Phosphoglucomutase 1 Contributes to Optimal Cyst Development in *Toxoplasma Gondii*

Emily V. Quach  
Laney College

Binh Cao  
University of California San Francisco

Edres Babacarkhial  
California State University East Bay

Daniel Ho  
Stanford University School of Medicine

Janak Sharma  
California State University East Bay

Pascale S. Guiton (✉ pascale.guiton@csueastbay.edu)  
California State University East Bay  https://orcid.org/0000-0002-8186-3515

Research Article

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Abstract

Objective: *Toxoplasma gondii* is a ubiquitous parasite of medical and veterinary importance; however, there exists no cure for chronic toxoplasmosis. Metabolic enzymes required for the production and maintenance of tissue cysts represent promising targets for novel therapies. Here, we use reverse genetics to investigate the role of *Toxoplasma* phosphoglucomutase 1, PGM1, in *Toxoplasma* growth and cystogenesis.

Results: We found that disruption of *pgm1* did not significantly affect *Toxoplasma* intracellular growth and the lytic cycle. *pgm1*-defective parasites could differentiate into bradyzoites and produced cysts containing amylopectin *in vitro*. However, cysts produced in the absence of *pgm1* were significantly smaller than wildtype. Together, our findings suggest that PGM1 is dispensable for in vitro growth but contributes to optimal *Toxoplasma* cyst development *in vitro*, thereby necessitating further investigation.

Introduction

*Toxoplasma gondii* is an obligate intracellular protozoan responsible for toxoplasmosis in humans and other warm-blooded animals. Infections occur mostly from consuming contaminated water, food, or undercooked meat from chronically infected animals [1]. Bradyzoites inside tissue cysts are released into the gastrointestinal tract where they invade enterocytes and convert to tachyzoites inside a parasitophorous vacuole (PV). Tachyzoites replicate rapidly, eventually lysing out of the host cell to disseminate throughout the body. In response to stressful stimuli, they convert back to bradyzoites which remain encysted in the brain and skeletal muscles for life [2]. Chronic toxoplasmosis is incurable and parasite reactivation life-threatening, particularly for the immunocompromised [3].

Bradyzoites are replete with cytoplasmic amylopectin granules [4]. The tight regulation of enzymes involved in metabolizing this polysaccharide is critical for tissue cyst production and survival [5–8]. Phosphoglucomutases (PGMs) catalyze the interconversion of glucose-1-phosphate to glucose-6-phosphate [9], effectively linking amylopectin metabolism to glycolysis in this parasite. Both PGM paralogs in *Toxoplasma* [10], PGM1, also known as parafusin-related *Toxoplasma* protein 1 (PRP1) [11], and PGM2, are upregulated during chronic infection in mice [12] and have been implicated in calcium (Ca$^{2+}$)-dependent signaling for microneme secretion [13–15].

Here, we used the CRISPR/Cas9 gene-editing system [16] to disrupt *pgm1* in a cyst-forming *Toxoplasma* strain. Our data show that this mutation did not prevent intracellular replication or the completion of the lytic cycle. While both strains could produce amylopectin-containing cysts, we found that *pgm1*-defective cysts are significantly smaller than the parent’s. Together, our findings corroborate previous reports that PGM1 is dispensable for *Toxoplasma* viability and demonstrate that the enzyme contributes to optimal cyst development *in vitro*.

Materials And Methods
Parasite and host cells

Human foreskin fibroblasts (HFFs) and Me49Δhxgprt, a Type II strain of *Toxoplasma* lacking hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT), were kind gifts from John Boothroyd at Stanford University. Parasites were maintained in HFFs in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 2.5 µg/ml fungizone, 100 U/ml penicillin, and 100 µg/ml streptomycin (cDMEM) at 37°C and 5% CO₂.

Disruption of *pgm1*

All primers used in this study are listed in Additional file 1. pSAG::Cas9-U6::sgPGM1 was obtained by substitution of sgUPRT with sgPGM1 in pSAG1::Cas9-U6::sgUPRT [16] using Q5 site-directed mutagenesis (New England Biolabs Inc, NEB). pUC19 modified to express *hxgprt* under the dihydrofolate reductase (DHFR) promoter using standard molecular cloning techniques to create pDHFR::*hxgprt*. Freshly released Me49Δhxgprt (WT) were transfected with pSAG1::Cas9-U6::sgUPRT and linearized pDHFR::*hxgprt* at a 1:3 molar ratio in a 4 mm gap cuvette in an BTX ECM 630 Exponential Decay Wave electroporator system (BTX Harvard Apparatus) [16]. Transgenic Me49ΔhxgprtΔpgm1 parasites (Δpgm1) were obtained after 10 days of selection in cDMEM containing 25 µg/ml of mycophenolic acid and 50 µg/ml xanthine and cloned by limiting dilutions [17]. Disruption of *pgm1* and integration of the selection cassette were confirmed by polymerase chain reaction (PCR) and DNA sequencing (Elim Biopharmaceuticals Inc).

