The FBPase Encoding Gene glpX Is Required for Gluconeogenesis, Bacterial Proliferation and Division In Vivo of Mycobacterium marinum

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Abstract

Lipids have been identified as important carbon sources for Mycobacterium tuberculosis (Mtb) to utilize in vivo. Thus gluconeogenesis bears a key role for Mtb to survive and replicate in host. A rate-limiting enzyme of gluconeogenesis, fructose 1, 6-bisphosphatase (FBPase) is encoded by the gene glpX. The functions of glpX were studied in M. marinum, a closely related species to Mtb. The glpX deletion strain (ΔglpX) displayed altered gluconeogenesis, attenuated virulence, and altered bacterial proliferation. Metabolic profiles indicate an accumulation of the FBPase substrate, fructose 1, 6-bisphosphate (FBP) and altered gluconeogenic flux when ΔglpX is cultivated in a gluconeogenic carbon substrate, acetate. In both macrophages and zebrafish, the proliferation of ΔglpX was halted, resulting in dramatically attenuated virulence. Intracellular ΔglpX exhibited an elongated morphology, which was also observed when ΔglpX was grown in a gluconeogenic carbon source. This elongated morphology is also supported by the observation of unseparated multi-nucleoid cell, indicating that a complete mycobacterial division in vivo is correlated with intact gluconeogenesis. Together, our results indicate that glpX has essential functions in gluconeogenesis, and plays an indispensable role in bacterial proliferation in vivo and virulence of M. marinum.

Introduction

Mycobacterium tuberculosis (Mtb), one of the most successful human pathogens, has evolved strategies to evade host immunity and cause tuberculosis (TB) [1,2]. During TB pathogenesis, central carbon metabolism (CCM) plays a critical role, and many CCM genes are considered
essential [3]. Unlike in vitro cultivation, growing evidence indicates that Mtb tends to utilize lipids in vivo, including fatty acids, cholesterol, cholesteryl ester, and triacylglycerides [3,4]. Thus, both the glyoxylate cycle and gluconeogenesis have important roles in converting metabolites from lipid catabolism to key precursors in various bacterial physiological processes, such as the synthesis of peptidoglycan (PG) and amino acids [5,6]. Therefore, intensive studies have been performed on several genes in these metabolic pathways. For example, icl1 and icl2 from the Mtb genome encode isocitrate lyases in the glyoxylate cycle and are jointly required for Mtb survival in macrophages and mice through infection [7,8]. Moreover, pckA encoding phosphoenolpyruvate carboxykinase that catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis plays a pivotal role for survival and proliferation of Mtb during mouse infection [9]. In addition, fba that modulates the switch between glycolysis and gluconeogenesis plays a considerable role in both acute and chronic mouse infections [10,11]. Therefore, gluconeogenesis is critical to TB pathogenesis and several gluconeogenic enzymes have been proposed as potential targets for chemotherapeutic interventions [10,11].

Fructose-1, 6-bisphosphatase (FBPase), encoded by glpX, is a key gluconeogenic enzyme catalyzing an irreversible reaction that converts fructose 1, 6-bisphosphate (FBP) to fructose 6-phosphate (F6P) [12,13]. F6P is a critical precursor for synthesis of cell envelope components, such as glycans and mannolipids [14,15,16]. Moreover, F6P is fed into the pentose phosphate pathway (PPP) for production of the precursors used in synthesis of nucleotides and aromatic amino acids. Thus, the roles of the FBPase encoding genes in CCM and virulence of Mtb has drawn much attention. An earlier report demonstrated that the overexpression of Mtb glpX rescued the growth defects of an Escherichia coli FBPase mutant [17]. In a recent report, glpX was demonstrated essential for both proliferation and virulence of Mtb [18]. In addition, gene gpm2 (Rv3214) was identified as a novel FBPase [19], and the gluconeogenesis and virulence were seriously affected by the disruption of both gpm2 and glpX. The importance of glpX in Mtb has been addressed through these studies. However, the mechanisms by which FBPase-encoding genes contribute to mycobacterial proliferation and virulence remains to be further investigated [19]. To address this issue, we performed a series of experiments using M. marinum which is a closely genetic relative to Mtb. The genomes between Mtb and M. marinum were compared earlier and display a high degree of homology [20]. Besides, as one of the natural hosts of M. marinum, zebrafish has been developed as an elegant model to investigate mycobacterial pathogenesis, especially in the angle of early TB pathogenesis [21,22,23].

