Conditional knock-out of lipoic acid protein ligase 1 reveals redundancy pathway for lipoic acid metabolism in *Plasmodium berghei* malaria parasite

Min Wang¹,², Qiong Wang², Xiang Gao² & Zhong Su¹,²,*

*Correspondence: su_zhong@gibh.ac.cn

¹University of Science and Technology of China, Fei Xi Road, Hefei, 230000, China

²Laboratory of Immunobiology, Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences, 190 Kai Yuan Road, Guangzhou, 510530, China
Abstract

**Background:** Lipoic acid is a cofactor for $\alpha$-keto acid dehydrogenase system that is involved in the central energy metabolism. In apicomplexan parasite *Plasmodium*, lipoic acid protein ligase 1 (LpL1) and LpL2 are identified that catalyse the ligation of acquired lipoic acid to the dehydrogenase complexes in mitochondrion. The enzymes LipB and LipA mediate lipoic acid synthesis and ligation to the enzymes in 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 utilized an anhydrotetracycline (ATc)-inducible transcription system to generate transgenic *P. berghei* parasites in which the *lplA1* gene was conditionally knocked out (LpL1-cKO). Phenotypic changes and the *lplA1* and *lplA2* gene expression profiles of cloned LpL1-cKO parasites were analyzed.

**Results:** LpL1-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 the 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 LpL1-cKO parasites that were not exposed to ATc.

**Conclusions:** *lplA2* gene can be activated as an alternative pathway to compensate for the loss of LpL1 activity and to 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 $\alpha$-ketoad id dehydrogenases (KADH) and the glycine cleavage system (GCS). In most organisms, these enzyme complexes are present in mitochondrion and involved in fatty acid biosynthesis, energy metabolism and amino acid degradation [1-4]. The cofactor LA that is ligated to the enzyme protein is supplied either by biosynthesis pathway or by salvage from environment. For LA biosynthesis, the octanoyl-acyl carrier protein (ACP) is first ligated to the E2-subunit of the KADHs and apo-H-protein of GCS under the catalysis by octanoyl-acyl carrier protein: protein N-octanoyltransferase (lipoic acid protein ligase B, LipB) [5]. It is followed by the insertion of two sulphurs at position 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 that is catalyzed by two enzymes, lipoate activating enzyme and lipoyltransferase[8-10]. *Plasmodium* parasite, an apicomplexa protozoan pathogen that causes malaria, also possesses a plastid-like organelle called apicoplast that contains the LA-requiring enzyme machinery with peculiar feature [11, 12]. It is now known that in *Plasmodium* parasite, the LA biosynthesis pathway and lipoylation of pyruvate dehydrogenase (PDH) only occurs in apicoplast, while the lipoylation of other two KADHs, $\alpha$-ketoglutarate dehydrogenase (KGDH) and branched chain-keto acid dehydrogenase (BCDH) as well as H-protein of GCS, by salvaged LA takes place in mitochondrion [13-16].

Unlike the situation in mammals, salvaged LA is ligated to E2-subunit of the enzyme complexes by lipoic acid protein ligase A (LplA) [17, 18]. Two forms of LplA have been
identified in *Plasmodium* parasite with LplA1 being localized in mitochondrion and LplA2 being present in both mitochondrion and apicoplast [15, 19]. The LA metabolism pathways in *Plasmodium* parasite that is distinct from that of the mammalian host make them promising targets for development of malaria intervention strategy.

Previous study by Dahl *et al.* showed that treatment of *P. falciparum* with antibiotic induced loss of apicoplast function and resulted in delayed death of the parasites [20], suggesting that this organelle is indispensable for the survival of parasite. Disruption of *lipB* gene did not affect the growth of *P. falciparum* although the LA level was significantly reduced. Further analysis showed that LplA2 compensated for the loss of LipB activity and lipolyation of PDH in apicoplast [19]. Furthermore, lipoic acid analogue 8-bromoocctanoate inhibited the activity of LplA1, blocked the salvage of LA and arrested growth of *P. falciparum* in vitro [18]. Günther *et al.* reported that the *lplA1* gene in murine malaria parasite *P. berghei* can be targeted by cross-over recombination but *lplA1* knockout parasite population was not possible to isolate, indicating that this gene is essential for survival of parasite [21]. These observations demonstrate that the LA metabolism machineries are critical for the survival and development of malaria parasites.

