The mitochondrion of *Plasmodium falciparum* generates essential acetyl-CoA for protein acetylation

Sethu C. Nair[1,*], Justin T. Munro[2,3,*], Alexis Mann[1], Manuel Llinás[2,3,4] and Sean T. Prigge[1].

[1]Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD, 21218, [2]Department of Chemistry PSU, [3]Huck Center for Malaria Research PSU, [4]Biochemistry and Molecular Biology Department Pennsylvania State University, University Park, PA 16802, USA.

Supplementary Information Appendix

Materials and Methods

P. falciparum culture and maintenance

Unless otherwise noted, blood-stage *P. falciparum* parasites were cultured in human erythrocytes at 1% hematocrit in CMA (Complete Medium with Albumax) containing RPMI 1640 medium with L-glutamine (US Biological Life Sciences), supplemented with 20 mM HEPES, 0.2% sodium bicarbonate, 12.5 µg/mL hypoxanthine, 5 g/L Albumax II (Life Technologies) and 25 µg/mL gentamicin. Cultures were maintained in 25 cm^2 gassed flasks (94% N_2, 3% O_2, 3% CO_2) and incubated at 37 °C. For cultures that require acetate supplementation, 5 mM sterile sodium acetate was supplemented in the media. Deidentified human O+ red blood cells were purchased from BioIVT (Westbury, NY) or obtained through an Institutional Review Board-approved protocol at the Johns Hopkins University.

Generation of knockout and knockdown constructs

We employed Cas9-mediated gene editing[1] using plasmid pCasG-LacZ[2] to express Cas9 and a gRNA and repair plasmid pRSng[3,4]. For generation of pRSng, homology arms of

~200-400 bp from the gene of interest were amplified using homology arm (HA) 1 and 2 forward and reverse primers (**Dataset S1)** using blood stage *P. falciparum* NF54^{attB} genomic DNA as template. HA1 and HA2 primers were designed to contain ~15 bp overhangs for insertion into pRSng using ligation-independent cloning (LIC) methods (In-Fusion, Clontech). pRSng plasmid was digested with *Not*I for insertion of HA1, and with *Ngo*MIV for HA2. Plasmid pCasG-LacZ was digested with *Bsa*I for insertion of double-stranded DNA adaptamers (generated from primers listed in **Dataset S1**) using LIC or standard ligation; positive colonies were selected using blue/white colony screening. To generate double knockout lines, the gene encoding human Dihydrofolate Reductase (*dhfr*) was excised from plasmid pRSng using *Bam*HI/*Hin*dIII and replaced using LIC (primers pRsBSD.F and pRsBSD.R) with a sequence encoding *Aspergillus terreus* Blasticidin-S Deaminase codon harmonized for expression in *P. falciparum*[5] to generate the pRSngB plasmid.

For knockdown constructs, we used a variant of plasmid pMG74[6] called pKD[2]. For generation of the ↓*mpdh$_{E2}$* (mPDH$_{E2}$ knockdown) plasmid, HA1 (253 bp) was amplified from bases 1592 to 1844 of the sequence encoding mPDH$_{E2}$. HA2 (327 bp) was amplified from the 3' UTR, starting from 471 bp after the stop codon. The HA2 and HA1 amplicons were concatenated by PCR using the HA2 forward primer and the HA1 reverse primer to generate a HA2-HA1 amplicon with *Eco*RV endonuclease sites between the two homology arms. The HA2-HA1 fragment was then inserted into pKD digested with *Asc*I and *Aat*II. The sequences of primers used for amplifying homology arms and the gRNA sequences are included in **Dataset S1**. For generation of the ↓*acs* (ACS knockdown) plasmid, HA1 (323 bp) was amplified from bases 3169 to 3491 of the sequence encoding ACS. HA2 (247 bp) was amplified from the 3' UTR region starting 65 bp after the stop codon. HA2 and HA1 were concatenated and inserted into the knockdown plasmid as described above for mPDH$_{E2}$. The guide RNA was cloned into the pCasG-LacZ vector as described above.

P. falciparum transfections to generate knockout and knockdown lines

For deletion of *mpdh$_{E1\alpha}$* (PF3D7_1312600), *mpdh$_{E2}$* (PF3D7_0303700), *e3* (PF3D7_1232200), *kdh$_{E2}$* (PF3D7_1320800) and *lipl2* (PF3D7_0923600), 300 µL of red blood cells were electroporated with 75 µg each of gene-specific pRSng and pCasG plasmids. Electroporated RBCs were mixed with ~2.5 mL NF54^{attB} parasites synchronized as schizonts and cultured in CMA (or CMA with 5 mM acetate for some experiments). After 48 hrs, transfected parasites were selected with 1.5 µM DSM1 and 2.5 nM WR99210 for seven days,

after which they were cultured in drug-free medium until parasites were observed (typically between 17 and 30 days after transfection). Once parasites were observed, they were switched to medium containing WR99210. For generation of double knockout parasites, we used the pRSngB plasmid containing *mpdh*$_{E2}$ homology arms. The Δ*kdh*$_{E2}$ and Δ*mpdh*$_{E1α}$ parasite lines were transfected with the pRSngB plasmid using the methods described above with WR99210 substituted with 2.5 µg/mL blasticidin. After parasites were observed, they were maintained in media containing WR99210 and blasticidin.

For the generation of *acs* (PF3D7_0627800) and *mpdh*$_{E2}$ (PF3D7_1314600) inducible knockdown lines, plasmid pKD was linearized by overnight digestion with *Eco*RV. Transfection was performed by electroporating 75 µg of linearized pKD along with 75 µg of the corresponding pCasG plasmid into 300 µL of uninfected red blood cells. Following electroporation, 2.5 mL of NF54^{attB} parasites were added to red blood cell cultures containing the plasmids. Starting 48 hrs post transfection, parasites were maintained in drug selection media containing 1.5 µM DSM1 and 2.5 µg/mL blasticidin for 7 days, after which drug-free media was used until parasites were observed (20-30 days post-transfection). After this point, parasites were maintained in medium containing blasticidin.

Confirmation of genotypes

Gene deletion parasite lines generated with the pRSng plasmid were validated with genotype PCR reactions. Primers were designed to screen for 5' integration (Δ5' reaction primers GOI.5'F and pRS.R) and 3' integration (Δ3' reaction primers pRS.F and GOI.3'R) of the gene of interest (GOI) disruption cassette, and the 5' region (primers GOI.5'F and GOI.5'WTR) and 3' region (primers GOI.3'WTF and GOI.3'R) of the unmodified gene. The parental NF54^{attB} line was used as a control for these reactions. For the ACS knockdown line, primers were designed to screen for 5' integration (Δ5' reaction primers iACS.5'F and pMG.3HA.R) and 3' integration (Δ3' reaction primers HSP86.F and iACS.3'R) of the pKD plasmid, and the unmodified parental gene (P reaction primers iACS.5'F and iACS.3'R). For the mPDH$_{E2}$ knockdown line, primers were designed to screen for 5' integration (Δ5' reaction primers imPDH.5'F and pMG.3HA.R) and 3' integration (Δ3' reaction primers HSP86.F and imPDH.3'R) of the pKD plasmid, and the unmodified parental gene (P reaction primers imPDH.5'F and imPDH.3'R). The parental NF54^{attB} line was used as a control for these reactions. The primer sequences are included in **Dataset S1**.