Replication assay

Freshly released parasites were centrifuged at 1500 rpm for 10 minutes and washed once with 1XPBS. Confluent HFFs on glass coverslips were infected with 1.2 x 10⁵ parasites in cDMEM for 24h. The number of parasites per vacuole was determined by immunofluorescence microscopy.

Plaque assay

WT and Δpgm1 tachyzoites were syringe-lysed through a 27G needle and passed through a 5 µm filter. Confluent HFFs were infected with 250 parasites in cDMEM and incubated at 37°C with 5% CO₂ for 10 days undisturbed. Following methanol fixation and crystal violet staining, plaque numbers and sizes were determined using a stereoscope (Leica EZ4) and ImageJ version 1.52A (National Institutes of Health).

Tachyzoite-to-bradyzoite differentiation

Tachyzoites were induced to differentiate into bradyzoites in HFFs as previously described [18]. Briefly, confluent HFFs on glass coverslips were infected with 4.8 x 10⁴ parasites for 3 hours in cDMEM before replacing the medium with Switch Medium (RPMI 1640 supplemented with 1% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin, 10 mg/mL HEPES, pH 8.2). Parasites were incubated for 4
days at 37ºC with ambient CO₂ and the medium was changed every 24 hours to maintain alkaline conditions.

**Immunostaining fluorescence assay and amylopectin staining**

Infected monolayers were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at room temperature (RT). Cells were permeabilized with 0.2% or 0.4% Triton X-100 for 20 minutes and incubated for 1 hour in 3% Bovine Serum Albumin (BSA; Fisher Scientific) in PBS. Primary antibodies diluted in 3% BSA/PBS (mouse α-SAG1 1:10000, rabbit α-GRA7 1:1000) were added to the monolayers, when indicated, and incubated overnight at 4ºC. Unbound antibodies were washed away with three 5-minute washes in 1XPBS. The cells were then stained with secondary antibodies in 3% BSA/PBS (Goat α-Mouse 546 or Goat α-Rabbit 488 at 1:5000) for 45 min at RT. *Dolichos biflorus* Agglutinin (DBA; Vector Laboratories) was used at 1:100 to detect the cyst wall. After washing as described above, the coverslips were mounted with VECTASHIELD Mounting Medium containing DAPI (Vector Laboratories). Amylopectin was stained with Periodic Acid Schiff (PAS; Fisher Scientific) according to the manufacturer's guidelines.

Immunofluorescence images were obtained using an inverted microscope (Leica DM IL LED) with 100X oil immersion objective. The number of parasites per vacuole and cyst areas, both selected from random fields of view, were determined using ImageJ version 1.52A and 1.53, respectively.

**Statistical methods**

Statistical analyses were performed using GraphPad Prism version 8.4.3. A *p*-value ≤ 0.05 was considered statistically significant difference between groups.

**Results**

*Toxoplasma* phosphoglucomutases are upregulated during chronic infection in mice

Comparative transcriptomic and proteomic analyses [19] revealed that *Toxoplasma* expresses stage-specific proteins which enable the parasite to survive and to be efficiently transmitted between hosts. We mined the transcriptional data from Pittman *et al.* [12] available on the *Toxoplasma* Informatics Resources database (ToxoDB) [10] to specifically identify metabolic enzymes involved in gluconeogenesis and glycolysis that are significantly upregulated at least 2 folds in chronic vs. acute infection. Of the 422 genes upregulated in chronic infection, our analysis revealed 21 that are specifically associated with carbohydrate metabolism (Fig. 1A-B, Additional File 1). As expected, these genes include well-known glycolytic isoenzymes involved in tissue cyst formation, such as lactate dehydrogenase 2 (*ldh2*) [20] and enolase 1 (*eno1*) [21]. Interestingly, unlike *ldh1/ldh2* and *eno1/eno2* which are expressed in a stage-dependent manner, both PGM isoforms (*pgm1* and *pgm2*) were upregulated 6.4 and 3.1 folds, respectively, in the chronic stage, 28 days post-infection (dpi) [12]. Transcriptional analyses of gene expression at 28, 90, and 120 dpi from Garfoot *et al.* [22] indicate that unlike *pgm2* whose expression
remained similar up to 120 dpi, pgm1 transcripts further increased from 28 to 120 dpi. Together, this analysis strongly suggests that transcriptional regulation of pgm1/pgm2 may be critical for the development and/or maintenance of tissue cysts in mice.

