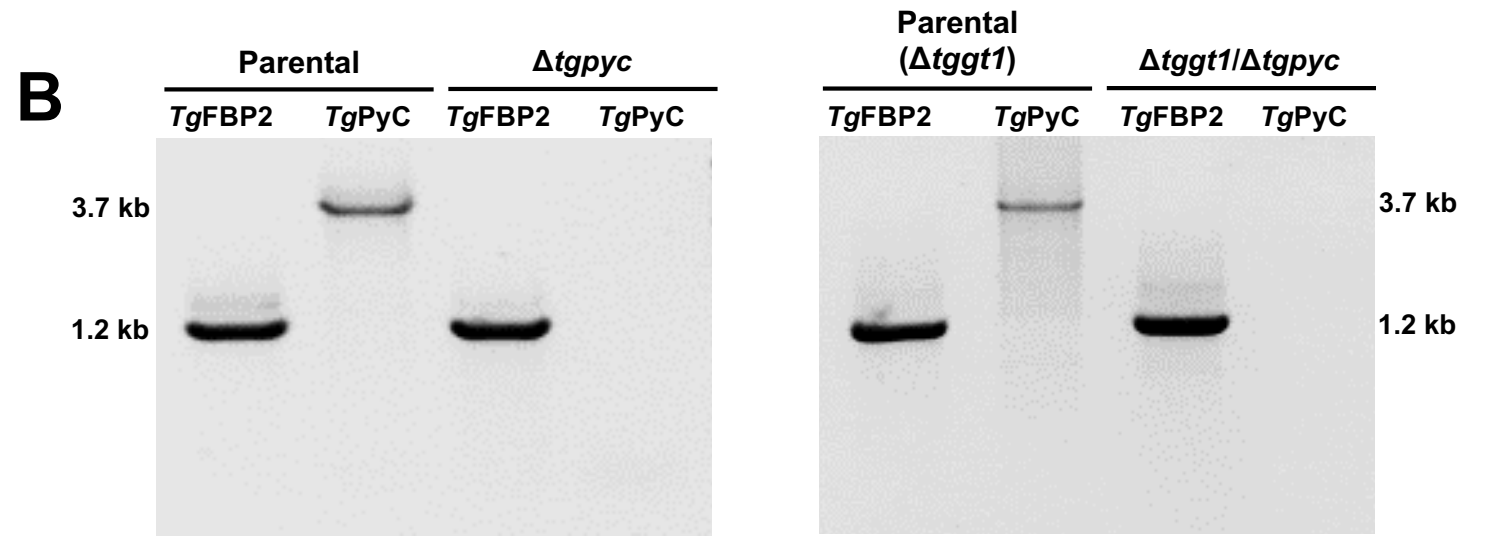
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Parental
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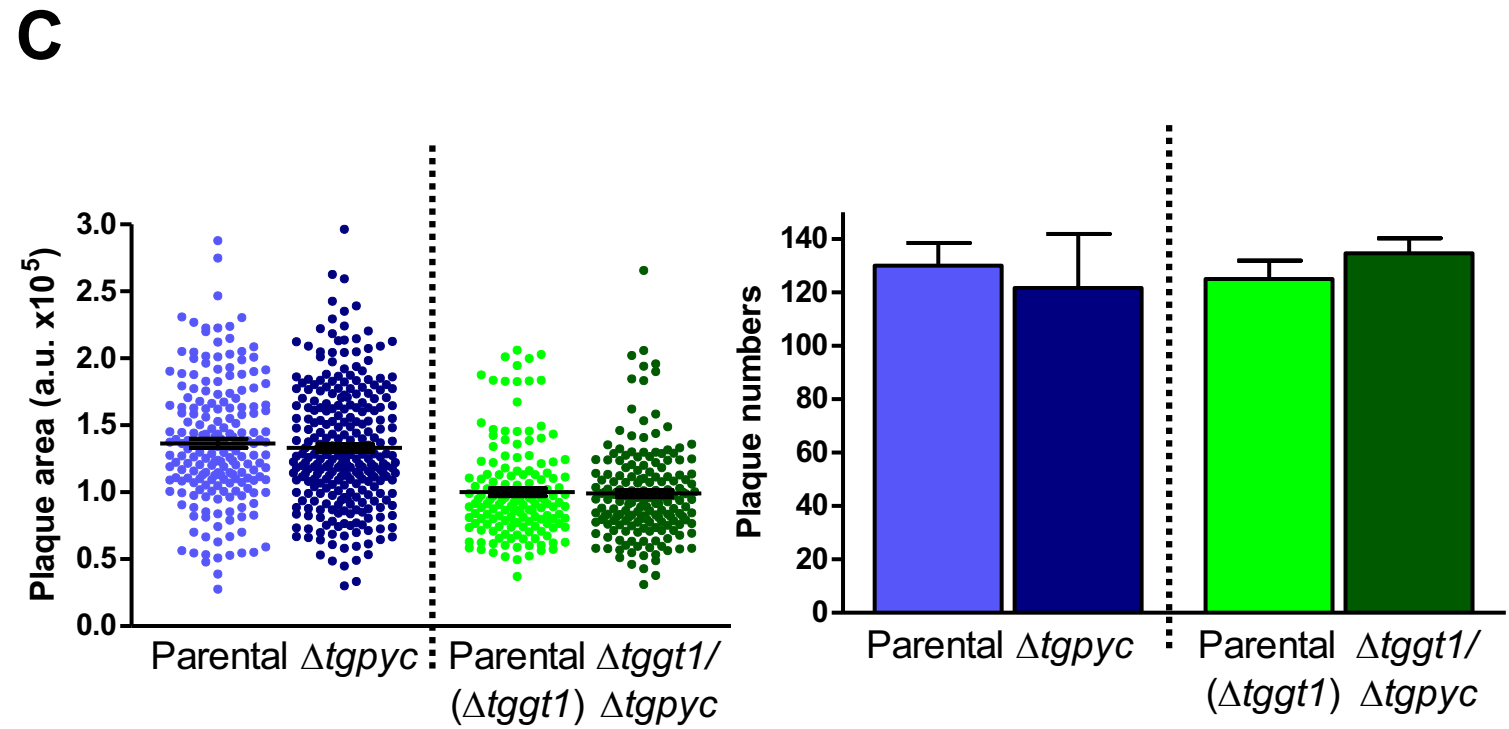
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Parental Δtggt1/
(Δtggt1) Δtgpyc
Plaque numbers
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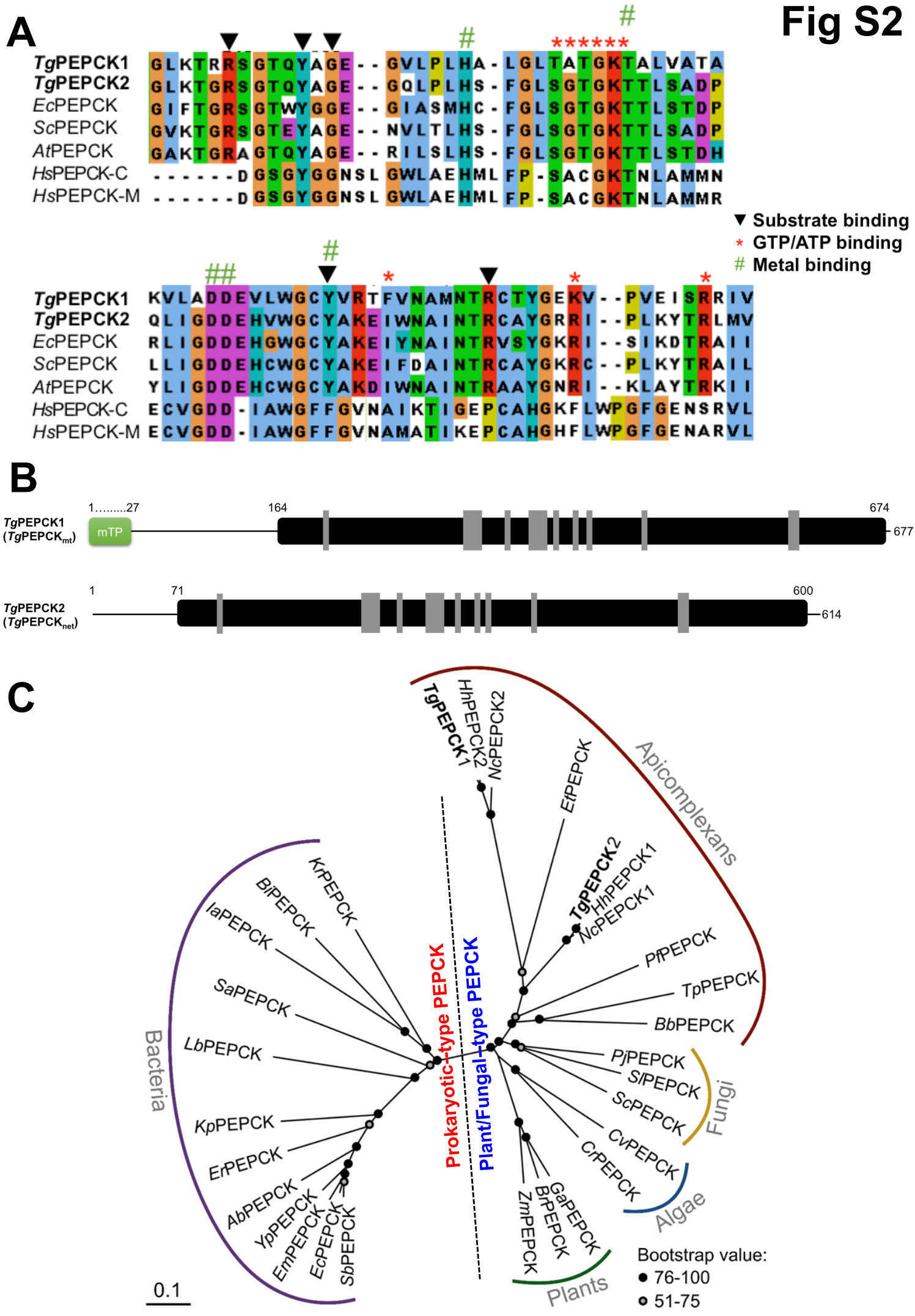