Parasite growth curve determination

For growth curve determination, parasites were stained with SYBR Green, and analyzed via flow cytometry. Parasite lines of interest were each seeded in quadruplicates in 96-well plates at about 0.5% parasitemia. Parasitemia was then measured at two-day intervals as previously described[4]. Parasites were diluted 1:10 in CMA and stored in a 96-well plate at 4 °C until analysis by flow cytometry. Parasites were stained by transferring 10 µL of the 1:10 dilutions to a 96-well plate containing 100 µL of 1x SYBR Green (S7563, Invitrogen) in PBS, and incubated for 30 minutes while shaking. Post-incubation, 150 µL of PBS was added to each well to dilute unbound SYBR Green dye. Samples were analyzed with an Attune Nxt Flow Cytometer (Thermo Fisher Scientific), with a 50 µL acquisition volume, and a running speed of 25 µL/minute with 10,000 total events collected.

Immuno-fluorescent microscopy

Parasites were resuspended in a 1:2 solution of 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 minutes at 37° C to fix cells. Next, 40 µL of fixed parasite sample was added to 11 mm wells in PTFE printed microscope slides (Electron Microscopy Sciences, Cat No: 63422-11) and incubated for half an hour. After 30 minutes, superficial fluid was aspirated and wells were washed once with 40 µL of PBS. Cells were then permeabilized for 10 minutes with 40 µL of 1% Triton X-100 solution followed by three washes with PBS. Cells were then reduced for 10 minutes by adding 40 µL of 0.1 g/L $NaBH_4$ in PBS and washed 3 times with PBS afterwards. Cells were blocked for 2 hrs in a solution of 3% (30 g/L) BSA in PBS, and then washed 3 times with PBS. Cells were then incubated with 40 µL of primary antibody (1:1,000 rat anti-HA mAb 3F10, Roche) at 4°C overnight in a solution containing 3% BSA in PBS. Cells were washed three times with PBS on the next day, and then incubated for 2 hrs in 40 µL of secondary antibody (1:1,000 anti-rat Alexa Fluor 488, Life Technologies) in 3% BSA in PBS. Cells were washed with PBS 3 times and stained with Gold DAPI antifade (Life Technologies) under a coverslip sealed with nail polish. Slides were then viewed using a Zeiss AxioImager M2 microscope. A series of images spanning 5 µm were acquired with 0.2 µm spacing and images were deconvolved with VOLOCITY software (PerkinElmer) to report a single combined z-stack image.

Western blot

For western blot analysis, 10 mL of parasite culture of about 10-12% parasitemia in 2% hematocrit was pelleted by centrifugation. Pelleted cells were saponin lysed in 0.1% saponin for 5 minutes on ice. Lysed parasites were pelleted and washed once to remove soluble red blood

cell contents. The parasite pellet was then resuspended in 50 µL NuPAGE LDS sample buffer (Thermo Fisher) and boiled and vortexed for 10 minutes. Proteins were resolved by SDS-PAGE on 4-12% gradient gels and transferred to nitrocellulose membranes. The membranes were then blocked for an hour in 5% milk powder/PBS solution and probed with 1:2,500 rat anti-HA mAb 3F10 antibody (Roche) in 5% milk powder/PBS solution overnight. After washing in PBS containing 0.5% Tween-20, the membranes were probed with 1:2,500 goat anti-rat HRP-linked secondary antibodies (GE healthcare, NA935) in 5% milk powder/PBS solution for 1 hour, and then washed again and the signal detected using Super Signal Chemiluminescent substrate (Thermo fisher scientific, 34577). For loading controls, the membranes were stripped in 200 mM Glycine, 1% Tween-20 for 15 min and washed in PBS containing 0.5% Tween-20. Aldolase was detected using the above protocol with 1:10,000 mouse anti-*P. falciparum* Aldolase monoclonal antibodies (a kind gift from David Sullivan and also available from CancerTools.com #156424) and 1:2,500 sheep anti-mouse HRP-linked secondary antibodies (GE healthcare, NA931).

Whole genome sequencing of the clonal lines

Parasites used for gDNA extraction were grown to 8-10% schizonts in a 50-mL culture at 4% hematocrit on the day of extraction. Cells were isolated by centrifugation (400 g for 3 minutes) before aspirating the supernatant and adding 50 mL of 0.1% saponin in PBS. Following 10 minutes of lysis, parasites were isolated by centrifugation (1,500 g for 10 minutes) and washed twice with 50 mL of PBS. The gDNA was extracted using a Qiagen DNeasy® kit according to provided protocol. Extracted gDNA was quantified using a Nanodrop spectrophotometer. For sequencing, 1 µg of gDNA per sample was analyzed using the TruSeq® DNA PCR-Free Low-Throughput Library Prep Kit (Illumina) according to the manufacturer's directions. Genomic sequence data for all genetically-modified lines are available at the NCBI Sequence Read Archive under submission PRJNA781991 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA781991).

^{13}C-Metabolic labeling experiments to detect acetylated proteins

Blood stage parasite cultures were initiated in 50 mL culture flasks at 2% hematocrit, at ~1% parasitemia and grown for four days. Wild-type parasites were grown in glucose-free media that was supplemented with 2 g/L of ^{13}C-glucose for all four days. For the knockdown experiments, the ↓*mpdh*$_{E2}$/*Δkdh*$_{E2}$ line (↓*p*$_{E2}$/*Δk*$_{E2}$) was cultured in permissive (0.5 µM aTc) or non-permissive (No aTc) conditions for four days. As shown in **Figure 8D**, acetate supplementation was not needed since the growth of ↓*p*$_{E2}$/*Δk*$_{E2}$ parasites in permissive and non-

permissive conditions is similar for up to four days. For the last two days of the growth period, parasites were cultured in glucose-free media that was supplemented with 2 g/L of ^{13}C-glucose. After the four-day growth period, cells were harvested through centrifugation at 2,000 g for 10 minutes. The cell pellet was resuspended in 1 mL of 0.05% saponin and incubated on ice for 5 minutes to selectively lyse red blood cells. After incubation, the lysis solution was diluted with 10 mL of PBS and centrifuged at 2,000 g for 10 minutes. Pelleted parasites were washed once with PBS to remove red blood cell residue. Parasite pellets were then resuspended in 1 mL of PBS and spun down again at 16,000 g for 5 minutes. Protein extraction was performed by resuspending the pellet in 50µL of guanidine-based extraction buffer (8 M guanidine HCl, 100 mM Tris, pH 8.0, 0.5 mM DTT), briefly vortexing, and incubating the solution on ice for 20-30 minutes. After the incubation, the solution was spun down and the supernatant containing extracted protein was stored at -80 °C for mass spectrometric analysis. The protein sample was quantified using Bio-Rad Protein Assay Dye Reagent (Cat No: 5000006) and 20 µg of each sample were sent for analysis.

