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Farnesyl pyrophosphate synthase is essential for the promastigote and amastigote stages in *Leishmania major*

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Abstract

Isoprenoid synthesis provides a diverse class of biomolecules including sterols, dolichols, ubiquinones and prenol groups. The enzyme farnesyl pyrophosphate synthase (FPPS) catalyzes the formation of farnesyl pyrophosphate, a key intermediate for the biosynthesis of all isoprenoids. In *Leishmania*, FPPS is considered the main target of nitrogen containing bisphosphonates, yet the essentiality of this enzyme remains untested. Using a facilitated knockout approach, we carried out the genetic analysis of FPPS in *Leishmania major*. Our data indicated that chromosomal null mutants for *FPPS* could only be generated in presence of an episomally expressed *FPPS*. Long-term retention of the episome by the chromosomal *FPPS*-null mutants in culture and in infected BALB/c mice suggests that FPPS is indispensable.

In addition, applying negative selection pressure failed to induce the loss of ectopic *FPPS* in the chromosomal *FPPS*-null mutants, although it led to significant growth delay in culture and in mice. Together, our findings have confirmed the essentiality of FPPS in both promastigotes and amastigotes in *L. major* and thus validate its potential as a drug target for the treatment of cutaneous leishmaniasis.

Graphical Abstract

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Author contributions

SM and KZ designed this study; SM and SB performed the experiments; SM and KZ wrote this manuscript.

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Conflict of interest

The authors declare no conflict of interest (financial or non-financial).

Keywords

Leishmania; isoprenoid; essentiality; drug; target; lipid; amastigote

1. Introduction

Leishmania parasites are trypanosomatid protozoans responsible for a group of diseases collectively known as leishmaniasis [1]. Current treatments are plagued with high toxicity and threatened by drug resistance [2]. Without a licensed vaccine to use in humans, a thorough and rigorous validation of potential drug targets is clearly needed for the development of new antileishmanials.

In many eukaryotes, isoprenoid synthesis is a complex pathway leading to production of sterols, dolichols, ubiquinones, heme a and prenylated proteins [3]. Despite their structural and functional diversity, all isoprenoids derive from the same two precursors: isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Fig. S1). Inhibitors of isoprenoid synthesis such as statins and bisphosphonates have been widely used in humans to treat hypercholesterolemia and bone diseases, respectively [4–8]. Bisphosphonates are pyrophosphate analogs in which the oxygen bridge between the two phosphorus atoms has been replaced by a carbon substituted with various side chains [4]. In mammalian cells, bisphosphonates inhibit farnesyl pyrophosphate synthase (FPPS, EC 2.5.1.10), which is required for the synthesis of farnesyl pyrophosphate (FPP), an important isoprenoid intermediate. (Fig. S1)[9]. FPPS catalyzes a two-step reaction. In the first step, IPP and DMAPP undergo condensation to form the 10-carbon compound geranyl pyrophosphate (GPP). In the second step, GPP is converted to the 15-carbon FPP after condensation with another molecule of IPP (Fig. S1). FPP is then utilized for the synthesis of sterols, ubiquinones, dolichols, heme a, and prenyl groups that bind proteins, or converted into longer isoprenoid intermediates.

In addition to treating bone disorders, nitrogen containing bisphosphonate compounds such as risedronate and pamidronate can inhibit the growth of a number of protozoan parasites including *Toxoplasma gondii*, *Plasmodium falciparum*, *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania donovani* [10–12]. When resistance to risedronate was selected in *Leishmania major* promastigotes by stepwise increase of drug concentration, the resulting parasites expressed high levels of FPPS [13]. The same study also indicates that *L. major* promastigotes overproducing FPPS were more resistant to risedronate [13]. In agreement with these findings, recombinant FPPS from *T. brucei* and *T. cruzi* were found to be

inhibited by risedronate [14, 15]. Similar inhibitory effects of nitrogen containing bisphosphonates were observed against FPPS from *T. gondii* and *P. falciparum* [16–18]. While these studies have highlighted the potential of FPPS as a drug target in protozoan parasites, alternative enzymes such as hexokinase [19], purine transferase [20] and exopolyphosphatase [21] have also been implicated for the antiparasitic effects of bisphosphonates. It is therefore necessary to critically evaluate the essentiality of FPPS in protozoan parasites.