Parental Δtggt1/
(Δtggt1) Δtgpyc

Fig S2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TgPEPCK2
EcPEPCK
ScPEPCK
AtPEPCK
HsPEPCK-C
HsPEPCK-M
▼ Substrate binding
* GTP/ATP binding
# Metal binding
B
TgPEPCK1 (TgPEPCK_mt)
mTP
1.....27
164
674
677
TgPEPCK2 (TgPEPCK_net)
1
71
600
614
C
Apicomplexans
Bacteria
Fungi
Algae
Plants
Prokaryotic-type PEPCK
Plant/Fungal-type PEPCK
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HhPEPCK2
NcPEPCK2
EtPEPCK
TgPEPCK2
HhPEPCK1
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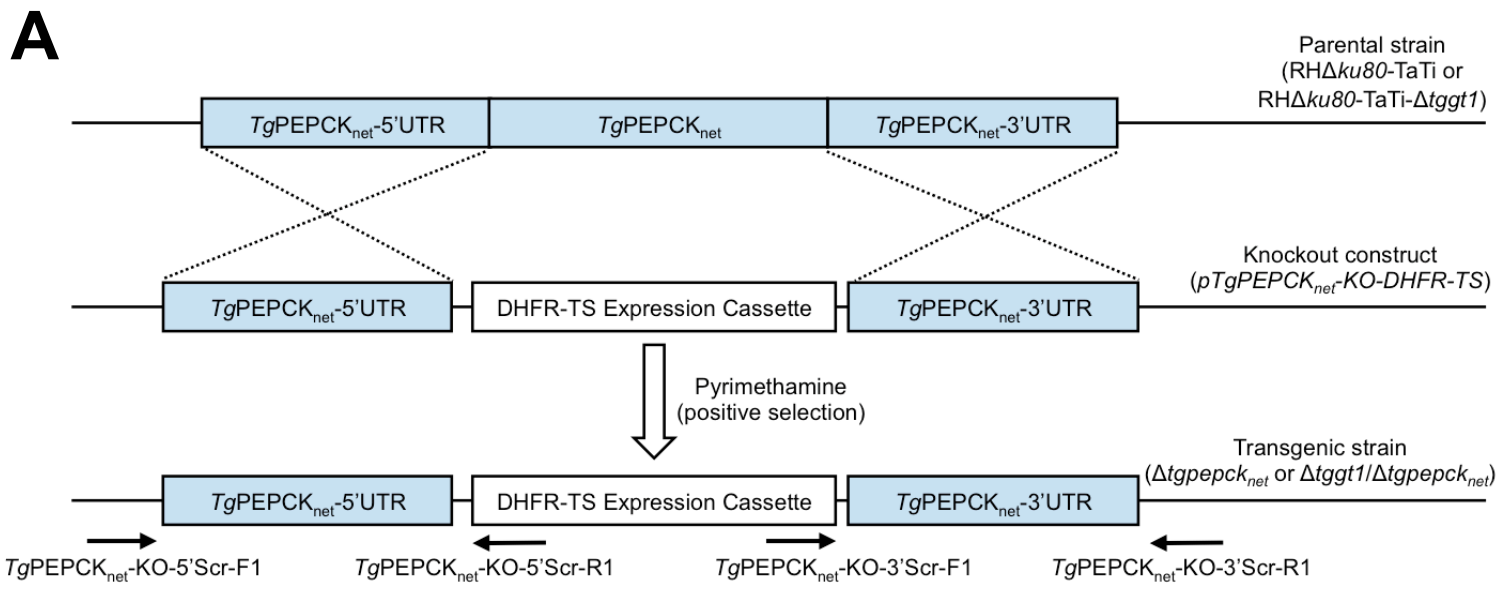
Fig S3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TgPEPCKnet-3'UTR
Knockout construct
(pTgPEPCKnet-KO-DHFR-TS)
