Conditional knockout of lipoic acid protein ligase 1 reveals a redundant pathway for lipoic acid metabolism in the malaria parasite Plasmodium berghei (Postprint)

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Abstract

Background: Lipoic acid is a cofactor for a-keto acid dehydrogenase system that is involved in the central energy metabolism. In the apicomplexan parasite, Plasmodium, lipoic acid protein ligase 1 (LplA1) and LplA2 catalyse the ligation of acquired lipoic acid to the dehydrogenase complexes in the mitochondrion. The enzymes LipB and LipA mediate lipoic acid synthesis and ligation to the enzymes in the apicoplast. These enzymes in the lipoic acid metabolism machinery have been shown to play important roles in the biology of Plasmodium parasites, but the relationship between the enzymes is not fully elucidated. Methods: We used an anhydrotetracycline (ATc)-inducible transcription system to generate transgenic P. berghei parasites in which the lplA1 gene was conditionally knocked out (LplA1-cKO). Phenotypic changes and the lplA1 and lplA2 gene expression profiles of cloned LplA1-cKO parasites were analysed.Results: LplA1-cKO parasites showed severely impaired growth in vivo in the first 8 days of infection, and retarded blood-stage development in vitro, in the absence of ATc. However, these parasites resumed viability in the late stage of infection and mounted high levels of parasitemia leading to the death of the hosts. Although lplA1 mRNA expression was regulated tightly by ATc during the whole course of infection, lplA2 mRNA expression was significantly increased in the late stage of infection only in the LplA1-cKO parasites that were not exposed to ATc. Conclusions: The lplA2 gene can be activated as an alternative pathway to compensate for the loss of LplA1 activity and to maintain lipoic acid metabolism.

Full Text

Preamble

Conditional Knockout of Lipoic Acid Protein Ligase 1 Reveals a Redundancy Pathway for Lipoic Acid Metabolism in the Plasmodium berghei Malaria Parasite

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Abstract

Background: Lipoic acid is a cofactor for the -keto acid dehydrogenase system involved in central energy metabolism. In the apicomplexan parasite *Plasmodium*, two enzymes—lipoic acid protein ligase 1 (LplA1) and LplA2—catalyze the ligation of acquired lipoic acid to dehydrogenase complexes in the mitochondrion. The enzymes LipB and LipA mediate lipoic acid synthesis and ligation to enzymes in the apicoplast. While these components of the lipoic acid metabolism machinery play important roles in *Plasmodium* parasite biology, the relationships between them remain incompletely understood.

Methods: We utilized an anhydrotetracycline (ATc)-inducible transcription system to generate transgenic *P. berghei* parasites in which the *lplA1* gene was conditionally knocked out (LplA1-cKO). Phenotypic changes and the expression profiles of *lplA1* and *lplA2* were analyzed in cloned LplA1-cKO parasites.

Results: In the absence of ATc, LplA1-cKO parasites showed severely impaired growth during the first 8 days of infection and retarded blood-stage development *in vitro*. However, these parasites regained viability in the late stage of infection, mounting high parasitemia levels that led to host death. Although *lplA1* mRNA expression was tightly regulated by ATc throughout the infection course, *lplA2* mRNA expression increased significantly in the late stage only in LplA1-cKO parasites not exposed to ATc.

Conclusions: The *lplA2* gene can be activated as an alternative pathway to compensate for loss of LplA1 activity and maintain lipoic acid metabolism.

Keywords: *Plasmodium berghei*, lipoic acid protein ligase, tetracycline-inducible transcription system, conditional knockout

Background

Lipoic acid (LA, 6,8-thiooctanoic acid) is a cofactor required for the function of -keto acid dehydrogenases (KADH) and the glycine cleavage system (GCS). In most organisms, these enzyme complexes reside in mitochondria and participate in fatty acid biosynthesis, energy metabolism, and amino acid degradation [1-4]. The cofactor LA is supplied either through biosynthesis or salvage from the environment.

For LA biosynthesis, octanoyl-acyl carrier protein (ACP) is first ligated to the E2-subunit of KADHs and the apo-H-protein of GCS under the catalysis of octanoyl-acyl carrier protein:protein N-octanoyltransferase (lipoic acid protein ligase B, LipB) [5]. This is followed by insertion of two sulfurs at positions C6 and C8 of the octanoyl moiety by lipoic acid synthase (LipA) to form the lipoyl arm required for KADH and GCS activity [6,7].