In trypanosomatids, isoprenoid precursors are synthesized through the mevalonate pathway (Fig. S1) and FPPS is mostly found in the cytosol [3, 13, 22]. RNA interference studies have demonstrated that FPPS is likely indispensable for both the procyclic and bloodstream forms of *T. brucei* [15]. Unlike trypanosomatids or mammals, synthesis of isoprenoid precursors in apicomplexans occurs in the apicoplast via a prokaryotic-type 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway [23–25]. In *T. gondii*, FPPS is localized to the mitochondria and null mutants for this enzyme show severe growth defects in macrophages but are able to grow normally in fibroblasts [16, 26]. Moreover, metabolite analysis of FPPS-null tachyzoites isolated from host fibroblasts revealed similar isoprenoid profiles between the host and the FPPS-null mutants, indicating efficient salvage of host isoprenoids by these intracellular parasites. Therefore, for intracellular parasites like *T. gondii*, the requirement for FPPS may depend on specific host cell types.

During the mammalian stage, *Leishmania* parasites persist and replicate as intracellular amastigotes in the phagolysosome of macrophages. Previous studies have elucidated an active exchange of metabolites including amino acids, hexose, amino sugars and fatty acids between *Leishmania* amastigotes and host macrophages [27–30]. However, whether this holds true for isoprenoids is not known. In *L. major*, deletion of sterol-14- α -demethylase (*C14DM*) or sterol methyltransferase (*SMT*) leads to depletion of endogenous ergostane-based sterols, yet the *C14DM*- and *SMT*-null mutants are viable in culture and capable of causing diseases in mice (albeit attenuated in comparison to wild type parasites) [31, 32]. Further analysis indicates that sterol synthesis is downregulated during the mammalian stage and amastigotes isolated from mice contain host-derived cholesterol as their dominant sterol species [31]. While these findings argue that endogenous sterol synthesis is not absolutely essential in *L. major*, FPPS is expected to provide isoprenoid intermediates for functions above and beyond sterol synthesis (Fig. S1). Thus, a rigorous evaluation of FPPS's essentiality is of significant interest for the development of new treatments and refinement of existing drugs.

To determine whether FPPS is required for survival in *L. major*, we carried out a facilitated knockout approach based on the segregational loss of complementing episome [33]. Results showed that chromosomal *FPPS* genes could only be deleted in presence of an episomal *FPPS*. This episome cannot be lost either in culture or in BALB/c mice, even when cells face strong negative selection pressure. Therefore, our findings confirm the essentiality of FPPS in *L. major* during both promastigote and amastigote stages and further endorse its potential as a drug target.

2. Materials and Methods

2.1 Materials

Female BALB/c mice (7–8 weeks old) were purchased from Charles River Laboratories International (Wilmington, MA). Blasticidin and hygromycin were purchased from Calbiochem. Nourseothricin (SAT) was purchased from Goldbio, USA. Ganciclovir was purchased from Acros Organics. [α - 32 P]-labeled dCTP (3000 Ci/mmol) was purchased from Perkin Elmer. All other chemicals and reagents are purchased from Thermo Fisher Scientific unless otherwise specified.

2.2 Molecular constructs

The two *FPPS* knock-out constructs (pUC18-KO-*FPPS::BSD* and pUC18-KO-*FPPS::HYG*) were generated as follows. First, the upstream and downstream regions (~1 kb each) of *L. major FPPS* (LmjF.22.1360) were identified from tritrypDB and amplified by PCR from *L. major* genomic DNA using primer pairs #577/#578 and #573/#574, respectively (oligonucleotides used in this study are summarized in Table S1). The upstream flanking region (FR) was digested with KpnI and SacI and ligated into pUC18 plasmid to generate pUC18–5'UTR. The downstream FR was digested with SmaI and BglII and the resultant product was ligated in pUC18–5'UTR to generate pUC18–5'UTR–3'UTR. Genes conferring resistance to blasticidin (*BSD*) and hygromycin (*HYG*) were inserted between the upstream and downstream FRs in pUC18–5'UTR–3'UTR to generate pUC18-KO-*FPPS::BSD* and pUC18-KO-*FPPS::HYG*, respectively. Finally, linear knockout cassettes for *BSD* and *HYG* were generated by restriction enzyme digestion of pUC18-KO-*FPPS::BSD* and pUC18-KO-*FPPS::HYG* followed by blunt ending and dephosphorylation. All DNA constructs were confirmed by restriction enzyme digestion and sequencing.

The open reading frame (ORF) of *L. major FPPS* was amplified by PCR using primer pair #579/#590. Following digestion with BglII, the obtained product was ligated into pXNG4-SAT vector [33] to generate pXNG4-*FPPS*. DNA fragments used as probes for Southern blot were synthesized by PCR using primer pairs #571/#572 (for the FR probe) and #633/#634 (for the *FPPS* ORF probe).

2.3 Leishmania culture, genetic manipulation and Southern blot

Leishmania major FV1 (MHOM/IL/81/Friedlin) wild type (WT) promastigotes were cultivated at 27 °C in M199 media with 10% fetal bovine serum (FBS) and other supplements as previously described [34]. To examine cell growth, promastigotes were cultured in complete M199 media with a starting density of 1.0×10^5 cells/ml. Cell density was determined using a hemocytometer at specific time points. In this study, log phase promastigotes refer to replicative parasites at densities lower than 1.0×10^7 cells/ml, and stationary phase promastigotes refer to mainly non-replicative parasites at densities higher than 2.0×10^7 cells/ml.