TgPEPCKnet-5'UTR
DHFR-TS Expression Cassette
TgPEPCKnet-3'UTR
Pyrimethamine
(positive selection)
Transgenic strain
(Δtgpepcknet or Δtggt1/Δtgpepcknet)
TgPEPCKnet-5'UTR
DHFR-TS Expression Cassette
TgPEPCKnet-3'UTR
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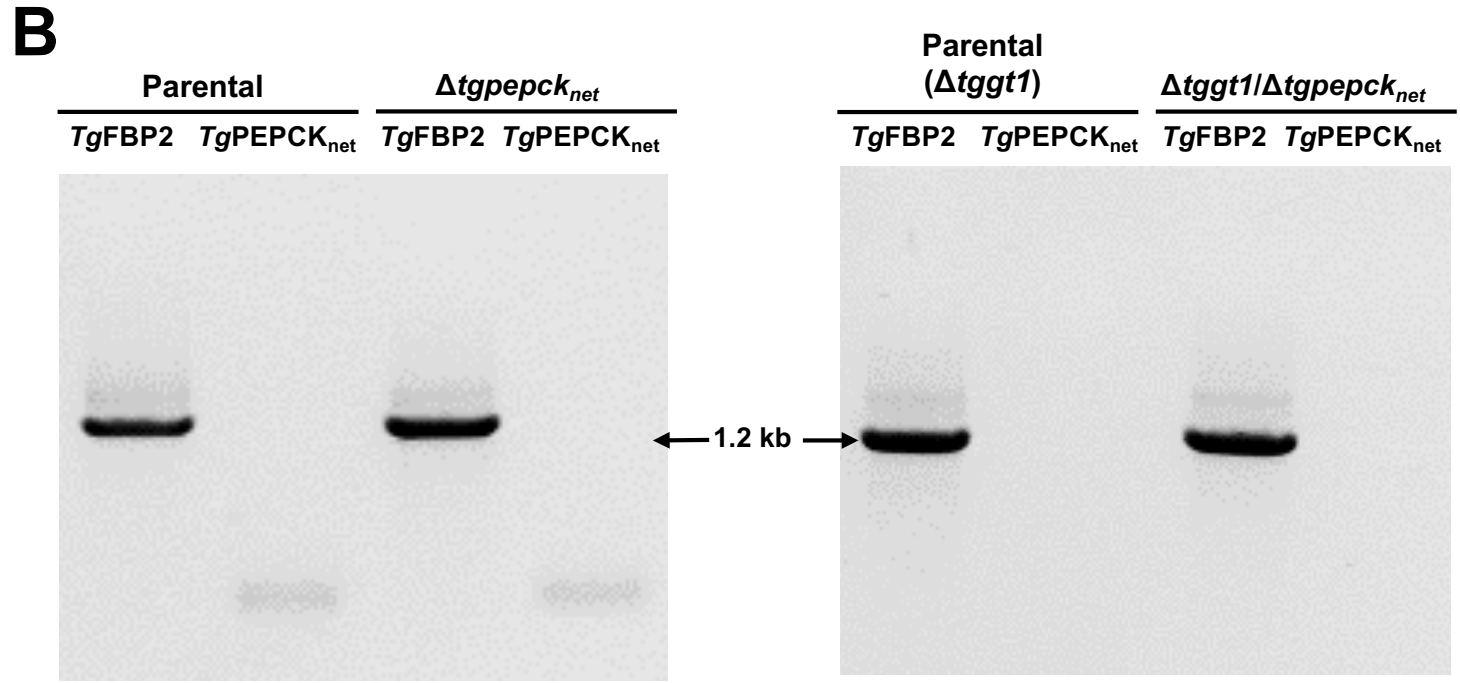
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Parental