In this study, we used an anhydrotetracycline (ATC)-inducible gene expression system to conditionally knockout *lplA1* (LplA1-cKO) gene in blood-stage *P. berghei* and analysed the phenotypical changes of the transgenic parasite. We observed that although the LplA1-cKO parasites showed arrested proliferation in vivo in the initial stage of infection in the absence of ATC, the transgenic parasites restored the viability in the late stage of
infection. Further analysis revealed that lplA2 gene expression was increased during the late stage of infection, suggesting a compensatory role of LplA2 for the 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) was purchased from Vital River Laboratories (Beijing, China). All mice were housed in specific-pathogen-free barrier facility. The blood stage infection of P. berghei was initiated by i.p. injection of 1x10^6 parasitized red blood cells (pRBCs) and the 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 the tetracycline (Tet) resistant Escherichia coli, were modified for regulation of gene expression in mammalian cells [22, 23]. We utilized these regulatory and responsive elements to generate an ATc-regulatable gene expression system for the blood-stage P. berghei parasite. Transfection vector was constructed using the plasmid pL0016 (MR4, BEI Resources Repository), that contains dihydrofolate reductase-thymidylate synthase derived from pyrimethamine-resistant Toxoplasma gondii (tgdhfr/ts) as selectable marker, was originally designed for P. berghei [24]. This plasmid contains the green fluorescence protein (gfp) gene under the control of elongation factor-1 alpha promoter in P. berghei (PbEF1) [25]. The 657-nucleotide sequence encoding TetR was cloned using the primer pair
TetR-1/TetR-657 (Additional file: Table S1) from the genomic DNA of the T-REx293 cell line that was transfected with pcDNA™6/TR (Invitrogen, Carlsbad, USA) [23] and inserted into pL0016 to replace the gfp, resulting in plasmid pL0016-TetR. The 3’UTR of lplA1 of P.berghei (PblplA1) amplified using primers 3’A41/3’A42 was cloned into pL0016-TetR to replace the ssurrna fragment resulting in pL0016-TetR-3’arm. The PbEF1 promoter that was modified to contain tandem repeats of two TetO sequences at the transcription start site was synthesized (GenScript, Nanjing, China), and then inserted into PMD 18-T simple vector (Takara, Shiga, Japan) to create pT-PbEF1-2TetO plasmid. The fragment of 2A (Table 1) with BamHI, EcoRI restriction site at 5’end and BglII, Xhol at 3’end was synthesized (GenScript), and introduced into pT-PbEF1-2TetO plasmid to produce pT-PbEF1-2TetO-2A plasmid. Subsequently, gfp fragment was amplified from pL0016 using the primers 2aGFP-1/2aGFP-717 with BglII and XhoI site, and inserted into pT-PbEF1-2TetO-2A to obtain pT-PbEF1-TetO-2A-GFP plasmid. The 5’UTR of PblplA1 amplified using primers 5’A41/5’A42 was cloned into pT-PbEF1-3TetO-2A-GFP plasmid to create pT-PbEF1-TetO-2A-GFP-5’arm plasmid. The lpLA1 open reading frame (PblplL1, PBANKA_1413000, http://plasmodb.org) was obtained from P. berghei cDNA by reverse-transcription PCR from P.berghei total RNA using the oligonucleotides LplA1-1/LplA1-2 with BamHI and EcoRI restriction site but without endogenous stop codon. This amplified fragment was sub-cloned into pT-PbEF1-TetO-2A-GFP-5’arm plasmid to generate pT-PbEF1-2TetO-LplA1-2A-GFP-5’UTR plasmid. This plasmid was subsequently inserted into pL0016-TetR-3’arm using SapI and HindIII restriction sites to generate a conditional knockout plasmid vector pATcon-LplA1-cKO. A control vector, pCTL-LplA1,
was also constructed that contains all elements of pATcon-LplA1-cKO but lacks TetO sequences. All these transfected fragments were verified by sequencing using 3730xl DNA Analyser (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 the parasitemia reached 1-3%, and passed through a CF11 cellulose column (Whatman, Maidstone, UK) to remove leukocytes and platelets. The total RBCs were washed twice with RPMI-1640 medium (HyClone, Beijing, China), loaded on 74% Percoll (Sigma-Aldrich) and centrifuged at 5000g for 20 min at 20°C. The layer containing pRBCs was collected, washed twice with complete RPMI-1640 medium containing 20% FBS (HyClone), 25mM HEPES (Sigma-Aldrich), 2mM glutamine, 2mg/ml glucose, 10μg/ml hypoxanthine (Sigma-Aldrich) and 50mg/ml neomycin sulphate (Invitrogen) and resuspended with complete RPMI-1640 medium to 2 x 10^7/ml. The pRBCs was cultured in 6-well culture plate in a candle jar at 37°C for 16 hours [26]. During the overnight culture, the majority of parasites developed into schizont stage.