In our study, a glpX deletion mutant (ΔglpX) of M. marinum was constructed, and the functions of glpX in carbon metabolism, bacterial proliferation and virulence were investigated through LC-MS analysis, and macrophage and zebrafish infections. The ΔglpX mutant displayed a disrupted gluconeogenesis when grown on gluconeogenic carbon source. Furthermore, glpX is required for virulence of M. marinum both in macrophages and zebrafish. The proliferation of ΔglpX was halted inside macrophage cells, and an elongated morphology appeared. We further sought the causes of this abnormal morphology and identified that glpX is required for intact division of M. marinum under gluconeogenic conditions and inside macrophage cells.

Materials and Methods

Strains and Culture Conditions

M. marinum M strain (ATCC BAA-535) was used as the wild type (WT) strain in this study. M. marinum was routinely cultivated in Middlebrook 7H9 broth or on 7H10 agar enriched with 10% OADC (oleic acid-albumin-dextrose-catalase) and 0.4% volume/volume (v/v) glycerol. When necessary, 40 μg/ml of kanamycin or 20 μg/ml of gentamycin or 50 μg/ml of
hygromycin was included. For growth measurement, strains were cultured in 7H9 broth with or without 0.2% weight/volume (w/v) glucose, 0.2% v/v glycerol or 0.2% w/v acetate separately. In addition, 0.02% v/v tyloxapol was added into 7H9 broth to reduce bacterial clumping. Minimal medium is used for cultivation of *M. marinum* strains for LC-MS analysis, and the composition is as following: 1.5 g/L KH2PO4, 1.0 g/L NH4Cl, 0.2 g/L MgSO4·7H2O, 0.02 g/L CaCl2·2H2O, 0.85 g/L NaCl, 8.99 g/L Na2HPO4·12H2O, and 0.014 g/L (NH4)2 Fe (SO4)2·6H2O. The carbon sources supplemented into minimal medium are either 1 g/L glucose or 1 g/L acetate.

The *glpX* Mutant Generation and Complementation

The *glpX* open reading frame (ORF) plus 1kb flanking region was PCR amplified from WT *M. marinum* genomic DNA using primers MM*glpX* KO_F and MM*glpX* KO_R, and the amplified fragment was ligated to T-vector. The 527–939 nt fragment of *glpX* was digested out with *SmaI*, and the hygromycin cassette from pHINT1 vector was inserted to obtain *glpX::hyg*, which was then digested with *SpeI* and cloned into *SpeI* site of the vector pPR27-*wasabi* (S1B Fig) to obtain pPR27*glpX* KO. The vector pPR27*glpX* KO was transformed into WT, and positive clones were first selected using hygromycin followed 10% w/v sucrose. Then, potential deletion mutants were selected by fluorescent microscopy for the loss of counter-selection marker *wasabi*. For complementation of Δ*glpX*, the MM*glpX* fragment containing the *glpX* gene together with 475 bp of upstream sequence was obtained by PCR amplification of *M. marinum* genomic DNA using primers MM*glpX* CO_F and MM*glpX* CO_R (S1 Table), and the fragment was digested with *EcoRI* and *SfiI*, ligated into *EcoRI* and *SfiI*-digested pMV306 vector, which is a single-copy attB integrating vector. The pMV306/C (*glpX*) vector was integrated into Δ*glpX* to get the complemented strain Δ*glpX/C* (*glpX*).