**Disruption of pgm1 does not hinder parasite growth in vitro**

To determine the contribution of PGM1 to *Toxoplasma* growth, we used the CRISPR-Cas9 gene-editing system to create an insertional mutant Me49ΔhxgprtΔpgm1 (Δpgm1) by introducing a hxgprt selection cassette at the pgm1 locus [16] (Fig. 2A-B). We assessed the intracellular growth of Δpgm1 parasites vs. WT 24 hours after infection of HFFs in glucose replete growth medium. SAG1-positive parasites inside GRA7-positive vacuoles were enumerated. We found similar numbers of Δpgm1 vacuoles with either 2, 4, or ≥8 parasites as WT (Fig. 2C). Likewise, no significant differences in plaque numbers and sizes were observed 10 days after infection (Fig. 2D-E). Thus, as previously reported for *Toxoplasma* RH strain [15, 23], our data indicate that PGM1 is dispensable for *Toxoplasma* intracellular growth and lytic cycle in vitro, albeit in glucose-rich conditions.

pgm1- defective parasites produced smaller amylopectin-containing cysts in vitro

Given the upregulation of pgm1 in chronic infection, we tested whether disruption of pgm1 would impede tissue cyst formation. We induced tachyzoites to differentiate into bradyzoites in nutrient-poor, alkaline conditions in ambient CO₂ [18]. After four days, we stained the monolayers with *Dolichos biflorus* agglutinin (DBA) to detect the cyst wall and Periodic Acid Schiff (PAS) to visualize amylopectin [8]. Both WT and Δpgm1 parasites produced PAS-positive cysts (Fig. 3A), suggesting that PGM1 is not essential for amylopectin accumulation during stage conversion in vitro. However, further studies are required to determine any differences in the relative amount of this polysaccharide between WT and Δpgm1 cysts. Interestingly, Δpgm1 cysts were on average ~4060 pixels² smaller than WT (p = 0.0362 by Mann-Whitney test, Fig. 3C). Together, our results indicate that although PGM1 is not required for stage conversion and amylopectin storage, the enzyme contributes to optimal cyst development in vitro.

**Discussion**

PGM1 is one of two PGM isoforms differentially expressed in *Toxoplasma* [10, 12, 24]. In this study, we showed that disruption of pgm1 in a cyst-forming *Toxoplasma* strain did not prevent intracellular growth or completion of the lytic cycle in glucose-replete conditions, corroborating previous studies in non-cyst forming Type I tachyzoites [15, 23]. Our observation that tachyzoites lacking pgm1 could differentiate into bradyzoites in the absence of glucose further supports the nonessential role of PGM1 and PGM1-dependent glucose-6-phosphate production in tachyzoites as suggested by Imada *et al.* [25]. Interestingly, PGM1 has been implicated in Ca²⁺-dependent microneme secretion in tachyzoites [11, 13, 15], and thus, like functionally characterized PGMs in other organisms [9, 26], it may play an unconventional role during *Toxoplasma* development.
Although glycolysis is not required for tachyzoite viability, it is critical for tissue cyst formation and pathogenesis in mice [27]. Parasites lacking hexokinase, the first enzyme in glycolysis that catalyzes the phosphorylation of glucose to glucose-6-phosphate, produce smaller cysts in vitro [27]. This phenotype was recapitulated in pgm1-defective parasites, further supporting the importance of glycolytic intermediates during cystogenesis. However, the absence of pgm1 did not abrogate amylopectin biosynthesis and storage, probably due to functional compensation with PGM2. While both pgm1 and pgm2 transcripts are higher in bradyzoites than tachyzoites [24], the proteins share only 25% homology. PGM2 has a significantly lower enzymatic activity than PGM1 [25]. Interestingly, Saha et al. [15] demonstrated that PGM2 didn’t compensate for the deletion of PGM1 in the context of Ca$^{2+}$-regulated microneme secretion in tachyzoites.

Overall, this study suggests that PGM1 is not critical for Toxoplasma growth and differentiation; however, it is required for optimal cyst maturation. Future studies is needed to parse out the interplay and diverse activities of Toxoplasma PGMs and understand how they affect central carbon metabolism and developmental differentiation in this ubiquitous parasites.

**Limitations**

Due to institutional infrastructure failures that resulted in the loss of all parasite lines, including the ones used here, we were unable to perform complementation studies or growth assays in the presence or absence of various carbon sources. We also did not quantify PAS staining to identify any difference in amylopectin accumulation between WT and mutant parasites.