Mass spectrometry (LC-MS/MS) based identification of acetylated peptides

Protein extracts (~20 µg) were reduced with 20 µL of 150 mM dithiothreitol for 45 mins at 60 °C, and subsequently alkylated with 20 µL of 250 mM iodoacetamide in dark for 15 minutes. Samples were digested by adding 100 µL trypsin solution (10 mM TEAB, pH 7.5, 2 µg Promega trypsin #V5111) and incubated overnight at 37 °C. Peptides were acidified and buffer exchanged using the Oasis Plate HLB (Waters) collected and dried by speedvac. Peptide fractions were analyzed by liquid chromatography interfaced with tandem mass spectrometry (LC/MSMS) using an Easy-LC 1100 HPLC system (Thermofisher) interfaced with an Orbitrap Fusion Lumos Mass Spectrometer (Thermofisher). Fractions were resuspended in 20 µL loading buffer (2% acetonitrile in 0.1% formic acid) and analyzed by reverse phase liquid chromatography coupled to tandem mass spectrometry. Peptides (20% each fraction) were loaded onto a C18 trap (S-10 µM, 120 Å, 75 µm x 2 cm, YMC, Japan) and subsequently separated on an in-house packed PicoFrit column (75 µm x 200 mm, 15 µm, +/-1 µm tip, New Objective) with C18 phase (ReproSil-Pur C18-AQ, 3 µm, 120 Å, Dr.Maisch, Germany) using 2-90% acetonitrile gradient at 300 nL/min over 120 min. Eluting peptides were sprayed at 2.0 kV directly into the Lumos. Survey scans (full MS) were acquired from 370-1400 m/z with data dependent monitoring with a 3 sec cycle time. Each precursor was individually isolated in a 1.2 Da window and fragmented using HCD activation collision energy 30 and 15 sec dynamic exclusion, first mass being 120 m/z. Precursor and fragment ions were analyzed at resolutions

of 120,000 and 30,000, respectively, with automatic gain control (AGC) target values at 4e5 with 60 ms maximum injection time (IT) and 1e5 with 100 ms maximum IT respectively. Isotopically resolved masses in precursor (MS) and fragmentation (MS/MS) spectra were processed in Proteome Discoverer (PD) software (v2.4, Thermo Scientific). All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.6.2). Mascot was set up to search the UP000001450_36329_3D7.fasta; RefSeq_83_Human_170919 database (118,734 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.015 Da and a parent ion tolerance of 3.0 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Deamidated products of asparagine and glutamine, oxidated methionine, and acetylated lysine (with 0, 1, or 2 ^{13}C-labeled carbons) were specified in Mascot as variable modifications. Scaffold (version Scaffold_4.11.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability by the Peptide Prophet algorithm[7] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm[8]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

^{13}C-Metabolic labeling experiments to detect small metabolites

Plasmodium falciparum parasites used for metabolomics experiments were tightly synchronized one cycle prior to performing the metabolite extraction procedure. To eliminate possible variation of culture conditions prior to performing metabolomics experiments, all parasite cultures used for metabolomics experiments were grown in media supplemented with 5 mM acetate. On the day of metabolite extraction, parasites were cultured to 5-10% early trophozoites at 4% hematocrit. Parasites were then magnetically purified from uninfected RBCs and the resulting culture was consolidated in fresh media without additional acetate. Parasite concentration was determined using a hemocytometer and triplicate samples were prepared with 1×10^8 cells in 5 mL of labeling media for each condition. The media used for labeling was deprived of labeled metabolite: glucose, glutamine, or acetate. Before initiating incubation, $^{13}C_6$-glucose, $^{13}C_5$-glutamine, or $^{13}C_2$-acetate were added to a concentration equivalent to standard culture conditions (11 mM for glucose, 2 mM for glutamine, and 5 mM for acetate). The only experiments in which acetate was returned to the growth medium were the experiments testing the labeled fraction of ^{13}C-acetate. In that case, unlabeled acetate was added to the samples

that were treated with heavy labeled glucose or glutamine. Parasites were incubated under labeling conditions for 2.5 hrs. The labeling media were then aspirated, and the parasite pellets were washed with PBS followed by quenching with 1 mL of 90% methanol containing 0.25 µM $^{13}C_4$,$^{15}N_1$-aspartate. These samples were vortexed for 30 seconds and centrifuged for 15 minutes at 15,000 g at 4 °C. Following centrifugation, these samples were dried under nitrogen gas and stored at -80 °C. To prepare these samples for HPLC/MS analysis, they were removed from storage and placed on ice for 10 minutes to thaw. Samples were resuspended in 100 µL of 3% HPLC-grade methanol:water containing 1 µM chlorpropamide to achieve a final concentration of 1×10^9 cells/mL in each sample. Samples were centrifuged and transferred to vials for HPLC/MS analysis. An equivalent portion of all samples was pooled into a separate vial to serve as a quality control for metabolite detection. Samples were run using a Thermo Scientific Exactive Plus mass spectrometer. Chromatographic conditions were similar to those used previously[9]. Briefly, the liquid chromatography column used was a Waters Xselect C18 HSS T3 column with 2.5 µm particle diameter. The mobile phase was composed of 3% methanol with 10 mM tributylamine, 2.5 µM medronic acid[10], and 15 mM acetic acid (solvent A) and methanol (solvent B). The mass spectrometer was operated exclusively in negative mode with a scan range from 85-1000 m/z. Data analysis was performed using El-Maven mass spectrometry analysis software[11]. Processed data for the metabolites presented in this manuscript are available in **Dataset S2**. Raw data for all metabolites are available at the NIH workbench under submission ST002024 (https://www.metabolomicsworkbench.org/data/DRCCMetadata.php?Mode=Study&StudyID=ST002024).