(Δtggt1)
Δtggt1/Δtgpepcknet
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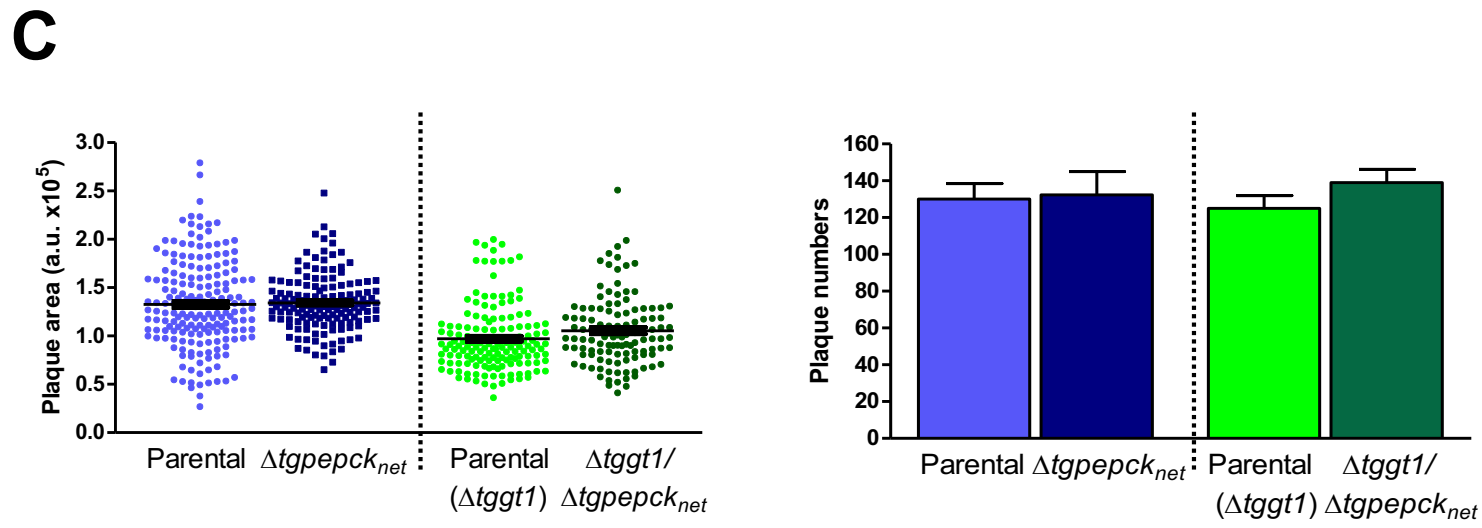
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2.5
2.0
1.5
1.0
0.5
0.0
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Δtgpepcknet
Parental
(Δtggt1)
Δtggt1/
Δtgpepcknet
Plaque numbers