Transfection was carried out by electroporation as previously described [35] using a Bio-Rad Gene Pulser Xcell by delivering two pulses at 1500 V and 25 μ F at 10 seconds interval. Parasites were transfected first with the *BSD* knockout cassette. The single allele knockout

FPPS/ *FPPS::BSD* clonal lines (*FPPS*^{+/-}) were selected with 15 µg/ml of blasticidin and confirmed by Southern blot. Two independent clones of *FPPS*^{+/-} were then transfected with pXNG4-*FPPS* and selected with nourseothricin (100 µg/ml) to generate *FPPS*^{+/-}+ pXNG4-*FPPS* lines. Finally, two independent lines of *FPPS*^{+/-}+ pXNG4-*FPPS* were transfected with the *HYG* knockout cassette and selected with a combination of blasticidin (15 µg/ml), hygromycin (50 µg/ml) and nourseothricin (100 µg/ml) to generate the chromosomal *FPPS*-null mutant lines carrying pXNG4-*FPPS* (*FPPS::HYG*/ *FPPS::BSD* + pXNG4-*FPPS* or *fpps*⁷⁺ + pXNG4-*FPPS*).

The loss of endogenous *FPPS* alleles was confirmed by Southern blot. Briefly, following digestion with restriction enzyme AlwNI, genomic DNA samples were resolved on a 0.7% agarose gel, transferred to a nitrocellulose membrane, and hybridized with a [³²P]-labeled DNA probe targeting ~600-bp upstream region of *FPPS* (the FR probe) or ~500-bp region in the *FPPS* ORF (the ORF probe).

2.4 In vitro ganciclovir (GCV) treatment and flow cytometry analysis

To induce the release of pXNG4-*FPPS*, promastigote cultures were inoculated at 1.0–2.0 × 10⁵ cells/ml and passaged every three days in the absence or presence of GCV (50 µg/ml). To monitor the loss of pXNG4-*FPPS*, GFP expression level was determined by flow cytometry (Accuri C6 plus, BD Biosciences) during the mid-log phase of each passage. For *fpps*⁷⁺ + pXNG4-*FPPS*, two independent clones were tested.

2.5 Mouse footpad infection and in vivo GCV treatment

Procedures involving mice were approved by the Animal Care and Use Committee at Texas Tech University (PHS Approved Animal Welfare Assurance No. A3629–01). Mice were housed and cared for in the facility operated by the Animal Care and Resources Center at Texas Tech University. To examine infectivity *in vivo*, day 3 stationary phase promastigotes were injected into the left hind footpad of BALB/c mice (1.0 × 10⁶ cells/mouse, 10 mice per group). GCV treatment was carried out as described previously [36]. Briefly, for each group, one half of the mice received GCV at 7.5 mg/kg/day (prepared in phosphate-buffered saline or PBS) through intraperitoneal injection. The other half received sterile PBS (same volume as GCV, 500 µl daily). GCV and PBS treatment started one day post infection and was continued for 14 consecutive days. Mouse body weights were taken once a week for three weeks post injection. Footpad lesions were measured weekly using a Vernier caliper. Parasite loads were determined at indicated times by limiting dilution assay as described previously [37] and by quantitative PCR analysis as described below.

2.6 Quantitative PCR (qPCR) analyses

To determine parasite loads, infected mice were euthanized and DNA was extracted from footpad homogenates by phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation. Extracted DNA samples were dissolved in molecular biology grade water and each qPCR reaction was performed in a 20 µl volume consisting of 2 µl of extracted DNA, 200 µM of each primer targeting the 28S rRNA gene of *L. major* (#699 and #700) and 1 x Power SYBR green PCR Master mix. Reactions were run in triplicates on an Applied Biosystems 7300 RT-PCR thermocycler using the following profile: initial

denaturation at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 15 sec, annealing and extension at 60 °C for 1 min. Each run included reactions that did have DNA template (no template control) and those with DNA extracted from uninfected mice tissue (negative control). The cycle threshold values (Ct) were determined by analyzing the melting curves. Samples with Ct > 30 were considered negative. To quantify parasite numbers in footpads, each run included a standard curve of 10-fold serially diluted DNA extracted from *L. major* promastigotes (from 2×10^6 cells to 0.2 cells per reaction). Standard curve samples were diluted in the presence of salmon sperm DNA as a carrier.