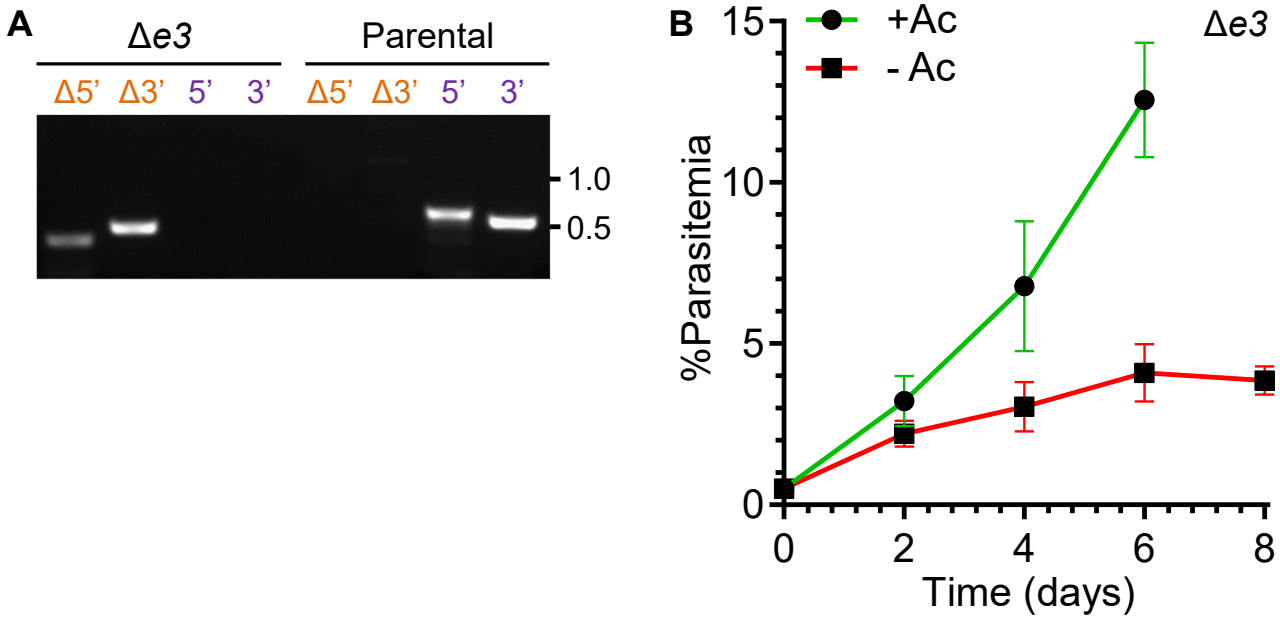

Figure S1

A. Genotyping PCR reactions confirming the deletion of the *e3* dehydrogenase gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S13** contains source data.

B. Growth curves comparing the growth of Δ*e3* parasites in the presence and absence of 5mM acetate (Ac). Error bars represent the standard deviation from two independent experiments, each conducted in quadruplicate.

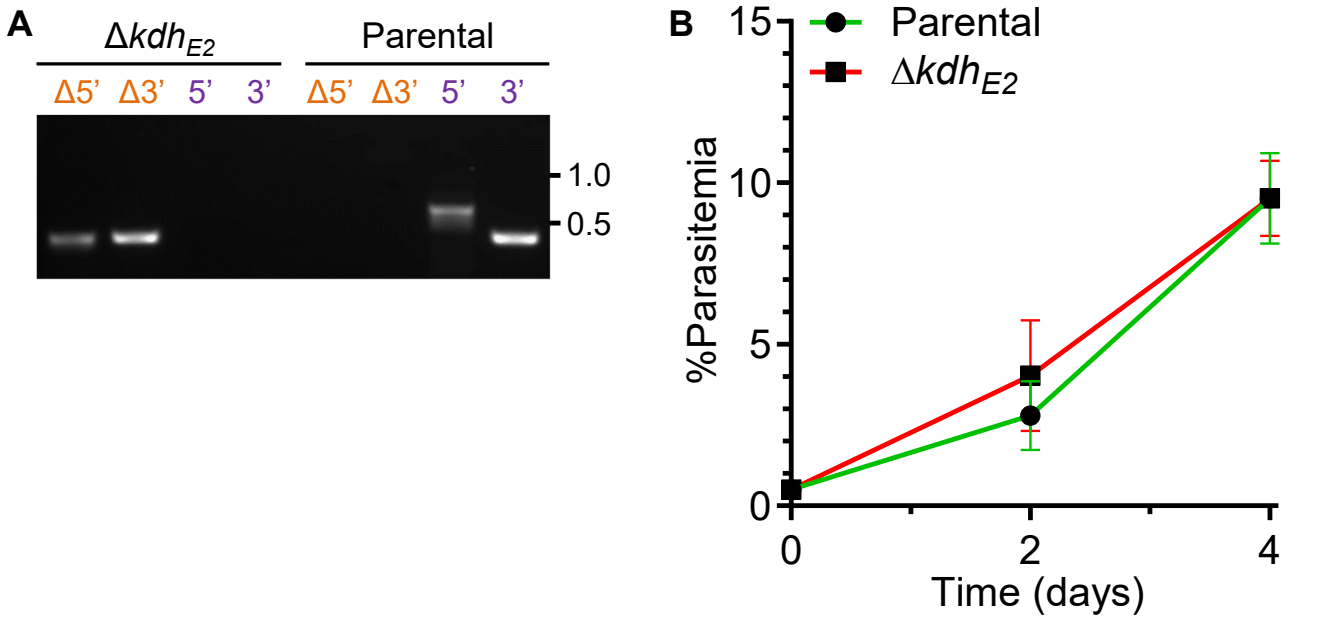

Figure S2

A. Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S14** contains source data.

B. Growth curves of Δ*kdh*$_{E2}$ parasites (red) compared to the parental NF54^{attB} line (green). Error bars represent the standard deviation from two independent experiments, each conducted in quadruplicate.

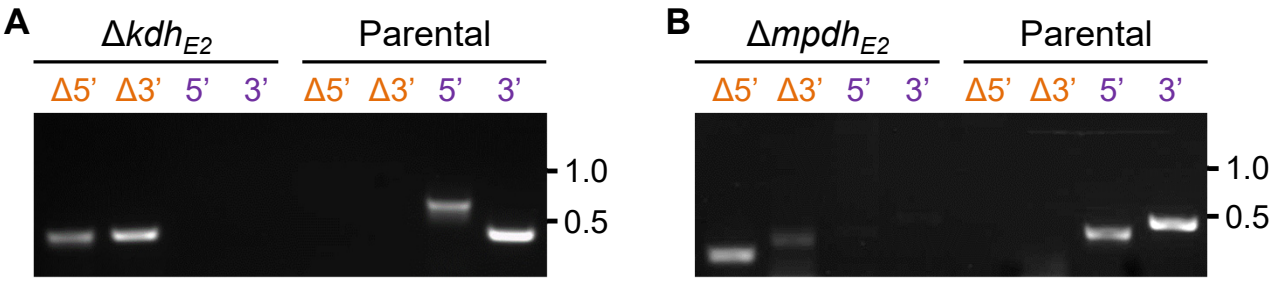

Figure S3

A, B. Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ and *mpdh*$_{E2}$ genes. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S15** contains source data.