**Abbreviations**

BSA: Bovine Serum Albumin  
DBA: *Dolichos biflorus* agglutinin  
DHFR: dihydrofolate reductase  
HFF: Human foreskin fibroblast  
HXGPRT: hypoxanthine-xanthine-guanine phosphoribosyltransferase  
PAS: Periodic Acid Schiff  
PBS: phosphate-buffered saline  
PCR: Polymerase Chain Reaction  
PGM: Phosphoglucomutase  
PV: parasitophorous vacuole
**RT**: room temperature

**WT**: Wildtype strain

### Declarations

#### Ethics approval and consent to participate

Not applicable

#### Consent for publication

Not applicable

### Availability of data and materials

The transcriptional dataset used in this study is publicly available on ToxoDB version 54 ([www.toxodb.org](http://www.toxodb.org)). The authors declare that all data generated supporting the findings of this study are available within the article and its supplementary information file. Plasmids, parasite and host cell strains (except for the mutant strain) are available from the corresponding author upon request.

### Competing interests

The authors declare that they have no competing interests.

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### Authors’ Contributions

Conceptualization: EVQ, PSG. Data collection: EVQ, BC, DH, JS. Data curation: EVQ, EB, BC PSG. Formal analysis: EVQ, EB, BC, PSG. Writing original manuscript: EVQ, EB, PSG. Manuscript editing and revision: PSG. Supervision: PSG. All authors have read and approved the final manuscript.

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**Figures**
Figure 1

Identification of upregulated metabolic genes during *Toxoplasma* chronic infection (A) Workflow for identification of genes associated with glycolysis and gluconeogenesis with higher expression in chronic vs. acute infection in dataset from Pittman *et al.*[12]; the analysis was performed on ToxoDB [10]. (B) Word cloud of enriched pathways among the 422 genes upregulated during chronic infection in mice. The image was generated on ToxoDB. (C) Transcript levels of differentially regulated glycolytic and
gluconeogenic enzymes in *Toxoplasma*. Values were obtained from Pittman *et al.* dataset available on ToxoDB version 54.

**Figure 2**

**Disruption of pgm1 and growth assays.** (A) Schematic representation of disruption of *pgm1* using CRISPR-Cas9 gene-editing system for nonhomologous insertion of the *hxgprt* selectable marker cassette. The dotted line represents the region in the first exon of *pgm1* targeted by the small guide RNA (sgPGM1). (B) Image of DNA gel electrophoresis of PCR1-3 performed using DNA from wildtype (WT) and mutant (*Δpgm1*) to demonstrate integration of the *hxgprt* expression cassette at the *pgm1* locus. The expected product for PCR1 (212bp) was obtained only for WT while products for PCR2 (813bp) and PCR3 (1185bp) were amplified only with *Δpgm1* DNA. (C) Intracellular growth. HFFs were infected with 1.2 x 10^5* WT or *Δpgm1* parasites for 24 hours in cDMEM. Monolayers were fixed and stained with antibodies raised against SAG1 (tachyzoite surface marker) and GRA7 (PV marker). Intracellular parasites were enumerated in at least 20 vacuoles/strain/experiment, N=3 independent experiments; error bars = standard error of the mean; *p*-value was determined by Chi-square test. (D) Total numbers of plaques counted 10 days after infection of HFFs with 250 WT or *Δpgm1* parasites. (E) Plaque areas were determined for 85 WT and 109 *Δpgm1* plaques using Fiji/ImageJ in pixels^2, N=3 replicates/strain in a single experiment, error bar = standard deviation; ns: *p*-value>0.05 by nonparametric Mann-Whitney test.

**Figure 3**

**In vitro stage conversion assay.** (A) Representative fluorescence images of amylopectin-containing WT and *Δpgm1* cysts at 4 days post-induction. Infected monolayers were stained with PAS to detect amylopectin (red), DBA to label the cyst wall (green), and DAPI for nuclei (blue); Scale bar = 10 microns. (B) Representative images of WT and *Δpgm1* cysts 4 days post-induction in vitro. The images are representative of the mean value of cyst areas for each strain. Cysts were stained with DBA (red), anti-GRA7 (green), and DAPI (blue); Scale bar = 10 microns. (C) Quantification of cyst sizes. The areas of 176 WT and 185 *Δpgm1* cysts were determined in pixels^2 at 4 days post-induction from 3 independent experiments; *p*=0.0362 by nonparametric Mann-Whitney test.

**Supplementary Files**

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