To determine pXNG4-*FPPS* plasmid copy number in each sample, 2 µl of extracted DNA (from infected mouse footpad or culture promastigotes) was analyzed by qPCR as described above using primer #703 and #704 targeting a specific region of the pXNG4 plasmid. A calibration curve was generated by serially diluting purified pXNG4-*FPPS* plasmid DNA from 2×10^6 molecules to 0.2 molecules per reaction. For each sample, the average plasmid copy number per cell was calculated by dividing the plasmid copy number with the parasite number.

FPPS transcript levels in WT promastigotes and lesion amastigotes were determined by quantitative reverse transcription-PCR (qRT-PCR) using primers specifically designed for *L. major FPPS* (#716/#717). Briefly, total RNA was extracted from log phase culture promastigotes or from freshly isolated lesion amastigotes. 1 µg of RNA was converted into complementary DNA (cDNA) using the high-capacity cDNA conversion kit followed by qPCR as described above. Controls included samples without leishmanial RNA and without reverse transcriptase. The relative expression level of *FPPS* was normalized to that of the endogenous α -tubulin mRNA using the comparative Ct approach, also known as 2^{-Ct} method [38].

2.7 Statistical analysis

All experiments were repeated for 2–3 times unless specified otherwise. Difference between two groups was evaluated by the Student's *t* test using Sigmaplot 11.0 (Systat Software Inc, San Jose, CA). *P* values indicating statistical significance were grouped into value of <0.05.

3. Results

3.1 Attempts to delete the endogenous *FPPS* alleles from *L. major* promastigotes.

L. major FPPS is encoded by a single copy gene on chromosome 22 (TritrypDB ID: LmjF.22.1360, 1089 bp)[13]. To test if *FPPS* is dispensable in *L. major*, we first tried a classic approach to delete the two alleles of *FPPS* from WT promastigotes [39]. After transfection with the *BSD* resistance cassette, we selected four putative heterozygous knockout clones based on resistance to blasticidin. Southern blot analyses of genomic DNA digested with AlwNI were performed using radioactive probes for the 5'-FR of *FPPS*. For the endogenous *FPPS* locus, this probe would detect a 2.7 kb fragment; and replacement of one allele with *BSD* would generate a 6.7 kb fragment (Fig. S2A–B). As shown in Fig. 1A, all four clones selected after the first round of gene replacement were verified as *FPPS*^{+/-}.

To delete the second *FPPS* allele, three *FPPS*^{+/-} clones were transfected with the *HYG* resistance cassette, followed by selection with both blasticidin and hygromycin. Initial efforts failed to generate any doubly resistant clonal transfectants (data not shown). Through repeated attempts, we eventually were able to isolate three clones that grew in presence of both drugs. Southern blot with the 5'-FR probe confirmed the integration of both *BSD* and *HYG* resistance genes in these clones as demonstrated by the 6.7 kb and 7.3 kb fragments (Fig. 1B and Fig. S2A–B); however, we could still observe the 2.7 kb fragment, indicating the presence of *FPPS* in these clones (Fig. 1B and Fig. S2A–B). Southern blot with the *FPPS* ORF probe also detected the same 2.7 kb band in these clones as in WT and *FPPS*^{+/-} parasites (Fig. 1A–B and Fig. S2A–B). Therefore, our BSD- and HYG-resistant clones still retained *FPPS*, presumably through gene amplification or generation of aneuploid parasites. This kind of finding had been observed previously in attempts to target essential genes in *Leishmania* [33, 40, 41].

Failure to generate the *FPPS*-null parasites (*fpps*⁷) with the classic gene replacement approach prompted us to apply a method developed by Murta SM et al that uses plasmid complementation to acquire conditional knockout mutants [33]. To do so, we introduced the pXNG4-*FPPS* plasmid (Fig. S2C) into a *FPPS*^{+/-} clone, generating *FPPS*^{+/-}+pXNG4-*FPPS*, and then targeted the second chromosomal *FPPS* allele with the *HYG* resistance cassette. With this approach, several clones were readily generated showing resistance to blasticidin, nourseothricin (selection marker for the pXNG4-*FPPS* plasmid) and hygromycin. As illustrated in Fig. 1C, both the ORF probe and 5'-FR probe confirmed the complete loss of chromosomal *FPPS* in the selected clones. These conditional knockout lines were referred to as *fpps*⁷+pXNG4-*FPPS*. Overall, the fact that chromosomal *FPPS*-null mutants can only be generated in presence of an episomal complementing gene suggests that *FPPS* is indispensable during the promastigote stage of *L. major*.

3.2 *FPPS* is essential for the viability of *L. major* promastigotes in culture.