For transfection, the pATcon-LplA1-cKO construct and the control vector pCTL-LplA1 were digested with *NotI*. The schizont-stage parasites were collected by centrifugation at 200g for 5 min. pRBCs (1x10^7) were resuspended with 100 μl Nucleofector Transfection Solution (Lonza, Basal, Switzerland) and 10μg linearized plasmid was added. The mixture was transfected by electroporation in the Nucleofector device (Lonza) using the recommended program U-033. Fifty μl complete medium was added immediately after transfection and the 150 μl mixture was intravenously (i.v.) injected into BALB/c mice. Forty
eight hours after injection, the mice were given 0.07mg/ml pyrimethamine (Sigma-Aldrich) in drinking water for 7~10 days until the parasitemia reached 3~4%, the drug-resistant transgenic parasites were harvested and GFP-positive pRBCs were enriched by FACS Aria II flow cytometer (FACS Aria II, BD Biosciences, San Jose, CA, USA) and stored in liquid nitrogen.

Cloning of transgenic parasites

Splenectomised BALB/c mice were rested for 10 days after surgery and then injected i.v. with 200 μl per mouse clodronate liposomes (Vrije Universiteit, The Netherlands) to deplete macrophages. The enriched GFP-positive parasites were first propagated normal BALB/c mice. The pRBCs were collected, washed with RPMI-1640 medium and the concentration of transgenic parasites was adjusted by limiting dilution to 0.7 pRBC/100 μl. One hundred μl of parasite suspension was i.v. injected into splenectomised BALB/c mice three days after macrophage depletion. These mice were then given ATc-containing water (0.2mg/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 by others [27]. Genomic DNA of transgenic and wild type parasites were obtained using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Integration of the vector into parasite genome was analysed by PCR using primers (Additional file: Table S1, Fig. 1). The nucleotide sequences were verified by sequencing using ABI 3730xl DNA analyser (Guangzhou IGE Biotechnology LTD).

Fluorescence microscopy
The enriched pRBCs were incubated for 10 min in RPMI-1640 medium containing 5µg/ml Hoechst 33258 (Sigma-Aldrich) at 37°C and washed twice with the medium. The samples were applied to glass slides and imaged immediately by Zeiss 710 NLO fluorescence microscope.