Alternatively, LA obtained from the environment is transferred to the E2-subunit of KADHs and apo-H-protein of GCS through the action of two enzymes: lipoate-activating enzyme and lipoyltransferase [8-10]. The *Plasmodium* parasite, an apicomplexan protozoan that causes malaria, possesses a plastid-like organelle called the apicoplast that contains LA-requiring enzyme machinery with unique features [11,12]. In *Plasmodium*, the LA biosynthesis pathway and lipoylation of pyruvate dehydrogenase (PDH) occur exclusively in the apicoplast, while lipoylation of the other two KADHs– -ketoglutarate dehydrogenase (KGDH) and branched-chain keto acid dehydrogenase (BCDH)—as well as the H-protein of GCS by salvaged LA takes place in the mitochondrion [13-16].

Unlike in mammals, salvaged LA is ligated to the E2-subunit of enzyme complexes by lipoic acid protein ligase A (LplA) [17,18]. Two forms of LplA exist in *Plasmodium*: LplA1 localized in the mitochondrion and LplA2 present in both mitochondrion and apicoplast [15,19]. These distinct LA metabolism pathways in *Plasmodium* compared to its mammalian host make them promising targets for malaria intervention strategies.

Previous work by Dahl et al. showed that antibiotic treatment of *P. falciparum* causing loss of apicoplast function resulted in delayed parasite death [20], suggesting this organelle is indispensable for parasite survival. Disruption of the *lipB* gene did not affect *P. falciparum* growth despite significantly reduced LA levels. Further analysis revealed that LplA2 compensated for loss of LipB activity and PDH lipoylation in the apicoplast [19]. Additionally, the lipoic acid analog 8-bromooctanoate inhibited LplA1 activity, blocked LA salvage, and arrested *P. falciparum* growth *in vitro* [18]. Günther et al. reported that the *lplA1* gene in the murine malaria parasite *P. berghei* could be targeted by crossover recombination, but *lplA1* knockout parasite populations could not be isolated, indicating this gene is essential for parasite survival [21]. These observations demonstrate that LA metabolism machinery is critical for malaria parasite survival and development.

In this study, we used an anhydrotetracycline (ATc)-inducible gene expression system to conditionally knockout *lplA1* (LplA1-cKO) in blood-stage *P. berghei* and analyzed phenotypic changes in the transgenic parasite. We observed that while LplA1-cKO parasites showed arrested proliferation *in vivo* during early infection in the absence of ATc, they restored viability in the late stage. Further analysis revealed increased *lplA2* gene expression during late-stage infection, suggesting a compensatory role for LplA2 in response to loss of LplA1 activity.

Methods

Mice and Parasites. The ANKA strain of *P. berghei* was obtained from BEI Resources (Manassas, VA, USA), propagated in BALB/c mice, and stored in liquid nitrogen. Female BALB/c mice (6-8 weeks of age) were purchased from Vital River Laboratories (Beijing, China). All mice were housed in a specific-pathogen-free barrier facility. Blood-stage *P. berghei* infection was initiated by intraperitoneal injection of 1×10 parasitized red blood cells (pRBCs), and parasitemia was monitored daily by examination of Giemsa-stained (Sigma-Aldrich, MO, USA) thin smears of tail blood.

Construction of ATc-Inducible Gene Expression Vector. The tetracycline repressor (TetR) protein and tetracycline operon (TetO) sequence, originally identified in tetracycline-resistant *Escherichia coli*, were modified for gene expression regulation in mammalian cells [22,23]. We utilized these regulatory elements to generate an ATc-regulatable expression system for blood-stage *P. berghei*. The transfection vector was constructed using plasmid pL0016 (MR4, BEI Resources Repository), which contains dihydrofolate reductase-thymidylate synthase derived from pyrimethamine-resistant *Toxoplasma gondii* (tgdhfr/ts) as a selectable marker and was originally designed for *P. berghei* [24]. This plasmid contains the green fluorescence protein (*gfp*) gene under control of the *P. berghei* elongation factor-1 alpha promoter (PbEF1) [25].