In addition to *FPPS*, the pXNG4-*FPPS* plasmid also contains genes for green fluorescence protein (GFP), thymidine kinase (TK), and nourseothricin resistance (SAT) (Fig. S2C). Adding ganciclovir (GCV) to these parasites will trigger the formation of GCV-triphosphate (through the activity of TK), a toxic guanosine analogue causing premature termination of DNA synthesis. Thus, in the presence of GCV and absence of nourseothricin, parasites would favor the elimination of pXNG4 plasmid during replication to avoid toxicity, if the episomal copy of *FPPS* is dispensable [33]. In contrast, when cultivated in the presence of nourseothricin but absence of GCV, *FPPS*^{+/-}+pXNG4-*FPPS* and *fpps*⁷+pXNG4-*FPPS* parasites showed high GFP expression levels, indicating robust plasmid retention (80–90% of cells were GFP +, Fig. 2A).

To examine the kinetics of episome loss, we cultured *FPPS*^{+/-}+pXNG4-*FPPS* and *fpps*⁷+pXNG4-*FPPS* parasites in the presence of nourseothricin; as cells entered late-log phase, they were inoculated into fresh media (without nourseothricin) in the absence or presence of 50 µg/ml of GCV; when these cultures entered stationary phase, the same process was repeated for eight passages; and during each passage, GFP expression levels were determined at the mid-log phase by flow cytometry. As indicated in Fig. 2B, without GCV,

the *FPPS*^{+/-}+pXNG4-*FPPS* parasites gradually lost GFP fluorescence from 82.5% GFP+ in passage 1 to <2% in passage 8 (■ in Fig. 2B). In comparison, the decrease of GFP fluorescence over time was much more rapid in GCV treated parasites (■ in Fig. 2B), indicating that the segregational loss of episome was effective. Apparently, the presence of endogenous *FPPS* allowed these parasites to lose the pXNG4-*FPPS* plasmid.

In contrast, the *fpps*7+pXNG4-*FPPS* parasites maintained a high level of GFP expression over multiple passages (82–86%, ● and ○ in Fig. 2B) when cultivated in absence of GCV, suggesting that the episomal *FPPS* is required in these parasites. Importantly, continuous GCV treatment did not significantly reduce the level of GFP fluorescence in these cells (71–83%, ▼ and △ in Fig. 2B), indicating that *fpps*7+pXNG4-*FPPS* parasites could not afford to lose the episome even when they were under strong negative selection pressure. GCV treatment did cause a ~24-hour growth delay in the *fpps*7+pXNG4-*FPPS* parasites (▼ in Fig. S3), which was consistent with its toxic effect in cells expressing TK. Together these data demonstrate that *FPPS* carries functions that are essential for promastigote viability in *L. major*.

3.3 *Fpps*⁻+pXNG4-*FPPS* parasites show attenuated virulence in BALB/c mice following treatment with GCV.

To determine whether *FPPS* is essential during the amastigote stage, we infected BALB/c mice with either WT or *FPPS*-mutant parasites via footpad infection. Mice were then split into two groups: one group received daily GCV treatment at a dose of 7.5 mg/kg/day (intraperitoneal injection) starting from day one post infection and continued for 14 days and the other group was injected with equal volume of sterile saline (PBS) as control. We did not detect any difference in body weights between the GCV- and PBS-treated mice (Fig. S4). In addition, we closely monitored these mice during and after the GCV treatment and did not observe any abnormality in appearance, movement or behavior. Thus, as previously reported [36], GCV treatment at such dosage had no obvious impact on mouse physiology.

As expected, mice infected with WT parasites developed lesions rapidly and GCV treatment had no significant effect (Fig. 3A). Very similar results were observed in mice infected with *FPPS*^{+/-}+pXNG4-*FPPS* parasites (Fig. 3A). Interestingly, although the *fpps*7+pXNG4-*FPPS* parasites caused lesions as efficiently as WT in PBS-treated mice, administration of GCV delayed disease development significantly (▼ in Fig. 3A). This observation suggests that under *in vivo* conditions, GCV was interfering with the proliferation of *fpps*7+pXNG4-*FPPS* parasites. Next, we determined parasite loads in infected footpads at 4, 6, and 10 weeks post infection by limiting dilution assay (LDA). All groups showed comparable parasite numbers except the *fpps*7+pXNG4-*FPPS* parasites in mice treated with GCV (Fig. 3B). In this group, there was a significant decrease in parasite loads at 4 and 6 weeks post infection, and it took 10 weeks for those parasites to reach comparable levels of lesion sizes and parasite loads as the other groups (Fig. 3A and B).

Parasite load determination by LDA depends on the conversion of lesion amastigotes into promastigotes and subsequent proliferation *in vitro*. Because *FPPS* is required for promastigote replication, detection of possible *fpps*⁻ parasites from *fpps*7+pXNG4-*FPPS*-infected mice after GCV treatment may be difficult. To overcome this potential problem, we

determined parasite loads by quantitative PCR (qPCR) analysis of amastigote DNA using primers targeting *L. major* 28S rRNA gene. With this approach, we again observed a substantial reduction in parasite number in *fpps*⁷+pXNG4-*FPPS*-infected, GCV-treated mice (Fig. 3C). This confirms that the delayed lesion development observed in this group of mice was due to slower proliferation of parasites. Together these data indicate that GCV was able to interfere with intracellular replication of *fpps*⁷+pXNG4-*FPPS* parasites without harming the host.