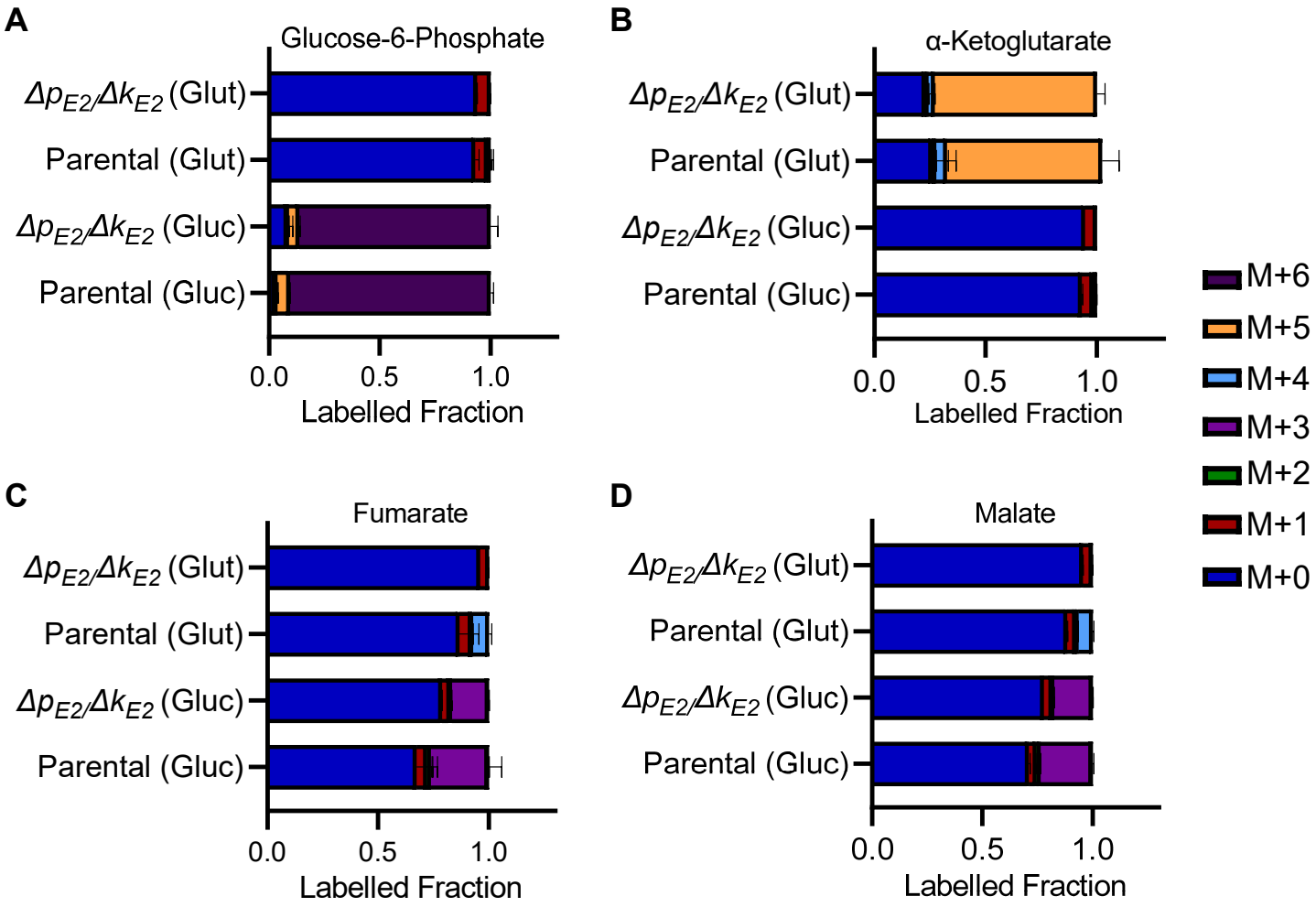

Figure S4

A-D. Fraction of isotopically labeled glucose 6-phosphate (**A**), α-ketoglutarate (**B**), fumarate (**C**), or malate (**D**) when incubated with labeled glutamine (Glut) or glucose (Gluc) in parental or $\Delta p_{E2}/\Delta k_{E2}$ parasites. For labeling experiments, color coding indicates the mass shift from the incorporation of heavy labeled carbon atoms in addition to the mass (M) of the parent compound. Labeling data are presented as the fraction of the total metabolite pool determined from N=3 experiments (parental) or N=2 experiments ($\Delta p_{E2}/\Delta k_{E2}$) with error bars representing the standard deviation (SD).