The 657-nucleotide sequence encoding TetR was cloned using primer pair TetR-1/TetR-657 (Additional file: Table S1) from genomic DNA of the T-REx293 cell line transfected with pcDNA™6/TR (Invitrogen, Carlsbad, USA) [23] and inserted into pL0016 to replace *gfp*, creating plasmid pL0016-TetR. The 3' UTR of *P. berghei lplA1* (PblplA1), amplified using primers 3'A41/3'A42, was cloned into pL0016-TetR to replace the *ssurrna* fragment, yielding pL0016-TetR-3'arm. The PbEF1 promoter, modified to contain tandem repeats of two TetO sequences at the transcription start site, was synthesized (GenScript, Nanjing, China) and inserted into pMD18-T simple vector (Takara, Shiga, Japan) to create pT-PbEF1-2TetO. A 2A peptide fragment (Table 1) with BamHI and EcoRI restriction sites at the 5' end and BglII and XhoI at the 3' end was synthesized (GenScript) and introduced into pT-PbEF1-2TetO to produce pT-PbEF1-2TetO-2A. Subsequently, a *gfp* fragment was amplified from pL0016 using primers 2aGFP-1/2aGFP-717 with BglII and XhoI sites and inserted into pT-PbEF1-2TetO-2A to obtain pT-PbEF1-TetO-2A-GFP. The 5' UTR of PblplA1, amplified using primers 5' A41/5' A42, was cloned into pT-PbEF1-3TetO-2A-GFP to cre-

ate pT-PbEF1-TetO-2A-GFP-5' arm. The *lplA1* open reading frame (PblipL1, PBANKA_1413000, http://plasmodb.org) was obtained from *P. berghei* cDNA by reverse-transcription PCR from total RNA using oligonucleotides LplA1-1/LplA1-2 with BamHI and EcoRI restriction sites but without the endogenous stop codon.

This amplified fragment was subcloned into pT-PbEF1-TetO-2A-GFP-5' arm to generate pT-PbEF1-2TetO-LplA1-2A-GFP-5' UTR. This plasmid was subsequently inserted into pL0016-TetR-3' arm using SapI and HindIII restriction sites to generate the conditional knockout vector pATcon-LplA1-cKO. A control vector, pCTL-LplA1, containing all elements of pATcon-LplA1-cKO but lacking TetO sequences was also constructed. All transfected fragments were verified by sequencing using a 3730xl DNA Analyzer (Thermo Fisher, Massachusetts, USA).

Parasite In Vitro Culture and Transfection. Blood was collected from *P. berghei*-infected BALB/c mice with heparin sodium 3-4 days post-infection when parasitemia reached 1-3%, and passed through a CF11 cellulose column (Whatman, Maidstone, UK) to remove leukocytes and platelets. Total RBCs were washed twice with RPMI-1640 medium (HyClone, Beijing, China), loaded onto 74% Percoll (Sigma-Aldrich), and centrifuged at 5000×g for 20 min at 20°C. The pRBC layer was collected, washed twice with complete RPMI-1640 medium containing 20% FBS (HyClone), 25 mM HEPES (Sigma-Aldrich), 2 mM glutamine, 2 mg/ml glucose, 10 g/ml hypoxanthine (Sigma-Aldrich), and 50 mg/ml neomycin sulfate (Invitrogen), and resuspended in complete RPMI-1640 medium to 2×10 /ml. pRBCs were cultured in 6-well plates in a candle jar at 37°C for 16 hours [26]. During overnight culture, the majority of parasites developed to schizont stage.

For transfection, pATcon-LplA1-cKO and control vector pCTL-LplA1 were digested with NotI. Schizont-stage parasites were collected by centrifugation at 200×g for 5 min. pRBCs (1×10) were resuspended in 100 l Nucleofector Transfection Solution (Lonza, Basel, Switzerland), and 10 g linearized plasmid was added. The mixture was transfected by electroporation using a Nucleofector device (Lonza) with program U-033. Immediately after transfection, 50 l complete medium was added, and the 150 l mixture was intravenously injected into BALB/c mice. Forty-eight hours after injection, mice received 0.07 mg/ml pyrimethamine (Sigma-Aldrich) in drinking water for 7-10 days until parasitemia reached 3-4%. Drug-resistant transgenic parasites were then harvested, GFP-positive pRBCs were enriched by FACS Aria II flow cytometer (BD Biosciences, San Jose, CA, USA), and stored in liquid nitrogen.