Detection of lplA1 and lplA2 mRNA expression by quantitative real-time PCR

Total RNA was extracted parasites using Trizol reagent (Invitrogen). Genomic DNA was removed and reverse transcription PCR was performed with the PrimeScript™ RT reagent kit with gDNA Eraser (Takara) according to the manufacturer’s instruction. Real-time PCR assay was performed using the SYBR® Premix Ex Taq™II (Takara). The oligonucleotides LpLA1-427F/LpLA1-482R and LpLA2-572F/LpLA2-655R were used. mRNA expression of lplA1 and lplA2 (PBANKA_0824500) were evaluated with actin (PBANKA_145930) as a housekeeping gene using the primer actin-430F/actin-546R (Additional file: Table S1). The relative mRNA levels of lplA1 and lplA2 were calculated using the $2^{\Delta\Delta CT}$ 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 ice bath (Scientz, Ningbo, China). Protein concentration was determined by BCA assay (Thermo Fisher). 8 µg Parasite proteins were separated on 10% sodium dodecyl sulphate polyacrylamide gel, and then blotted onto Immobilon-P transfer membranes (Millipore). Membranes were incubated with rabbit polyclonal anti-serum specific for protein-attached lipoate (1:10000, Calbiochem), mouse monoclonal antibody (mAb) specific for β-actin as a loading control (1:1000,
Sigma-Aldrich) or mouse mAb to TetR protein (1:1000, Clontech). Sheep anti-rabbit or rabbit anti-mouse antibodies conjugated with horseradish peroxidase (1:4000) were used for detection with Supersignal west pico chemiluminescence kit (Thermo Fisher).

Parasite growth in vitro and in vivo

To detect whether ATc regulates the blood stage growth of transgenic parasites in vivo, groups of mice were infected with $1 \times 10^6$ pRBC of wild type or transgenic *P. berghei*, and provided with normal drinking water or water containing 0.2mg/ml ATc [29]. Parasitemia was monitored daily by examination of Giemsa-stained thin blood smears.

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

Statistical analysis

All infection experiments were performed three times and each experimental group consists of three to five mice as indicated in the figure legends. Data shown 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* that contains a regulatory unit expressing the repressor protein TetR controlled by PbEF1 promoter and a responsive unit consisting of the modified PbEF1 to control the expression of the target gene as well as the *gfp* reporter. The PbEF1 promoter was modified by insertion of two TetO sequences at the transcription start sites (TSS) that were determined by 5′RACE and nested PCR (to be reported in a separate paper). The expressed TetR binds to the TetO sequences to inactivate the PbEF1 promoter. In the presence of ATc, the TetR dissociates from TetO and PbEF1 resumes the activity to drive the target gene expression (ATc-on system). Using this system, we constructed a plasmid vector, pATcon-LplA1-cKO, to conditionally knockout *lplA1* by double cross-over homologous recombination. Blood-stage *P.berghei* parasites were transfected with the vector and drug-resistant population was obtained. The pRBC was cloned by limiting dilution and injection into splenectomised and macrophage-depleted BALB/c mice. Parasitemia emerged in 12 of 60 cloning mice. Analysis of genotype of the 12 clones by PCR revealed that one clone showed mixed genotype (data not shown), and the other 11 clones showed correct integration of the vector into parasite genome and no episome was detected (Additional file: Figure S1a, Fig. 2a). In addition, control parasites that carry the control vector pCTL-LplA1 was generated and cloned in the same manner. To determine the regulatory activity of ATc-on system, groups of mice were infected with the cloned transgenic parasites and were provided with normal or ATc-containing water. The pRBCs were collected 6 days after infection and analyzed by flow cytometry to determine *gfp*
reporter expression. It was observed that, although the 11 parasite clones showed different 
gfp expression profiles, the parasites exposed to ATc in vivo expressed higher levels of GFP 
compared with the parasites of the same clone that was not exposed to ATc. As expected, the 
parasites transfected with control vector pCTL-LplA1 expressed high level of GFP regardless 
of ATc exposure (Additional File: Figure S1b and S1c, Fig. 2b and 2c). Two clones, clone 21 
and 56 that showed greatest difference in gfp expression with or without ATc treatment, were 
selected for further analysis. It was also observed by microscopy that the parasites of clone 21 
and 56 exposed to ATc in vivo expressed abundant GFP that was absent in the parasites 
without exposure to ATc (Fig. 2d).