LC-MS Analysis

*M. marinum* strains were precultured in 7H9 broth with 10% OADC to exponential phase. The cultures were started with an optical density at 600 nm (OD600) of 0.01 and performed in minimal medium with 1 g/L [U-13C] glucose or 1 g/L [U-13C] acetate. When OD600 reached 1.0, bacteria were collected by centrifugation and cell pellets were resuspended in 2 ml of 80:20 (v/v) methanol/water at -20°C separately and lysed by mechanical vortexing. After a centrifugation at 4°C for 3 min at 16200 × g, the supernatants were collected. Metabolite and isotope analyses were performed with a UPLC system (Waters) coupled by negative mode electrospray ionization to an orbitrap mass spectrometer (Thermo Scientific). Injection volume was 20 μl. Solvent A was 50 mM ammonium acetate adjusted to pH 9.0 with ammonium hydroxide and solvent B was acetonitrile. Metabolites were separated with a Luna NH2 column (10-cm length, 2-mm diameter, 3-μm particle size; Phenomenex). The column was maintained at 15°C with a solvent flow rate of 0.2 ml min⁻¹, and the gradient of B was as follows: 0 min, 85%; 3 min, 30%; 12 min, 2%; 15 min, 2%; 16 min, 85%; 23 min, 85%. The mass spectrometer scanned from *m/z* 70–1,000 at 1 Hz at 100,000 resolution. Data were analyzed using the Xcalibur software.

Quantitative Real-Time PCR (qRT-PCR)

The transcriptional expression of selected genes was measured by qRT-PCR using primers listed in S1 Table. The gene *sigA* was applied as an internal control. The cDNA used in these experiments was prepared from RNA isolations obtained from three independent biological replicates. qRT-PCRs were carried out in triplicate using iQ SYBR green supermixture kit (Bio-Rad) and a CFX96™ Real-Time PCR System (Bio-Rad).
Zebrafish Infection

Adult zebrafish were infected with M. marinum strains as described previously [24]. For larval infection, experiments were performed as described in a recent paper [25], and bacterial burdens in larvae were determined by fluorescence pixel counts (FPC) through the ImageJ software. Animal work was approved by IACUC of Fudan University (20120105–001). To minimize animal suffering and distress, adult or larval zebrafish were injected under anesthesia. The animal survival study was performed using adult fish, the specific criteria regarding humane endpoints is set as adult fish show irregularities in food consumption, hemorrhaging, and buoyancy loss, removal of animal whose illness or condition makes them nonproductive to the study. Fish are euthanized with an excess dosage of tricaine (MS-222) on ice, with a final concentration 0.2% w/v in fish water. Larval fish showed no signs of morbidity during the experimental period. We performed daily observations for fish health and mortality. The number of unexpected deaths during the experiment was zero. The dead or euthanized fish were disposed following the requirement of IACUC of Fudan University.

Macrophage Infections and Length Measurement of Intracellular M. marinum

Raw264.7 macrophages were infected with M. marinum strains at multiplicity of infection (MOI) of 1, and intracellular bacterial load was counted as described in a recent paper [25]. For length measurement of intracellular bacteria, infected macrophage monolayers were washed with phosphate buffered saline (PBS) and lysed with 0.1 ml of 0.1% Triton X-100 (Sigma) to release intracellular bacteria. The intracellular M. marinum cells were fixed on glass slides and photographed utilizing an EVOS FL color imaging system (Invitrogen). Images were analyzed using ImageJ software. An MOI of 10 was applied only for the measurement of phagocytosis ratio of M. marinum.

DAPI Staining to Detect the Nucleoid of M. marinum

Experiments were performed as referred to a published paper [23,26]. Briefly, WT and ΔglpX strains were cultivated to log phase in 7H9 broth supplemented with 0.2% v/v glycerol, harvested by centrifugation, washed in PBS, fixed in 4% paraformaldehyde (PFA) and stained with 0.2 μg/ml DAPI for 15 min. 5 μl stained culture was smeared on a glass slide mounted with a coverslip. Fluorescence signals were detected by CLSM at an excitation wavelength of 375 nm. Images were analyzed using LAS AF Lite software.

Colony Morphology and Antimicrobial Susceptibility Assay

Experiments were performed similarly as described in a paper by Wang et al [25]. For colony morphology observation, log phase M. marinum cultures were diluted to an OD600 of 0.01, and 5 μl of each dilution was incubated on 7H10 agar plates. The colonies formed after 7 days of cultivation, and colony morphology was photographed. For antimicrobial susceptibility assay, M. marinum strains were cultured in 7H9 broth with 10% OADC in 96-well plates. Selected antimicrobials were added into cultures at serial two-fold dilutions. The minimal inhibitory concentration (MIC) was defined as the minimal concentration at which no visible growth was observed after 7 days of cultivation.
Results