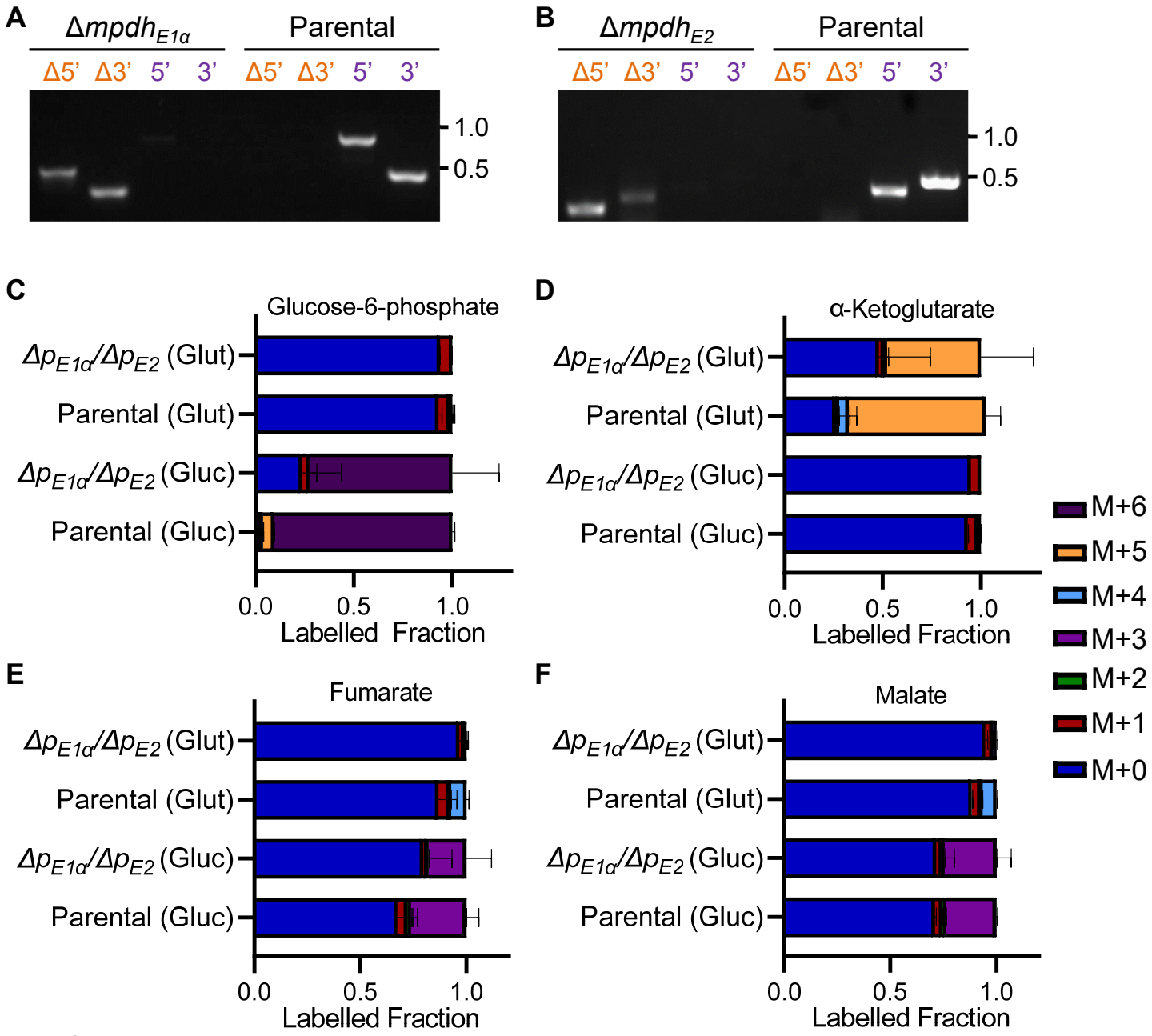

Figure S5

A, B. Genotyping PCR reactions confirming the deletion of the *mpdh*$_{E1α}$ and *mpdh*$_{E2}$ genes. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S16** contains source data.

C-F. Fraction of isotopically labeled glucose-6-phosphate **(C)**, α-ketoglutarate **(D)**, fumarate **(E)**, or malate **(F)** when incubated with labeled glutamine (Glut) or glucose (Gluc) in parental or Δ$p_{E1α}$/Δp_{E2} parasites. For labeling experiments, color coding indicates the mass shift from the incorporation of heavy labeled carbon atoms in addition to the mass (M) of the parent compound. Labeling data are presented as the fraction of the total metabolite pool determined from N=3 experiments (parental) or N=2 experiments (Δ$p_{E1α}$/Δp_{E2}) with error bars representing the standard deviation (SD).

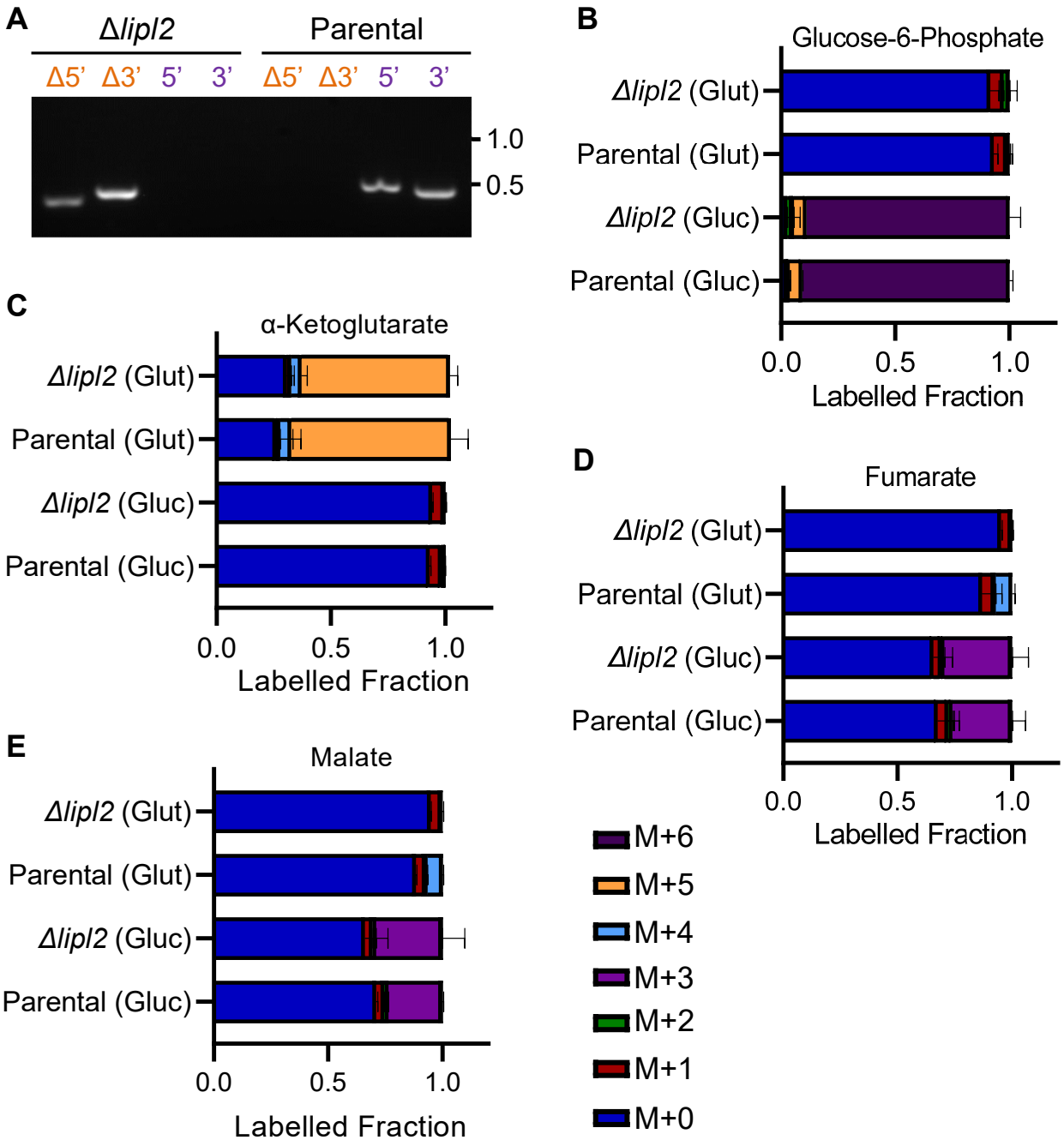

Figure S6

A. Genotyping PCR reactions confirming the deletion of the *lipl2* gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S17** contains source data.

B-E. Fraction of isotopically labeled glucose-6-phosphate **(B)**, α-ketoglutarate **(C)**, fumarate **(D)**, or malate **(E)** when incubated with labeled glutamine (Glut) or glucose (Gluc) in parental or Δ*lipl2* parasites. For labeling experiments, color coding indicates the mass shift from the incorporation of heavy labeled carbon atoms in addition to the mass (M) of the parent compound. Labeling data are presented as the fraction of the total metabolite pool determined from N=3 experiments (parental) or N=2 experiments (Δ*lipl2*) with error bars representing the standard deviation (SD).