Phenotypes of LplA1-cKO parasites

To determine whether repressed expression of lplA1 affects the viability of the parasite, 
groups of mice were infected with clone 21 and 56 transgenic parasites, as well as the control 
vector-transfected parasites, provided with normal or ATc-containing drinking water. Parasite 
growth was monitored daily. The mice infected with parasites of clone 21 and 56 and 
provided with ATc developed high levels of parasitemia and succumbed at day 15-17 post 
infection. The mouse groups infected with the same clones of transgenic parasites and given 
normal water showed low levels of parasitemia in the early stage (up to day 8) of infection. 
However, in the late stage of infection, the LplA1-cKO transgenic parasites resumed the 
proliferation ability and high levels of parasitemia developed in the mice (Fig. 3a, and insert 
figure). It was also noticed that the LplA1-cKO and control transgenic parasites showed 
slower growth in the presence of ATc when they were compared with the WT parasites (Fig. 
3a).
We then performed an experiment to examine the development and differentiation of the transgenic parasites in vitro. pRBCs were collected from the mice at early (day 6) or late (day 14) stages of infection and cultured in vitro for 16 h in the presence or absence of ATc. The LplA1-cKO parasites of clone 21 exposed to ATc in vivo collected at day 6 of infection developed from early ring stage to schizony stage in vitro in the presence or absence of ATc. The same transgenic parasites that were not exposed to ATc in vivo and collected at day 6 of infection were not able to develop to schizony stage in vitro in the absence of ATc. However, the LplA1-cKO parasites that were not exposed to ATc in vivo and collected at day 14 showed normal development from ring to schizony stage in vitro when ATc were not provided (Fig. 3b). These results demonstrate that the LplA1-cKO parasites were able to resume their viability in the absence of ATc in the late stage of infection.

**Redundant pathway of lipoic acid metabolism**

The observation that LplA1-cKO parasites resumed the proliferation ability in the late stage of infection in the absence of ATc suggests existence of a redundant pathway for lipoic acid metabolism. We collected the parasites at early (day 6) and late (day 14) stages of infection and determined mRNA expression of *lplA1* and *lplA2* genes by quantitative PCR. The parasites exposed to ATc in vivo showed *lplA1* mRNA levels that were higher than that of wild type parasites. The levels of *lplA1* mRNA expressed by the parasites that were not exposed to ATc in vivo were significantly lower than the levels of ATc-exposed transgenic parasites and the wild-type parasites. It was also observed that the mRNA expression profile of the parasites collected at late stage were comparable to that of parasites of early stage infection (Fig. 4a), indicating that the *lplA1* gene expression is under the control of ATc-on
system in the course of infection. The pattern of \( lplA2 \) mRNA expression was different from that of \( lplA1 \). The parasites at early stage of infection showed minimum expression of \( lplA2 \) mRNA. However, at the late stage of infection the parasites that were not exposed to ATc produced remarkably high levels of \( lplA2 \) mRNA which was not observed in the parasites exposed to ATc (Fig. 4b). We then determined lipoylation of the \( \alpha \)-keto acid dehydrogenases (KADH) and H-protein of GCS in the transgenic parasites from early and late stage of infection by immune blotting. At the early stage (day 6) of infection, the transgenic parasites that were not exposed to ATc showed reduced levels of lipoylated KGDH and BCDH in both clone 21 and 56, and reduced PDH in clone 56 than the parasites exposed to ATc. However, these differences in the levels of lipoylated KADH were not detected between parasites with and without ATc exposure at the late stage of infection (Fig. 4c). In addition, no difference in TetR protein expression was detected in parasites at early and late of infection (Fig. 4c).