A Functional Gluconeogenic Pathway Is Dependent on glpX

MMAR_4367, the annotated glpX gene in M. marinum was successfully deleted by homologous recombination as illustrated in S1 Fig. Both PCR and qRT-PCR analyses were applied to validate the mutant strain, ΔglpX. We first investigated the effects of glpX ablation on carbon metabolism of M. marinum. WT, ΔglpX and the complemented strain ΔglpX/C (glpX) were cultivated in 7H9 broth without or with supplementation of glycolytic (glucose) or gluconeogenic (glycerol or acetate) carbon substrates. WT and ΔglpX grew similarly in 7H9 broth without additional carbon source (Fig 1A), or with a glycolytic carbon source, 0.2% glucose (Fig 1B), or enriched with OADC and 0.4% glycerol (Fig 1C). However, ΔglpX grew considerably slower than WT in 7H9 broth supplemented with 0.2% glycerol or acetate as gluconeogenic carbon sources (Fig 1D and 1E), and the complementation by expressing M. marinum glpX rescued these growth defects in ΔglpX.

To further investigate the effect of glpX deletion on carbon metabolism in M. marinum, we compared the intracellular concentration and 13C labeling of metabolites among WT, ΔglpX and the complemented strain ΔglpX/C (glpX). The strains were grown on the minimal medium containing 1 g/L of [U-13C] acetate or 1 g/L of [U-13C] glucose and harvested at an OD600 of 1.0. After rapid quenching and extraction, the metabolites upstream and downstream of the FBPase-catalyzed reaction in gluconeogenesis were analyzed by LC-MS. When grown on acetate as the sole carbon source, the intracellular concentration of the FBPase substrate fructose-1, 6-bisphosphate (FBP) in ΔglpX was about 4-fold higher than that in WT (Fig 2A). The 3-phosphoglycerate (3-PGA) and phosphoenolpyruvate (PEP) levels in ΔglpX were also increased about 3-fold and 8-fold, respectively, compared to the WT. On the other hand, ΔglpX showed decreased pool sizes of downstream metabolites including fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P). The intracellular concentration of PPP intermediates ribulose-5-phosphate (Ru5P), ribose-5-phosphate (R5P) and xylulose-5-phosphate (X5P) in ΔglpX were also reduced over 10-fold compared with WT. Moreover, incorporation of 13C from [U-13C] acetate into F6P and R5P was decreased (Fig 2B). These results indicate that the gluconeogenic carbon flow is blocked at the FBPase reaction in ΔglpX. Complementation of ΔglpX by expressing M. marinum glpX led to a decrease in upstream metabolite concentrations and increases in the pool sizes and fractional 13C labeling of downstream metabolites (Fig 2A and 2B), indicating the restoration of the gluconeogenic flux through FBPase upon expression of glpX. When ΔglpX and WT strains were grown on glucose as the sole carbon source, no significant differences in intracellular concentrations and 13C labeling of the central pathway intermediates were observed (S2 Fig). This is consistent with the similar growth rate of WT and ΔglpX on glucose and indicates that glycolysis is not affected in ΔglpX. Therefore, the results of metabolite profiling and 13C labeling strongly suggest that glpX inactivation caused a disruption of gluconeogenesis at the FBPase reaction, resulting in an impairment of growth on gluconeogenic carbon sources.

M. marinum ΔglpX Is Attenuated in Zebrafish

To investigate the role of glpX in mycobacterial pathogenesis, a zebrafish infection model was utilized [27]. Adult zebrafish were infected at an initial dosage of 1×10⁴ colony forming unit (CFU) per fish. As shown in Fig 3A, the median lethal time (LT₅₀) of WT infected zebrafish was 12 days, whereas only one fish died in the ΔglpX group throughout the whole experimental period (21 days). The complementation by expressing M. marinum glpX rescued the virulence...
defects of ΔglpX, and the survival curve of infected zebrafish was indistinguishable from the WT infected group.