Cloning of Transgenic Parasites. Splenectomized BALB/c mice were rested for 10 days after surgery, then injected intravenously with 200 l clodronate liposomes (Vrije Universiteit, The Netherlands) per mouse to deplete macrophages. Enriched GFP-positive parasites were first propagated in normal BALB/c mice. pRBCs were collected, washed with RPMI-1640 medium, and the concentration of transgenic parasites was adjusted by limiting dilution to 0.7 pRBC/100

l. One hundred microliters of parasite suspension was intravenously injected into splenectomized BALB/c mice three days after macrophage depletion. These mice were then given ATc-containing water (0.2 mg/ml). Positive clones were detected 7-8 days after infection.

Genotype Analysis of *P. berghei*. Parasites were isolated from erythrocytes by saponin lysis as described previously [27]. Genomic DNA from transgenic and wild-type parasites was obtained using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Vector integration into the parasite genome was analyzed by PCR using primers (Additional file: Table S1, Fig. 1 [Figure 1: see original paper]), and nucleotide sequences were verified by sequencing using an ABI 3730xl DNA analyzer (Guangzhou IGE Biotechnology LTD).

Fluorescence Microscopy. Enriched pRBCs were incubated for 10 min in RPMI-1640 medium containing 5 g/ml Hoechst 33258 (Sigma-Aldrich) at 37°C and washed twice with medium. Samples were applied to glass slides and imaged immediately using a Zeiss 710 NLO fluorescence microscope.

Detection of *lplA1* and *lplA2* mRNA Expression by Quantitative Real-Time PCR. Total RNA was extracted from parasites using Trizol reagent (Invitrogen). Genomic DNA was removed and reverse transcription was performed with PrimeScript™ RT reagent kit with gDNA Eraser (Takara) according to manufacturer instructions. Real-time PCR was performed using SYBR® Premix Ex Taq™ II (Takara) with oligonucleotides LpLA1-427F/LpLA1-482R and LpLA2-572F/LpLA2-655R. mRNA expression of *lplA1* and *lplA2* (PBANKA_0824500) was evaluated using actin (PBANKA_145930) as a housekeeping gene with primers actin-430F/actin-546R (Additional file: Table S1). Relative mRNA levels were calculated using the 2^(-ΔΔCT) method [28].

Western Blot Analyses. Protein was extracted from parasites using RIPA lysis buffer (Beyotime, Shanghai, China) with protease inhibitor (Sigma-Aldrich) and sonicated in an ice bath (Scientz, Ningbo, China). Protein concentration was determined by BCA assay (Thermo Fisher). Eight micrograms of parasite protein were separated on 10% SDS-polyacrylamide gels and blotted onto Immobilon-P transfer membranes (Millipore). Membranes were incubated with rabbit polyclonal antiserum specific for protein-attached lipoate (1:10,000, Calbiochem), mouse monoclonal antibody (mAb) specific for -actin as loading control (1:1,000, Sigma-Aldrich), or mouse mAb to TetR protein (1:1,000, Clontech). Sheep anti-rabbit or rabbit anti-mouse antibodies conjugated with horseradish peroxidase (1:4,000) were used for detection with SuperSignal West Pico chemiluminescence kit (Thermo Fisher).

Parasite Growth *In Vitro* and *In Vivo*. To assess ATc regulation of blood-stage growth *in vivo*, groups of mice were infected with 1×10 pRBCs of wild-type or transgenic *P. berghei* and provided with normal drinking water or water containing 0.2 mg/ml ATc [29]. Parasitemia was monitored daily by examination of Giemsa-stained thin blood smears.

For *in vitro* analysis, blood was collected from mice at early (day 6) and late (day 14) infection stages. Blood samples were passed through a CF11 column and centrifuged on 74% Percoll at 5000×g for 20 min at 20°C. The pRBC layer was collected, washed twice with complete medium, and incubated in complete medium with or without 1 g/ml ATc for 16 hours in a candle jar. Cultured parasites were collected, thin smears were prepared on glass slides, stained with Giemsa solution, and analyzed by microscopy.