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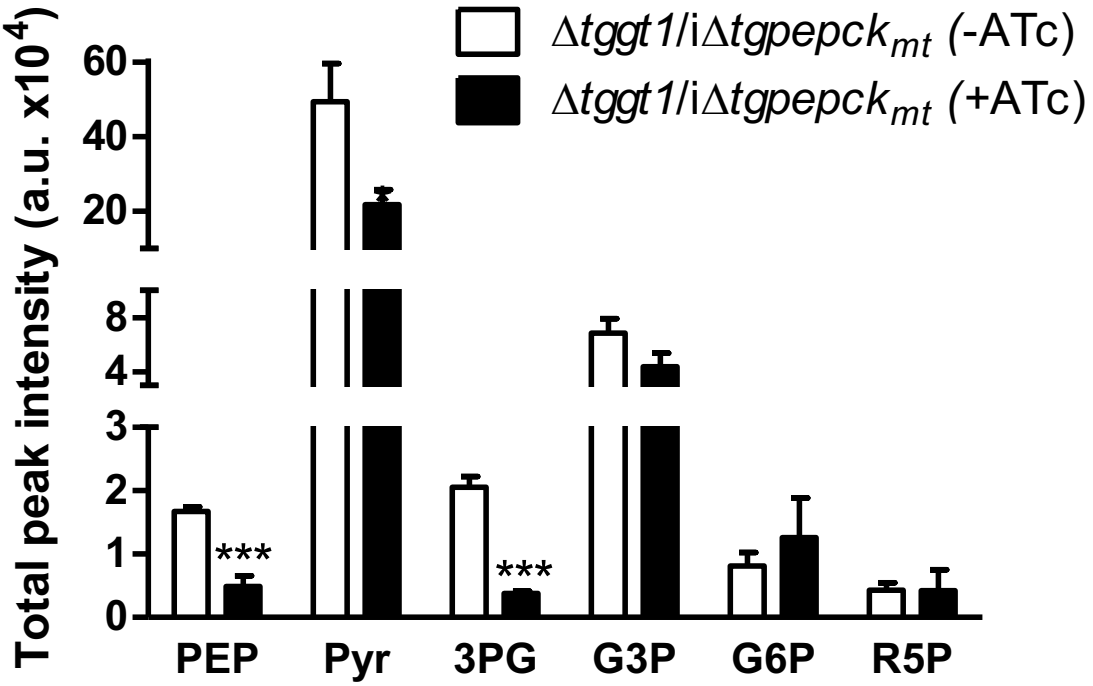
140
120
100
80
60
40
20
0
Parental
Δtgpepcknet
Parental
(Δtggt1)
Δtggt1/
Δtgpepcknet

Fig S4

A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Δtggt1/iΔtgpepck$_{mt}$ (+ATc)
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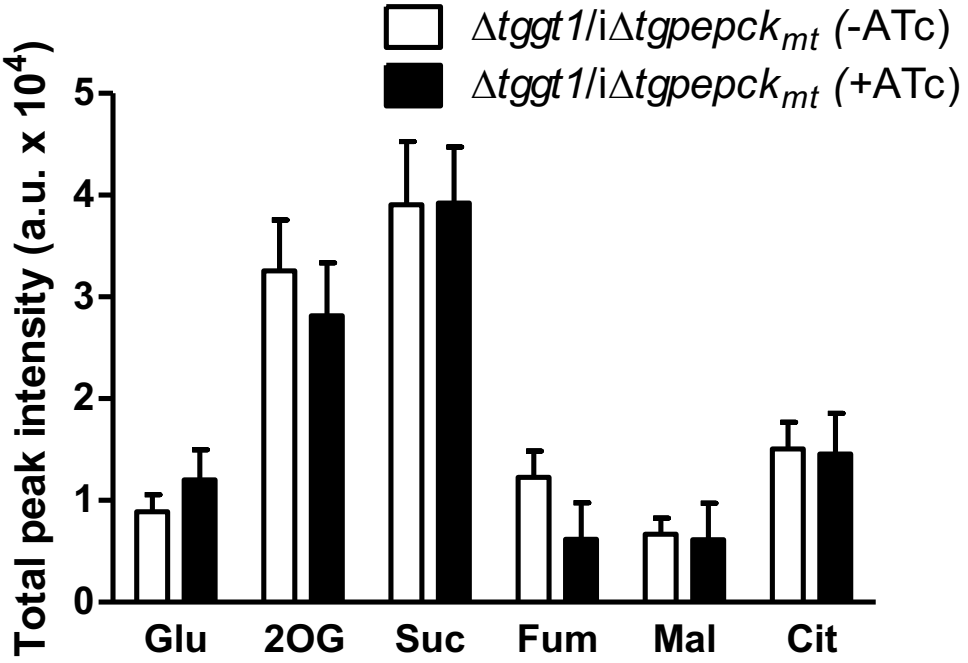
40
20
8
4
3
2
1
0
***
***
PEP
Pyr
3PG