To further inspect if glpX sub serves M. marinum to resist host innate immunity, 30 hpf zebrafish larvae were infected with WT, ΔglpX and the complemented strain ΔglpX/C (glpX) at

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**Fig 1.** *M. marinum* ΔglpX grows on gluconeogenic and glycolytic carbon sources. WT, ΔglpX and complemented strain ΔglpX/C (glpX) were cultivated in 7H9 broth containing (A) no carbon source; (B) 0.2% glucose; (C) 10% OADC + 0.4% glycerol; (D) 0.2% glycerol; (E) 0.2% acetate. Data are representative of three independent experiments.

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Fig 2. Effect of glpX disruption on gluconeogenic metabolism in *M. marinum*. The intracellular concentration (A) and unlabeled fraction (B) of metabolites in WT, ΔglpX, and complemented strain ΔglpX/C (glpX) were determined by LC-MS. The strains were grown on the minimal medium containing 1 g/L of [U-13C]acetate and harvested at an OD600 of 1.0. Data represent means ± standard deviation of three independent experiments. ND, not detectable. G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-phosphate.

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initial dosages of 56, 168, and 26 CFU per fish separately. The proliferation of *M. marinum* was measured by FPC analysis, and expression of several pro-inflammatory or anti-inflammatory cytokines was examined by qRT-PCR, including IL-1β, IFNγ, TNFα, and IL-10. Representative pictures of infected larvae were illustrated in Fig 3B. WT proliferated normally in infected
larvae, whereas ΔglpX displayed a significantly decreased bacterial load and defective proliferation in zebrafish larvae. Complementation by expressing *M. marinum* glpX restored the virulence of ΔglpX (Fig 3C and 3D). On 6 dpi, fish were lysed and total RNA was extracted for measuring cytokine levels. Among the tested cytokines, the elevation of TNFα transcription in zebrafish infected by WT was 3.5 fold relative to the levels seen in uninfected fish, which indicates a strong inflammatory response of the host post infection (Fig 3E). By contrast, TNFα transcription in zebrafish infected by ΔglpX was only 1.8 fold normalized to uninfected fish, displaying a significant drop compared to zebrafish infected by WT. Expression of in zebrafish infected by ΔglpX/C (glpX) was 3.4-fold normalized to uninfected fish, a similar level to zebrafish infected by WT. For the other three cytokines tested, no significant induction was observed. The difference of TNFα expression might reflected the different bacterial burden of zebrafish larvae infected with various strains.

**glpX Is Required for *M. marinum* Proliferation in Macrophages**

The innate immune system is crucial for host to detect invading *Mtb*, and macrophage is a primary cell of the innate immune system and the major site of residence for *Mtb* [28,29]. To further investigate how glpX assists mycobacterium coping with innate immunity, macrophages were infected by *M. marinum* strains and two key events during infection were examined, the phagocytosis of mycobacterium and the proliferation of intracellular bacilli. It was demonstrated that the inactivation of glpX did not affect the phagocytosis ratio of *M. marinum* by RAW264.7 macrophage cells (Fig 4A), with a phagocytosis ratio of 10% similar to WT and ΔglpX/C (glpX). Intracellular proliferation of *M. marinum* was monitored by enumerating the bacterial load at different time points post infection. The intracellular bacterial load of WT gradually increased from 10^4 (0 dpi) to 10^6 CFU/ml (4 dpi), whereas ΔglpX lost its ability to proliferate inside macrophages yet remained at a load of 10^4 CFU/ml (Fig 4B), the complementation by expressing *M. marinum* glpX rescued the proliferation defect of ΔglpX. Taken together, these results indicate that *M. marinum* proliferation inside macrophages requires a functional glpX gene.

**ΔglpX Exhibits an Elongated Morphology both In Vitro and In Vivo**

It was previously reported that *Mtb* inside macrophages display an elongated morphology compared to *in vitro* cultivation, which might be correlated with intracellular stress conditions that the bacilli encounters [30]. Since altered cell length could be correlated to the change of replication rate in mycobacteria [31], we examined the morphology of ΔglpX inside macrophage. RAW264.7 macrophages were infected with *M. marinum* strains carrying a GFP containing vector pTEC15 at an MOI of 1. As shown in Fig 4C and 4D, the average length of intracellular ΔglpX (10.4 μm) was significantly longer than WT (4.9 μm) at 4 dpi, and the length of ΔglpX/C (glpX) was 4.3 μm.