Statistical Analysis. All infection experiments were performed three times, with each experimental group consisting of three to five mice as indicated in figure legends. Data represent mean (+SD). Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., CA, USA).

Results

Generation of *lplA1* Conditional Knockout (LplA1-cKO) Parasites. We developed an ATc-regulated expression system for blood-stage *P. berghei* containing a regulatory unit expressing repressor protein TetR under PbEF1 promoter control and a responsive unit with modified PbEF1 to regulate target gene expression and *gfp* reporter expression. The PbEF1 promoter was modified by inserting two TetO sequences at transcription start sites (TSS) determined by 5' RACE and nested PCR (to be reported separately). Expressed TetR binds TetO sequences to inactivate the PbEF1 promoter; in the presence of ATc, TetR dissociates from TetO and PbEF1 resumes activity to drive target gene expression (ATc-on system). Using this system, we constructed plasmid vector pATcon-LplA1-cKO to conditionally knockout *lplA1* via double crossover homologous recombination. Blood-stage *P. berghei* parasites were transfected with this vector, and drug-resistant populations were obtained. pRBCs were cloned by limiting dilution and injection into splenectomized, macrophage-depleted BALB/c mice. Parasitemia emerged in 12 of 60 cloning mice. Genotype analysis of these 12 clones by PCR revealed one mixed genotype (data not shown) and 11 clones with correct vector integration and no detectable episomes (Additional file: Figure S1a, Fig. 2a [Figure 2: see original paper]). Control parasites carrying vector pCTL-LplA1 were generated and cloned similarly.

To determine ATc-on system regulatory activity, groups of mice were infected with cloned transgenic parasites and provided with normal or ATc-containing water. pRBCs collected 6 days after infection were analyzed by flow cytometry for GFP reporter expression. Although the 11 parasite clones showed different GFP expression profiles, parasites exposed to ATc *in vivo* expressed higher GFP levels compared to the same clone without ATc exposure. As expected, parasites transfected with control vector pCTL-LplA1 expressed high GFP levels regardless of ATc exposure (Additional File: Figure S1b and S1c, Fig. 2b and 2c). Two clones (21 and 56) showing the greatest difference in GFP expression with or without ATc treatment were selected for further analysis. Microscopy confirmed that clone 21 and 56 parasites exposed to ATc *in vivo* expressed abundant GFP that was absent in parasites without ATc exposure (Fig. 2d).

Phenotypes of LplA1-cKO Parasites. To determine whether repressed *lplA1* expression affects parasite viability, groups of mice were infected with clone 21 and 56 transgenic parasites or control vector-transfected parasites, provided with normal or ATc-containing drinking water, and parasite growth was monitored daily. Mice infected with clone 21 and 56 parasites and provided ATc developed high parasitemia and succumbed at days 15-17 post-infection. Mice infected with the same transgenic clones but given normal water showed low parasitemia during early infection (up to day 8). However, in the late infection stage, LplA1-cKO transgenic parasites resumed proliferative ability and developed high parasitemia (Fig. 3a [Figure 3: see original paper] and insert figure). LplA1-cKO and control transgenic parasites also showed slower growth in the presence of ATc compared to WT parasites.

We next examined transgenic parasite development and differentiation *in vitro*. pRBCs were collected from mice at early (day 6) or late (day 14) infection stages and cultured *in vitro* for 16 h with or without ATc. Clone 21 LplA1-cKO parasites exposed to ATc *in vivo* and collected at day 6 developed from early ring to schizont stage *in vitro* regardless of ATc presence. The same transgenic parasites not exposed to ATc *in vivo* and collected at day 6 could not develop to schizont stage *in vitro* without ATc. However, LplA1-cKO parasites not exposed to ATc *in vivo* and collected at day 14 showed normal development from ring to schizont stage *in vitro* without ATc (Fig. 3b). These results demonstrate that LplA1-cKO parasites can resume viability in the absence of ATc during late-stage infection.