3.4 Plasmid retention by *fpps*⁷+pXNG4-*FPPS* amastigotes in BALB/c mice.

The fact that GCV interfered with the proliferation of *fpps*⁷+pXNG4-*FPPS* parasites in mice suggested that these parasites were retaining the pXNG4 plasmid during the amastigote stage. To confirm this, we performed qPCR analysis of DNA obtained from either culture promastigotes or lesion amastigotes using pXNG4 plasmid-specific primers. The average plasmid copy number per cell was determined by dividing the total plasmid copy number with the total number of parasites (determined by qPCR as described in *Materials and Methods*). For culture promastigotes that were grown in the presence of nourseothricin, both *FPPS*^{+/-}+pXNG4-*FPPS* and *fpps*⁷+pXNG4-*FPPS* parasites retained 19–22 copies of pXNG4-*FPPS* per cell (Fig. 4). In contrast, qPCR analysis of *FPPS*^{+/-}+pXNG4-*FPPS* lesion amastigotes showed that parasites from both GCV- and PBS-treated mice had lost most of the plasmid after 4–6 weeks post infection (Fig. 4). The minute retention in both groups (0.2–0.4 copies per cell) could represent a small fraction of parasites in which the plasmid was not yet released. Importantly, the *fpps*⁷+pXNG4-*FPPS* amastigotes from PBS-treated mice contained 10–13 copies of plasmid per cell after 4–6 weeks of infection (Fig. 4), suggesting that *FPPS* is required for amastigotes survival. For *fpps*⁷+pXNG4-*FPPS* amastigotes recovered from GCV-treated mice, the average plasmid copy numbers per cell at 4, 6, and 10 weeks post infection were 7, 8, and 11, respectively (Fig. 4), highlighting the retention of pXNG4-*FPPS* in amastigotes even after negative selection. The slight increase in plasmid copy number at 10 weeks in GCV-treated mice could be attributed to the removal of residual GCV by mice. We also recovered the pXNG4-*FPPS* plasmid DNA from lesion amastigotes and culture promastigotes (after 8–10 passage in GCV); and no changes were detected by restriction enzyme digestion or sequencing (data not shown).

Finally, we compared the relative *FPPS* transcript levels in WT culture promastigotes and lesion amastigotes extracted from BALB/c mice. Although we observed a ~two-fold reduction in comparison to promastigotes (Fig. S5), the fact that lesion amastigotes still synthesize *FPPS* transcripts suggests *FPPS* has important roles during the intracellular stage. Together our findings clearly indicate that *FPPS* is indispensable for both promastigotes and amastigotes of *L. major*.

4. Discussion

In *Leishmania*, the isoprenoid pathway and its downstream sterol biosynthesis pathway have offered multiple candidates as potential drug targets [42–46]. Despite the promise, the absolute requirement for certain sterol intermediates and end products remains an open question. Our recent characterization of the sterol methyltransferase (*SMT*)-null parasites

indicated that the C24-methylated sterols are not required during the promastigote stage of *L. major* [32]. The cholesterol-like sterol intermediates accumulated in the *SMT*-null mutants could compensate the loss of endogenous sterols under normal culture conditions. The *L. major* sterol C14 α -demethylase (*C14DM*)-null parasites display hypersensitivity to heat and fail to maintain membrane rigidity, yet they are replicative in culture, which again indicate the dispensability of sterol end products in the promastigote stage *in vitro* [31]. This was in sharp contrast with another study which failed to generate *C14DM*-knockouts in *L. donovani*, suggesting species-specific differences [45]. Although attenuated, both *C14DM*- and *SMT*-knockout mutants could cause diseases that were eventually comparable to *L. major* WT parasites [31, 32]. Unlike promastigotes, *L. major* amastigotes acquire large amounts of cholesterol from the host, which may reduce their dependence on endogenous sterol synthesis [31]. This may contribute to the low efficacy of ergosterol synthesis inhibitors in the mammalian host.

These and other studies have emphasized the importance of stringent genetic validation of drug targets for the development of antimicrobial agents. The lack of a robust and tightly controlled RNAi system in several *Leishmania* species limits the options for such analysis. To demonstrate essentiality in *Leishmania*, one strategy is to generate homozygous knockout parasites for the target gene in presence of a complementing episome derived from the pXNG4 vector [33]. The herpes simplex virus thymidine kinase gene from pXNG4 would render the transfectants sensitive to GCV. Thus, long term retention of the pXNG4 vector in mutant parasites in the presence of GCV would indicate the essential nature of target gene. This genetic approach has been used to probe the druggability of a number of *Leishmania* proteins [33, 45, 46]. In addition to controlling gene expression at the DNA or RNA level, another option is to regulate protein abundance through the use of conditionally destabilized fusion proteins [47, 48].