To inspect if the elongated phenotype of ΔglpX is correlated with altered gluconeogenesis, *M. marinum* WT and ΔglpX were cultivated *in vitro* with the addition of glycolytic or gluconeogenic substrates. ΔglpX exhibited a elongated morphology when cultured in 7H9 broth containing 0.4% glycerol (S3A and S3B Fig). By contrast, no elongated morphology was observed when ΔglpX was cultured in 7H9 broth containing 0.4% glycerol and an additional 0.2% glucose(S3C and S3D Fig). Under both cultivation conditions, WT displayed a normal cell length. As shown in S3E Fig, the average length of ΔglpX (6.1 μm) was significantly longer than WT (3.1 μm) when cultured in 7H9 broth containing 0.4% glycerol. The average lengths of WT and ΔglpX were 2.7 and 2.4 μm respectively (S3F Fig) in 7H9 broth containing 0.4% glycerol and an additional 0.2% glucose. Collectively, these results suggest that the proliferation defects of
ΔglpX both in vivo and in vitro is reflected by the elongated morphology and partially correlated to the altered gluconeogenesis.

**glpX Is Indispensable for Cell Division of *M. marinum* under Conditions of Gluconeogenesis**

In agreement with these findings, ΔglpX displays a multi-nucleoid (more than three) morphology when cultivated in 7H9 broth containing 0.2% glycerol (Fig 5). There are 46 out of 84 ΔglpX bacterial cells containing more than three nucleoids, and the percentage of this multi-nucleoid
population is 54.8%. In WT, bacterial cell with over three nucleoids is not identified, except that some dividing cells contain two nucleoids. In addition, several fts genes which are components of either elongation complex or division apparatus were down-regulated in ΔglpX compared to WT and ΔglpX/C (glpX) under gluconeogenic but not enriched cultivation conditions (S4A–S4C Fig). These observations strongly suggest that glpX is required for the normal mycobacterial division under conditions of gluconeogenesis or during infection.

ΔglpX Exhibits an Altered Antimicrobial Susceptibility

The susceptibility of M. marinum strains to various PG targeting antimicrobials were examined. MIC values of vancomycin and penicillin were 8-fold higher for ΔglpX compared to WT (S5A Fig), and the complementation by expressing M. marinum glpX restored the susceptibility of ΔglpX to WT. As a control, there was no susceptibility difference between ΔglpX and WT to other three tested antimicrobials, streptomycin, ethambutol, and erythromycin. In addition, ΔglpX exhibited a rough and dry colony morphology without the halo around the colony as in WT (S5B Fig), which also indicates possible alteration in cell wall integrity. The complementation by expressing M. marinum glpX restored the colony morphology of ΔglpX to WT.

Discussion

FBPase is a crucial gluconeogenic enzyme catalyzing the irreversible conversion of FBP to F6P. Our results demonstrate that the glpX gene is required for the growth of M. marinum on gluconeogenic carbon sources, due to the dependency of a gluconeogenic pathway relying on FBPase encoded by glpX. The glpX gene is essential and non-redundant for the virulence of M. marinum in zebrafish. Moreover, glpX is needed for the normal cell morphology, cell division and proliferation of M. marinum in macrophages.
Carbon metabolism is important for intracellular bacteria to survive and proliferate in host [32], and mycobacterium displays a great flexibility in carbon metabolism [33]. F6P/G6P and PPP intermediates are vital precursors for the synthesis of cell wall components and amino acids, and the considerably reduced pool size of these metabolites in ΔglpX (Fig 2) might cause profound effects on mycobacterial proliferation. In support of this, the halted proliferation of ΔglpX inside macrophage cells (Fig 4) strengthens the importance of gluconeogenesis for M. marinum to replicate intracellularly. To understand why the intracellular proliferation ceased in ΔglpX, the elongated morphology (Fig 4) attracted our attention, and its correlation to gluconeogenesis was validated in vitro (S3 Fig). The elongated morphology is frequently correlated to dysfunction of cell division apparatus, which made us to wonder if cell division in ΔglpX does not function properly. This speculation was supported in vitro as unseparated individual cells with multi-nucleoid was only observed for ΔglpX, not in WT (Fig 5). Thus, the impaired gluconeogenesis led to the cessation of cell division in M. marinum. Collectively, FBPase activity is essential for normal division of M. marinum under gluconeogenic conditions and inside macrophages, and abnormal division contributes to the halted cell division upon disruption of glpX.