Redundant Pathway of Lipoic Acid Metabolism. The observation that LplA1-cKO parasites resumed proliferative ability in late-stage infection without ATc suggests existence of a redundant lipoic acid metabolism pathway. We collected parasites at early (day 6) and late (day 14) infection stages and determined *lplA1* and *lplA2* mRNA expression by quantitative PCR. Parasites exposed to ATc *in vivo* showed *lplA1* mRNA levels higher than those of wild-type parasites. Parasites not exposed to ATc *in vivo* expressed *lplA1* mRNA at levels significantly lower than both ATc-exposed transgenic parasites and wild-type parasites. The *lplA1* mRNA expression profile of parasites collected at late stage was comparable to that of early-stage parasites (Fig. 4a [Figure 4: see original paper]), indicating *lplA1* gene expression remains under ATc-on system control throughout infection. The *lplA2* mRNA expression pattern differed from *lplA1*. Early-stage parasites showed minimal *lplA2* mRNA expression, while late-stage parasites not exposed to ATc produced remarkably high *lplA2* mRNA levels not observed in ATc-exposed parasites (Fig. 4b).

We then determined lipoylation of -keto acid dehydrogenases (KADH) and GCS H-protein in transgenic parasites from early and late infection stages by immunoblotting. At early stage (day 6), transgenic parasites not exposed to ATc showed reduced lipoylated KGDH and BCDH in both clones 21 and 56, and reduced PDH in clone 56 compared to ATc-exposed parasites. However, these differences in lipoylated KADH levels were not detected between parasites

with and without ATc exposure at late-stage infection (Fig. 4c). No difference in TetR protein expression was detected between early and late infection stage parasites (Fig. 4c).

Discussion

LA-dependent multienzyme complexes are involved in energy metabolism, making the enzymes that catalyze LA synthesis and ligation critical for organism survival. Previous studies in apicomplexan *Plasmodium* parasites have revealed unique features of LA-related metabolic machinery that offer opportunities for identifying potential malaria intervention targets [13,14,16,30].

LplA1 plays important roles in salvaging LA from the environment and ligating LA to KADH in *P. falciparum* mitochondria [18]. Gene deletion studies in *P. berghei* also showed this gene is essential for parasite survival [21]. In the present study, we demonstrated that conditionally switching off *lplA1* expression significantly impaired transgenic *P. berghei* proliferative ability during the early phase (first 8 days) of blood-stage infection, indicating LplA1 is critically required for parasite survival. However, transgenic parasites without ATc regained viability in late-stage infection and developed high parasitemia leading to host death. Loss of the defective phenotype in late-stage infection was not due to cKO vector malfunction, as parasites collected at this stage produced TetR repressor protein (Fig. 4c) and *lplA1* mRNA expression remained efficiently regulated by ATc (Fig. 4a). Further examination revealed that LplA1-cKO parasites not exposed to ATc and collected in late-stage infection expressed high levels of *lplA2* mRNA not observed in ATc-exposed transgenic parasites. We propose that LplA2 represents a redundant mechanism that is induced to compensate for loss of LplA1 activity and maintain the critical LA metabolism pathway for parasite survival.

The Tet-on gene expression system established here in blood-stage *P. berghei* showed efficient regulatory activity. We observed that in the presence of ATc, transgenic parasites expressed *lplA1* mRNA at 1.5-1.8-fold higher levels than WT parasites, possibly due to PbEF1 being highly active throughout parasite development [25]. The potential effects of *lplA1* overexpression on parasite biology were not evaluated in detail. However, we observed that LplA1-cKO and CTL-cKO parasites treated with ATc showed altered growth profiles compared to WT *P. berghei*. Unlike WT parasites that developed rapidly and caused early host death from cerebral malaria, ATc-treated transgenic parasites proliferated at lower rates, eventually reaching severe parasitemia and causing late-stage host death due to anemia.

Günther et al. demonstrated the critical role of LplA1 for *P. berghei* viability, as permanent disruption of this gene impeded parasite survival [21]. Our study observed that LplA1-cKO parasites without ATc treatment showed severely impaired proliferation during the first 8 days of infection, supporting LplA1' s importance for parasite growth. The low-level growth seen in transgenic para-

sites is attributable to residual LplA1 from leaky expression of the Tet-on vector in the absence of ATc. Under these stressful conditions, surviving parasites may activate the LplA2 redundancy pathway for LA metabolism to support growth.

Conclusions

We utilized a conditional gene knockout approach to investigate the role of *lplA1* in blood-stage *P. berghei* survival. Our results reveal that LplA1 is required for parasite growth under normal conditions; however, *lplA2* expression can be activated as a redundancy pathway to compensate for loss of LplA1 function. We also demonstrated that the ATc-regulated gene expression system is a valuable tool for investigating gene function, analyzing phenotypic changes, and determining potential alternative pathways in genetically modified malaria parasites.