G3P
G6P
R5P

B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Δtggt1/iΔtgpepck$_{mt}$ (+ATc)
Total peak intensity (a.u. x 10^4)
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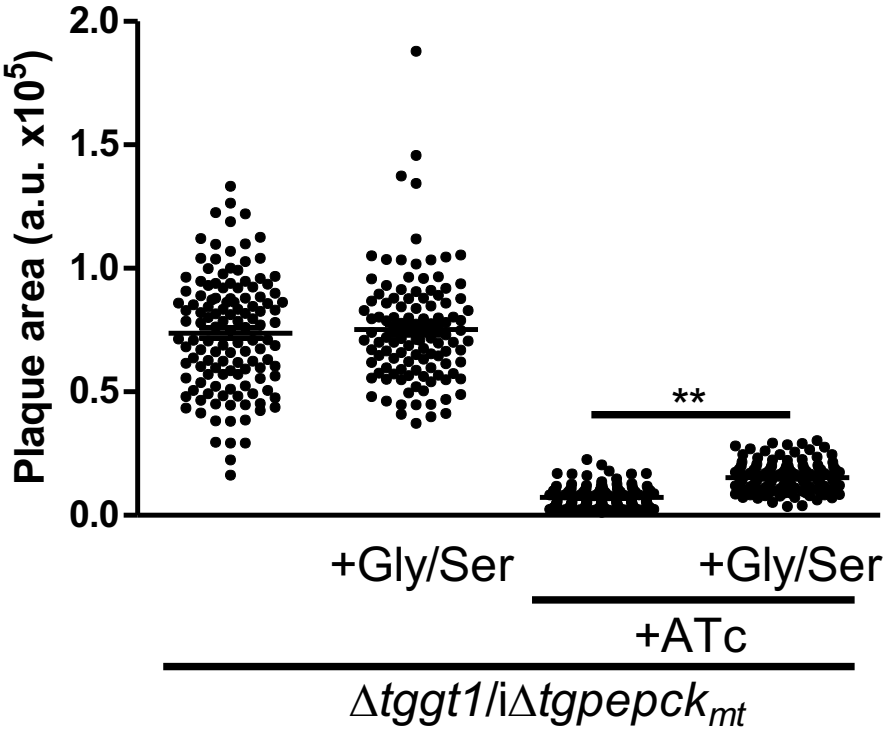
4
3
2
1
0
Glu
2OG
Suc
Fum
Mal
Cit

Fig S5


Plaque area (a.u. x10^5)
2.0
1.5
1.0
0.5
0.0
**
+Gly/Ser
+Gly/Ser
+ATc
Δtggt1/iΔtgpepck_mt