How bacteria adjust cell size and division according to its metabolic status is a fundamental yet poorly understood question. Recently, light shed on how nutritional status mediates bacterial cell size and growth rate [34]. In Bacillus subtilis, a conserved glucolipid biosynthesis pathway starting with the enzyme PgcA which interconverts Glc-1-P to G6P coordinates cell size with growth rate by transferring nutritional information directly to the division apparatus [35]. Additionally, defective glucosyltransferase OpgH increased the frequency of FtsZ ring formation over incompletely segregated nucleoids and reduced size of E. coli cells [36]. In both cases, UDP-glucose was proposed to indicate the nutritional status of bacteria, whose biosynthesis is dependent on G6P as the precursor during gluconeogenesis. Since the concentration of F6P/G6P was significantly lower in ΔglpX compared to WT growing on gluconeogenic carbon source (Fig 2), we speculated that such metabolic alteration might affect the division apparatus of M. marinum, yet to be validated. In addition, we cannot exclude the possibility that certain buildup products upon the loss of a functional FBPase may contribute to the lack of cell division in ΔglpX.

Two recent studies confirmed the important roles of FBPase encoding genes in gluconeogenesis and pathogenesis of Mtb, and concluded that glpX encodes an FBPase, yet the FBPase activity was not entirely eliminated in ΔglpX [18]. The gene glpX was demonstrated essential for proliferation and virulence of Mtb [18], and another gene gpm2 (Rv3214) was identified to encode a novel functional FBPase [19]. Notably, MMAR_1343 which is annotated as a homologous gene to Rv3214 in M. tuberculosis, exhibited a higher relative expression in the absence of glpX under gluconeogenic culture conditions (S4D and S4E Fig). It indicated that MMAR_1343 might also function as an FBPase in M. marinum upon further validation. However, the decreased growth on gluconeogenic carbon sources (Fig 1) and virulence attenuation in zebrafish (Fig 3) of ΔglpX indicates that MMAR_1343 cannot substitute glpX in M. marinum. Furthermore, the significantly attenuated virulence of ΔglpX in zebrafish (Fig 3) could be due to the declined bacterial proliferation in vivo upon the loss of glpX, and the lower induction of a pro-inflammatory cytokine TNFα in larvae might reflect the decreased bacterial burden. On one side, the loss of a functional FBPase turned bacilli into a non-proliferating state, which was also reported in the studies on icl and pckA mutants of Mtb [7,9]. On the other side, the alteration of metabolites might pose regulatory effects and finally affected the fate of M. marinum. For example, FBP may work as an effector on transcriptional regulation in model bacteria. In E. coli, FBP may work as a “flux-signaling metabolite” working with the transcription factor Cra during flux-dependent regulation [37]. In B. subtilis, FBP modifies
DNA binding activity of a glycolytic regulator, CggR [38,39]. Whether FBP plays a direct or moonlighting role in regulatory or signaling pathways to mediate mycobacterial division \textit{in vivo} and proliferation thereafter remains to be answered in future work. Through this, the mechanisms by which gluconeogenesis modulates mycobacterial virulence will be further elucidated.

**Supporting Information**

S1 Fig. Deletion and validation of \textit{glpX} in \textit{M. marinum}. (TIF)

S2 Fig. Effect of \textit{glpX} disruption on glycolytic metabolism in \textit{M. marinum}. (TIF)

S3 Fig. \textit{M. marinum} \textit{ΔglpX} exhibits an elongated morphology under gluconeogenic cultivation \textit{in vitro}. (TIF)

S4 Fig. \textit{M. marinum} \textit{ΔglpX} displays the altered relative expression of division genes and a second probable FBPase encoding gene, \textit{gpm2}. (TIF)

S5 Fig. \textit{M. marinum} \textit{ΔglpX} displays altered MIC and colony morphology. (TIF)

S1 Table. The primers used in this study. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: JT LDL CW QG CY CN. Performed the experiments: JT LM XW LL YL. Analyzed the data: JT XW LL CY CN. Wrote the paper: JT CY CN.

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