Abbreviations

LA: Lipoic acid; LipA: Lipoic acid synthase; LipB: Octanoyl-[acyl carrier protein]:protein N-octanoyltransferase; LplA1: Lipoic acid protein ligase 1; LplA2: Lipoic acid protein ligase 2; ATc: Anhydrotetracycline; KGDH: -ketoglutarate dehydrogenase; BCDH: Branched-chain ketoacid dehydrogenase; PDH: Pyruvate dehydrogenase; GCS: Glycine cleavage system; FACS: Fluorescence-activated cell sorting.

Declarations

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Availability of Data and Material. All data supporting the conclusions are included within the article.

Competing Interests. The authors declare no competing interests.

Consent for Publication. Not applicable.

Authors' Contributions. Z.S. and M.W. conceived the research and designed experiments. M.W. and Q.W. performed molecular biology experiments. M.W. and X.G. conducted parasitological experiments in mice. M.W. and Z.S. prepared the manuscript.

Ethics Approval and Consent to Participate. Experimental protocols were approved by the Institutional Animal Care and Use Committee.

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Figure Legends

Fig. 1 Schematic illustration of *lplA1* wild-type locus, the pATcon-LplA1-cKO construct for transfection, and the recombinant locus with the *lplA1* locus replaced by double crossover homologous recombination. Primers for PCR verification are indicated.

Fig. 2 Genotype confirmation and GFP expression profile of clone 21 (C-21) and 56 (C-56) of LplA1-cKO transgenic parasites. (a) Genotypes of cloned transgenic parasites were analyzed by PCR using primer pairs gfp-73/4-1053 (lane 1) to detect a 1.6 kb fragment crossing parasite genome and vector, LplA1-10/LplA1-11 (lane 2) to amplify a 2.5 kb fragment crossing the *lplA1* locus in WT parasite, and 4-1885/4-5083 (lane 3) to amplify a 0.99 kb fragment of the plasmid vector or episome (also see Fig. 1 legend). Fragments were verified by sequencing. (b) Flow cytometric profiles of GFP expression by cloned LplA1-cKO or control transgenic parasites with or without *in vivo* ATc exposure. (c) Mean GFP fluorescence intensity of cloned LplA1-cKO parasites. Mean (+SD) from triplicate flow cytometric analyses are presented. (d) Fluorescence photomicrographs showing GFP expression by transgenic parasites with or without ATc exposure *in vivo*.

Fig. 3 Proliferation *in vivo* and development *in vitro* profiles of cloned LplA1-cKO parasites in the presence or absence of ATc. (a) Parasitemia levels in mice

infected with cloned LplA1-cKO parasites and provided with normal or ATc-containing water. Results shown are from one of two experiments. Data are mean of 3-5 mice per group. The insert shows parasitemia during the first 8 days of infection. (b) *In vitro* development of LplA1-cKO parasites collected at early (D6) and late (D14) infection stages. Photomicrographs of clone 21 (C-21) parasites are shown.

Fig. 4 Levels of *lplA1* and *lplA2* mRNA expression and lipoylated KADHs in cloned LplA1-cKO parasites collected at early and late infection stages. (a) *lplA1* mRNA levels determined by quantitative PCR. Data are mean+SD from three analyses. (b) *lplA2* mRNA levels determined by quantitative PCR. (c) Levels of lipoylated PDH, KGDH, BCDH, and H-protein were analyzed by protein blotting using specific antibodies. TetR protein levels were analyzed in parallel. Actin was used as a sample loading control.

Additional File

Additional file: Figure S1. Genotype and phenotype analysis of LplA1-cKO transgenic parasite clones. (a) PCR verification of genotypes of LplA1-cKO parasite clones. (b) Regulation of GFP expression by ATc in cloned LplA1-cKO parasites. (c) Mean GFP fluorescence intensity of 9 LplA1-cKO clones. Each clone was analyzed by flow cytometry in triplicate and mean+SD was reported. Table S1. Nucleotide sequences of primers and the 2A peptide used in the study.

Note: Figure translations are in progress. See original paper for figures.