In this study, we assessed the essentiality of *FPPS* in the promastigote and amastigote stages of *L. major* using the pXNG4-based conditional knockout approach. Localized in the cytosol, *FPPS* is responsible for condensing IPP and DMAPP into FPP, a precursor for the synthesis of sterols, dolichols, ubiquinones, and prenyl groups (Fig. S1). Although we could delete one of the endogenous alleles of *FPPS* in WT *L. major* promastigotes, attempts to generate *FPPS*-null mutants by homologous recombination were unsuccessful. As indicated by Southern blot, repeated attempts yielded independent mutant lines bearing two expected replacements and extra copies of *FPPS* (Fig. 1A–B), possibly through gene duplication [40, 41]. In contrast, we could easily obtain both the heterozygous and homozygous *FPPS* knockout lines following the ectopic expression of *FPPS* (Fig. 1C) (*FPPS*^{+/-}/+pXNG4-*FPPS* and *fpps*⁷+pXNG4-*FPPS*). Treating the *FPPS*^{+/-}/+pXNG4-*FPPS* parasites *in vitro* with GCV led to a near complete loss of the plasmid after six passages (Fig. 2). However, despite strong selective pressure, the *fpps*⁷+pXNG4-*FPPS* parasites still maintained a high level of the pXNG4-*FPPS* plasmid (Fig. 2). Supplementation of *fpps*⁷+pXNG4-*FPPS* with farnesol, dolichols or coenzyme Q (5–10 μ M each) failed to facilitate plasmid loss in GCV-treated parasites (data not shown), suggesting that *FPPS* is needed in multiple pathways or *Leishmania* cannot sufficiently take up/metabolize these substrates. Overall, our genetic analysis provides the definitive proof that *FPPS* is indispensable for *L. major* promastigotes in culture.

In the mammalian host, *L. major* amastigotes are less dependent on the *de novo* synthesis of lipid metabolites and instead salvage them from the host [31, 49]. This adds more complexity in the process of validating potential drug targets. Indeed, certain genes and gene products that were found to be important for promastigotes have been turned out to be dispensable for amastigotes [49–52]. To assess the essentiality of FPPS during the mammalian stage of *L. major*, we infected susceptible BALB/c mice with WT or pXNG4-*FPPS*-transfected mutants, and followed up with daily GCV (or PBS) treatment for 14 consecutive days. Compared to all other groups, mice infected with the *fpps*⁷+pXNG4-*FPPS* parasites displayed a slower rate of lesion development after GCV treatment (Fig. 3A). This indicates possible growth inhibition of GCV with this group of parasites, which is confirmed by parasite load analyses (Fig. 3B–C). QPCR assays revealed that *FPPS*^{+/-}+pXNG4-*FPPS* amastigotes lost most of the plasmid (0.2–0.3 copies per cell) after 4–6 weeks of infection (Fig. 4). However, *fpps*⁷+pXNG4-*FPPS* amastigotes were maintaining 7–13 copies of the plasmid per cell irrespective of GCV treatment, up to 10 weeks post infection (Fig. 4; all mice must be euthanized after reaching humane endpoints so we could not continue the experiment after 10 weeks). The fact that *fpps*⁷+pXNG4-*FPPS* parasites retain the plasmid after GCV treatment strongly suggests neither promastigotes nor amastigotes can afford to lose FPPS. The reduced replication rate (Fig. 3 and S3) and lowered plasmid copy number in cells treated with GCV (Fig. 4) would allow parasites to alleviate the toxic effect. In theory, long-term GCV treatment could induce adaptive changes that affect TK activity or the uptake/efflux of GCV, although we did not detect mutations in the pXNG4-*FPPS* plasmid recovered from promastigotes (after 8 passages, Fig. 2) or amastigotes (10 weeks post infection, Fig. 4). Finally, analysis of *FPPS* transcript level in WT promastigotes and lesion amastigotes by qRT-PCR confirmed its expression in both stages (Fig. S5). The decreased expression in amastigotes could be related to the slower growth rate in the mammalian host. Together, these experiments clearly indicate that similar to promastigotes, *L. major* amastigotes are critically dependent on FPPS-mediated isoprenoid synthesis.