**Discussion**

LA-dependent multienzyme complexes are involved in energy metabolism. The enzymes that catalyze the synthesis of LA or ligation of LA to the multienzyme complexes, therefore, are critical for survival of the organisms. Previous studies in apicomplexan *Plasmodium* parasites have revealed many unique features of the LA-related metabolism machineries that offer the opportunity for identification of potential targets for intervention of malaria [13, 14, 16, 30]. It has been shown that LplA1 plays important roles in salvage of LA from environment and ligation of LA to KADH in the mitochondrion in *P. falciparum* parasites [18]. Gene deletion study in *P. berghei* also showed that this gene is essential for the survival of the parasite [21]. In the present study, we demonstrate that conditionally switching-off \( lplA1 \) gene expression
resulted in significant impairment of proliferative ability of the transgenic *P. berghei* parasites in the early phase (first 8 days) of blood-stage infection. This result indicates that LplA1 is critically required for the survival of parasites. However, the transgenic parasites in the absence of ATc regained the viability in the late stage of infection and developed high levels of parasitemia leading to the death of hosts. Loss of the defective phenotype of the transgenic parasites in the late stage of infection is not due to the malfunctioning of the cKO vector because the parasites collected in this stage of infection were able to produce the TetR repressor protein (Fig. 4c) and the *lplA1* mRNA expression was efficiently regulated by ATc (Fig. 4a). Further examination showed that the LplA1-cKO parasites that were not exposed to ATc and collected in the late stage of infection expressed high level of *lplA2* mRNA which was not observed in the transgenic parasites exposed to ATc. We propose here that LplA2 might be a redundant mechanism that is induced and compensates for the loss of LplA1 activity to maintain the critical LA metabolism pathway for the survival of the parasites.

The Tet-on gene expression system established and used here in blood-stage *P. berghei* parasite showed an efficient regulatory activity. We observed that in the presence of ATc, the transgenic parasites expressed *lplA1* mRNA level that was 1.5-1.8-fold higher than the level detected in WT parasites. This is possibly due to the PbEF1 that is known to be highly active in all development stages of the parasite [25]. The possible effect of over-expression of *lplA1* gene on the parasite biology was not evaluated in detail. However, we observed that the LplA1-cKO and CTL-cKO parasites treated with ATc showed differences in the growth profile in comparison with WT *P. berghei*. Unlike the WT parasites that developed rapidly and caused death of host due to cerebral malaria early during infection, the transgenic
parasites with treatment of ATc proliferated in lower rates and eventually reached severe parasitemia, and mice died in the late stage of infection due to anemia.

Günther et al. have demonstrated the critical role of LplA1 for viability of *P. berghei* because permanent disruption of this gene impeded the parasite survival [21]. We observed in our study that the LplA1-cKO parasites with no ATc treatment showed severely impaired proliferation ability in the first 8 days of infection, an evidence to support the important role of LplA1 for parasite growth. The low level of growth seen in the transgenic parasites is attributable to the residual level of LplA1 resulting from the leaky expression of the Tet-on vector in the absence of ATc. Under this stressful condition, the surviving parasites may activate LplA2 redundancy pathway for LA metabolism to support the parasite growth.

**Conclusions**

We utilized a conditional gene knock out approach to investigate the role of *lplA1* gene for survival of blood-stage *P. berghei* parasite. Our results revealed that LplA1 is required for the growth of the parasite in normal condition. However, the *lplA2* expression can be activated as a redundancy pathway to compensate for the loss of LplA1 function. We also demonstrated that the ATc-regulated gene expression system is a valuable tool not only for investigation of gene function, but also for analysis of phenotypic changes and determination of potential alternative pathway(s) of the 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: α-ketoglutarate dehydrogenase; BCDH: branched
chain-ketoacid dehydrogenase; PDH: pyruvate dehydrogenase; GCS: glycine cleavage system; FACS: Fluorescence-activated cell sorting.

**Declarations**

**Acknowledgements**

We thank the technicians at the GIBH Instrument Center for assistance in flow cytometry, and the staff at the GIBH Animal Center for care of laboratory animals. We thank Dr. Ling Chen at GIBH for providing the antibodies. We also thank BEI Resources Repository for providing the plasmid pL0016 originally constructed and deposited by A. P. Waters.

**Funding**

This work was supported by research fund from the Guangzhou Institute of Biomedicine and Health (GIBH) and grants from National Natural Science Foundation of China (81341058 and 81201308).

**Availability data and material**

All data supporting the conclusions of the article are included within the article.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

Not applicable

**Authors’ contributions**

Z.S. and M.W. conceived the research proposal and designed the experiment. M.W. and Q.W. performed the molecular biology experiments. M.W. and X.G. conducted the parasitological experiment in mice, M.W. and Z.S. prepared the manuscript.
**Ethics approval and consent to participate**

The experiment protocols were approved by Institutional Animal Care and Use Committee.