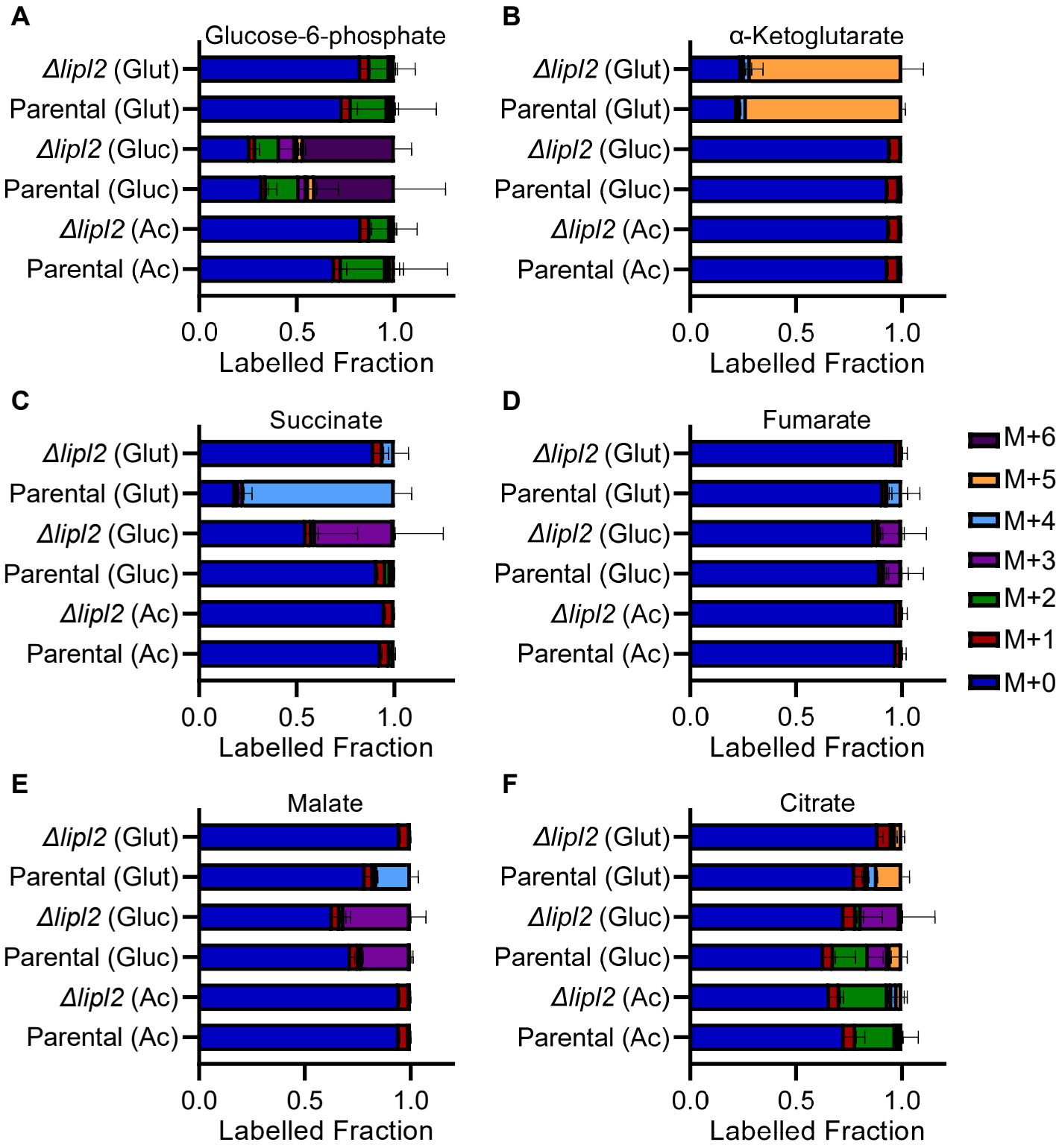

Figure S7

A-F. Fraction of isotopically labeled glucose-6-phosphate **(A)**, α-ketoglutarate **(B)**, succinate **(C)**, fumarate **(D)**, malate **(E)** or citrate **(F)** when incubated with labeled glutamine (Glut), glucose (Gluc) or acetate (Ac) in parental or Δ*lipl2* parasites. For labeling experiments, color coding indicates the mass shift from the incorporation of heavy labeled carbon atoms in addition to the mass (M) of the parent compound. Labeling data are presented as the fraction of the total metabolite pool determined from N=2 experiments with error bars representing the standard deviation (SD).