As *Leishmania* amastigotes predominantly reside in macrophages, the retention of episomal *FPPS* by *fpps*⁷+pXNG4-*FPPS* may reflect the poor availability of isoprenoids in the phagolysosome, or the inability of amastigotes to salvage isoprenoids. A similar observation was made in *T. gondii* (an apicomplexan), as the intracellular tachyzoites were capable of salvaging isoprenoids to support growth in fibroblasts but not macrophages [26]. Such discoveries have promoted the development of drug combinations that act synergistically by inhibiting both host and parasite isoprenoid synthesis [53].

In mammals, after the acute phase symptoms subside, *Leishmania* can persist indefinitely as quiescent intracellular amastigotes with a stringent metabolic state [54, 55]. While the overall replication rate is low, a subpopulation of persistent parasites undergo active proliferation [54], which may be important for disease reactivation. Future studies will explore whether FPPS is required for *Leishmania* survival during the chronic phase of infection.

In summary, our analyses confirmed the essentiality of FPPS during the promastigote and amastigote stages, thereby endorse its potential as a valuable drug target in *Leishmania* and possibly other trypanosomatid parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Farnesyl pyrophosphate synthase (FPPS) is a central enzyme in isoprenoid synthesis
- FPPS is a potential drug target against protozoan parasites
- In *Leishmania major*, FPPS is essential for both the vector and mammalian stages

Figure 1. Targeted deletion of *FPPS* in *L. major*.

Genomic DNA samples were digested with AlwNI and analyzed by Southern blot using radioactive probes for either an upstream flanking region (FR) or the open reading frame (ORF) of *FPPS*. A schematic representation of the Southern blot is illustrated in Fig. S2. (A) WT and four clones selected after the first round of deletion. (B) WT, *FPPS*^{+/-} #1, and three clones selected after the second round of deletion. (C) WT, *FPPS*^{+/-} #1, and five clones selected after the second round of deletion performed in presence of pXNG4-*FPPS*.

Figure 2. *FPPS* is essential for *L. major* promastigotes.

(A) WT (black), *FPPS*^{+/-}/+pXNG4-*FPPS* (dark gray, grown in 100 µg/ml of nourseothricin), and *fpps*⁻/+pXNG4-*FPPS* (light gray, grown in 100 µg/ml of nourseothricin) parasites were cultivated to mid-log phase and GFP expression was analyzed by flow cytometry. The dashed line separates GFP positive population from GFP negative population. (B) Retention of pXNG4-*FPPS* following GCV treatment. Promastigotes were continuously cultivated for 8 passages in presence or absence of GCV (50 µg/ml) and percentages of GFP positive cells were analyzed by flow cytometry. ●: *fpps*⁻/+pXNG4-*FPPS* #1; ▼: *fpps*⁻/+pXNG4-*FPPS* #1 with GCV; ○: *fpps*⁻/+pXNG4-*FPPS* #22; △: *fpps*⁻/+pXNG4-*FPPS* #2 with GCV; ■: *FPPS*^{+/-}/+pXNG4-*FPPS*; and □: *FPPS*^{+/-}/+pXNG4-*FPPS* with GCV. Error bars represent standard deviations.

Figure 3. *Fpps*^{+/pXNG4-FPPS} parasites show attenuated virulence in BALB/c mice following treatment with GCV.

BALB/c mice were infected in the footpads with stationary phase promastigotes and treated with either GCV (+) or PBS (–) from day 1 to day 14 post infection as described in *Materials and Methods*. (A) Footpad lesions (5 mice per group) were recorded weekly. Δ : WT, $\bullet$: WT + GCV, $\blacksquare$: *FPPS*^{+/–}/+pXNG4-*FPPS*, $\circ$: *FPPS*^{+/–}/+pXNG4-*FPPS* + GCV, $\square$: *fpps*[–]/+pXNG4-*FPPS*, $\blacktriangledown$: *fpps*[–]/+pXNG4-*FPPS* + GCV. Except for those receiving *fpps*[–]/+pXNG4-*FPPS* parasites and GCV, all infected mice were euthanized after 6 weeks. (B)–(C) Parasite numbers in the infected footpads were determined at the indicated times by limiting dilution assay (B) and qPCR (C). Black bars: WT, white bars: *FPPS*^{+/–}/+ pXNG4-*FPPS*, grey bars: *fpps*[–]/+ pXNG4-*FPPS*. Error bars represent standard deviations (*: $p < 0.05$).

Figure 4. *Fpps*^{+/+}pXNG4-FPPS amastigotes retain the pXNG4-FPPS plasmid in BALB/c mice. Following infection, mice treated with GCV (+) or PBS (-) were euthanized at the indicated times and DNA was extracted from lesion-derived amastigotes. Plasmid retention was determined by qPCR analysis as described in *Materials and Methods* using primers specifically designed for the pXNG4 vector. Plasmid copy numbers in promastigote cultures were also determined. Error bars represent standard deviations (**: $p < 0.01$).