**References**

1. Perham RN, Jones DD, Chauhan HJ, Howard MJ. Substrate channelling in 2-oxo acid dehydrogenase multienzyme complexes. Biochemical Society Transactions. 2002;30(2):47-51.
2. Reed JL, Hackert ML. Structure-function relationships in dihydrolipoamide acyltransferases. Journal of Biological Chemistry. 1990;265(16):8971-8974.
3. Fujiwara K, Ikeda KO, Motokawa Y. Chicken Liver H-protein, a Component of the Glycine Cleavage System. Journal of Biological Chemistry. 1986;261(19):8836-8841.
4. Perham RN. Swinging arms and swinging domains in multifunctional enzymes: catalytic machines for multistep reactions. Annu Rev Biochem. 2000;69:961~1004.
5. Zhao X, Miller JR, Cronan JE. The reaction of LipB, the octanoyl-[acyl carrier protein]:protein N-octanoyltransferase of lipoic acid synthesis, proceeds through an acyl-enzyme intermediate. Biochemistry. 2005;44(50):16737-16746.
6. Hayden MA, Huang IY, Iliopoulos G, Orozco M, Ashley GW. Biosynthesis of lipoic acid: characterization of the lipoic acid auxotrophs Escherichia coli W1485-lip2 and JRG33-lip9. Biochemistry. 1993;32(14):3778-3782.
7. Vanden Boom TJ, Reed KE, Jr CJ. Lipoic Acid Metabolism in Escherichia coli: Isolation of Null Mutants Defective in Lipoic Acid Biosynthesis, Molecular Cloning and Characterization of the E. coli lip Locus, and Identification of the Lipoylated Protein of the Glycine Cleavage System. Journal of bacteriology. 1991;173(20):6411-6420.
8. Fujiwara K, Okamuraikeda K, Motokawa Y. Purification and characterization of lipoyl-AMP:N epsilon-lysine lipoyltransferase from bovine liver mitochondria. Journal of Biological Chemistry. 1994;269(2):16605-16609.
9. Fujiwara K, Okamuraikeda K, Motokawa Y. Lipoylation of acyltransferase components of alpha-ketoacid dehydrogenase complexes. Journal of Biological Chemistry. 1996;271(22):12932-12936.
10. Tsunoda JN, Yasunobu KT. Mammalian lipoic acid activating enzyme. Archives of Biochemistry & Biophysics. 1967;118(2):395-401.
11. Ralph SA, van Dooren GG, Waller RF, Crawford MJ, Fraunholz MJ, Foth BJ, Tonkin CJ, Roos DS, McFadden GI. Tropical infectious diseases: metabolic maps and functions of the Plasmodium falciparum apicoplast. Nat Rev Microbiol. 2004;2(3):203-216.
12. Foth BJ, Stimmler LM, Handman E, Crabb BS, Hodder AN, McFadden GI. The malaria parasite Plasmodium falciparum has only one pyruvate dehydrogenase complex, which is located in the apicoplast. Mol Microbiol. 2005;55(1):39-53.
13. Gunther S, McMillan PJ, Wallace L, Muller S. Plasmodium falciparum possesses organelle-specific α-keto acid dehydrogenase complexes and lipoylation pathways. Biochemical Society Transactions. 2005;33(5):977~980.
14. Gunther S, Storm J, Muller S. *Plasmodium falciparum*: organelle-specific acquisition of lipoic acid. Int J Biochem Cell Biol. 2009;41(4):748-752.
15. Wrenger C, Muller S. The human malaria parasite *Plasmodium falciparum* has distinct organelle-specific lipoylation pathways. Mol Microbiol. 2004;53(1):103-113.
16. McMillan PJ, Stimmerl LM, Foth BJ, McFadden GI, Muller S. The human malaria parasite *Plasmodium falciparum* possesses two distinct dihydrolipoamide dehydrogenases. Mol Microbiol. 2005;55(1):27-38.
17. Thomsen-Zieger N, Schachtner J, Seeber F. Apicomplexan parasites contain a single lipoic acid synthase located in the plastid. FEBS Letters. 2003;547(1-3):80-86.
18. Allary M, Lu JZ, Zhu L, Prigge ST. Scavenging of the cofactor lipoate is essential for the survival of the malaria parasite *Plasmodium falciparum*. Mol Microbiol. 2007;63(5):1331-1344.
19. Gunther S, Wallace L, Patzewitz EM, McMillan PJ, Storm J, Wrenger C, Bissett R, Smith TK, Muller S. Apicoplast lipoic acid protein ligase B is not essential for *Plasmodium falciparum*. PLoS Pathog. 2007;3(12):e189.
20. Dahl EL, Rosenthal PJ. Multiple antibiotics exert delayed effects against the *Plasmodium falciparum* apicoplast. Antimicrob Agents Chemother. 2007;51(10):3485-3490.
21. Gunther S, Matuschewski K, Muller S. Knockout studies reveal an important role of *Plasmodium* lipoic acid protein ligase A1 for asexual blood stage parasite survival. PLoS One. 2009;4(5):e5510.
22. Yao F, Svensjo T, Winkler T, Lu M, Eriksson C, Eriksson E. Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. Human Gene Therapy. 1998;9(13):1939-1950.
23. Yao F, Pomahac B, Visovatti S, Chen M, Johnson S, Augustinova H, Svensjo T, Eriksson E. Systemic and localized reversible regulation of transgene expression by tetracycline with tetR-mediated transcription repression switch. J Surg Res. 2007;138(2):267-274.
24. Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, van der Linden R, Sinden RE, Waters AP, Janse CJ. A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. Mol Biochem Parasitol. 2004;137(1):23-33.
25. de Koning-Ward TF, Speranca MA, Waters AP, Janse CJ. Analysis of stage specificity of promoters in *Plasmodium berghei* using luciferase as a reporter. Molecular and Biochemical Parasitology. 1999;100(1):141-146.
26. Moll K, Ljungström I, Perlmann H, Scherf A, Wahlgren M. Methods in Malaria Research. 5rd ed. USA: American Type Culture Collection Press; 2008.
27. Umlas J, Fallon JN. New thick-film technique for malaria diagnosis. Use of saponin stromatolytic solution for lysis. American Journal of Tropical Medicine & Hygiene. 1971;20(4):527-529.
28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-408.
29. Pino P, Sebastian S, Kim EA, Bush E, Brochet M, Volkmann K, Kozlowski E, Llinas M, Billker O, Soldati-Favre D. A tetracycline-repressible transactivator system to study essential genes in malaria parasites. Cell Host Microbe. 2012;12(6):824-834.

30. Storm J, Mueller S. Lipoic Acid Metabolism of Plasmodium - A Suitable Drug Target. Current Pharmaceutical Design. 2012;18(24):3480-3489.

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 cross-over 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. Genotype of cloned transgenic parasites were analysed by PCR using the primer pairs gfp-73/4-1053 (lane 1) to detect a 1.6 kb fragment crossing parasite genome and the vector, LplA1-10/LplA1-11 (lane 2) to amplify a 2.5 kb fragment crossing 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 legend to Fig. 1). The fragments were verified by sequencing. b. Flow cytometric profiles of gfp expression by cloned LplA1-cKO or control transgenic parasites with or without exposure in vivo to ATc. 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 the parasitemia during the first 8 days of infection. b. In vitro development of LplA1-cKO parasites collected in the early (D6) and late (D14) stage of infection. Photomicrographs of clone 21 (C-21) parasites are shown.

Fig. 4 Levels of \( lplA1 \) and \( lplA2 \) mRNA expression and lipoylated KADHs by cloned LplA1-cKO parasites collected in early and late stage infection. 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 analysed by protein blotting using specific antibodies. TetR protein levels were analysed in parallel. Actin was used for control of sample loading.

**Additional file**

**Additional file: Figure S1.** Genotype and phenotype analysis of LplA1-cKO transgenic parasite clones. a. PCR verification of genotypes of the 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 the mean+SD was reported. **Table S1.** Nucleotide sequences of primers and the 2A peptide used in the study.