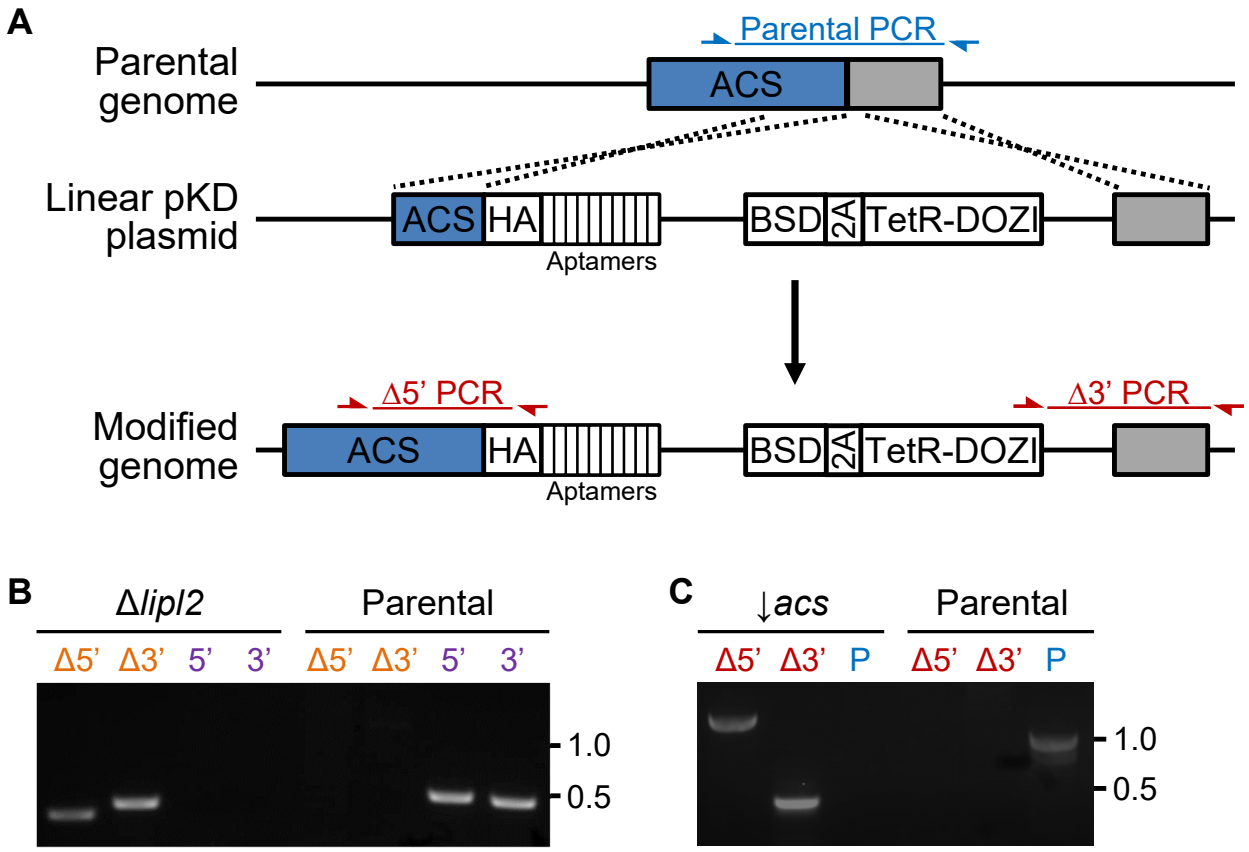

Figure S8

A. Schematic representation showing how the gene encoding acetyl-CoA synthetase (ACS) was modified using linearized pKD plasmid. Integration of the pKD plasmid appended a region encoding a c-terminal hemagglutinin tag (HA) and an array of ten aptamers. The modified genome also contained an expression cassette that produces tetracycline repressor (TetR) fused to the DOZI mRNA silencing protein (TetR-DOZI) and blasticidin-S deaminase (BSD). The positions of genotyping PCR products designed to identify the parental locus (blue) or the recombinant locus (red) are shown.

B. Genotyping PCR reactions confirming the deletion of the *lipl2* gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S18** contains source data.

C. Genotyping PCR reactions confirming the modification of the *acs* gene for knockdown experiments. Based on the scheme shown in **A**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify parental (P) locus). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S18** contains source data.

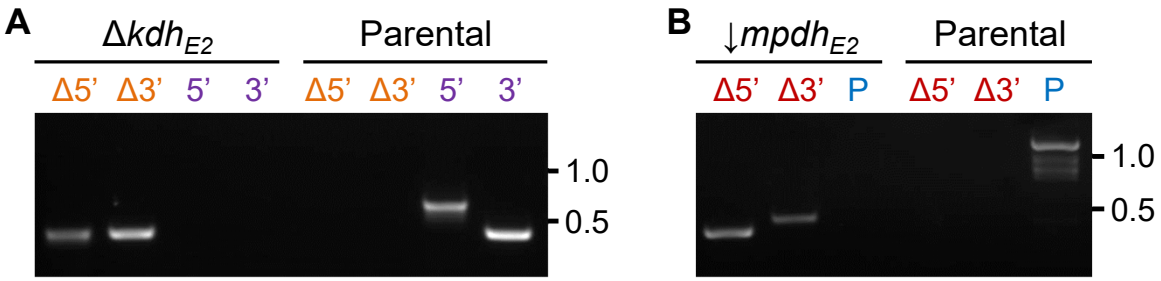

Figure S9

A. Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S19** contains source data.

B. Genotyping PCR reactions confirming the modification of the *mpdh*$_{E2}$ gene for knockdown experiments. Based on the scheme shown in **Figure S8A**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify parental (P) locus). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. **Figure S19** contains source data.

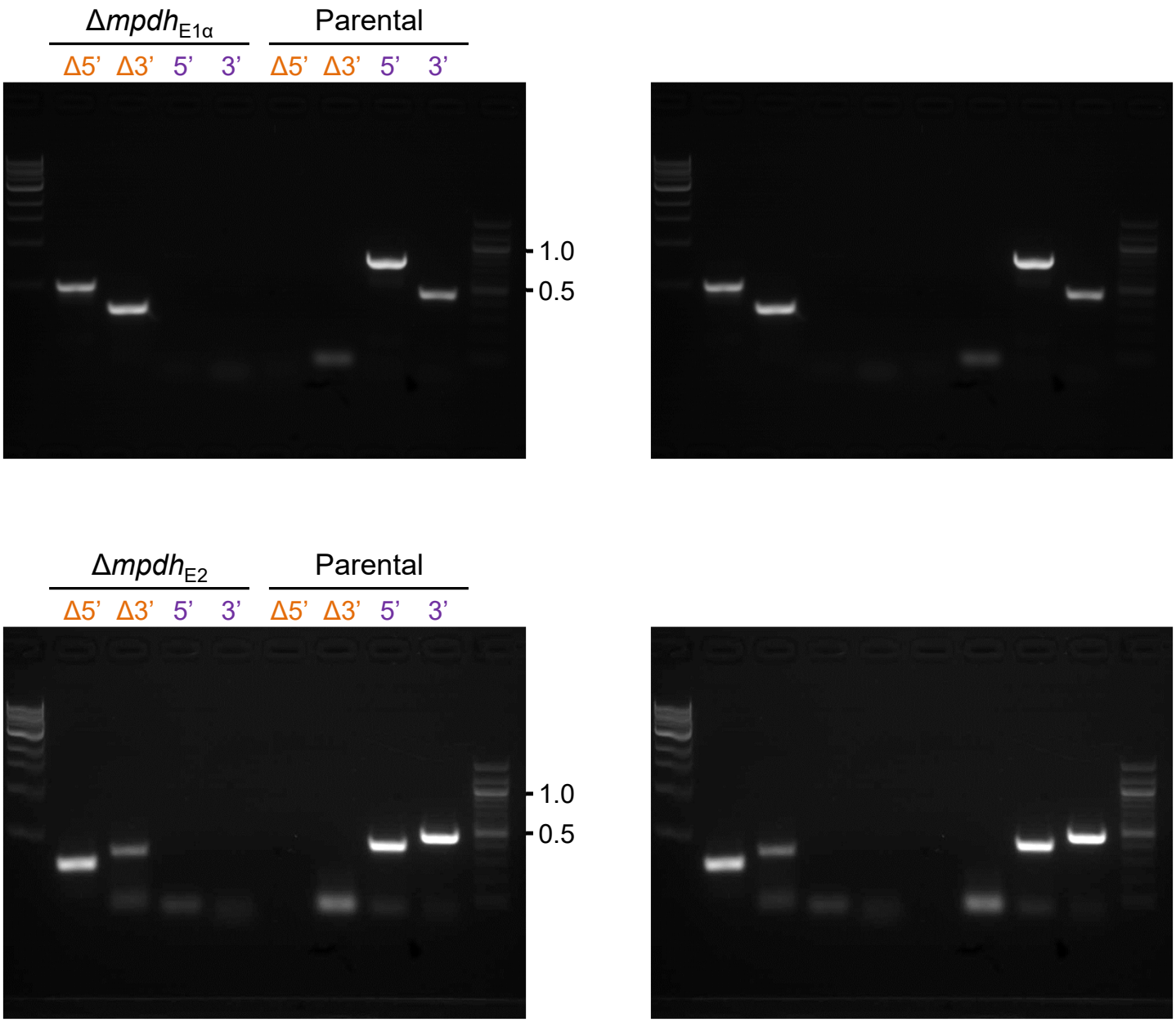

Figure S10

Figure 1-source data. Genotyping PCR reactions confirming the deletion of *mpdh*$_{E1α}$ (Top) and *mpdh*$_{E2}$ (bottom). Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

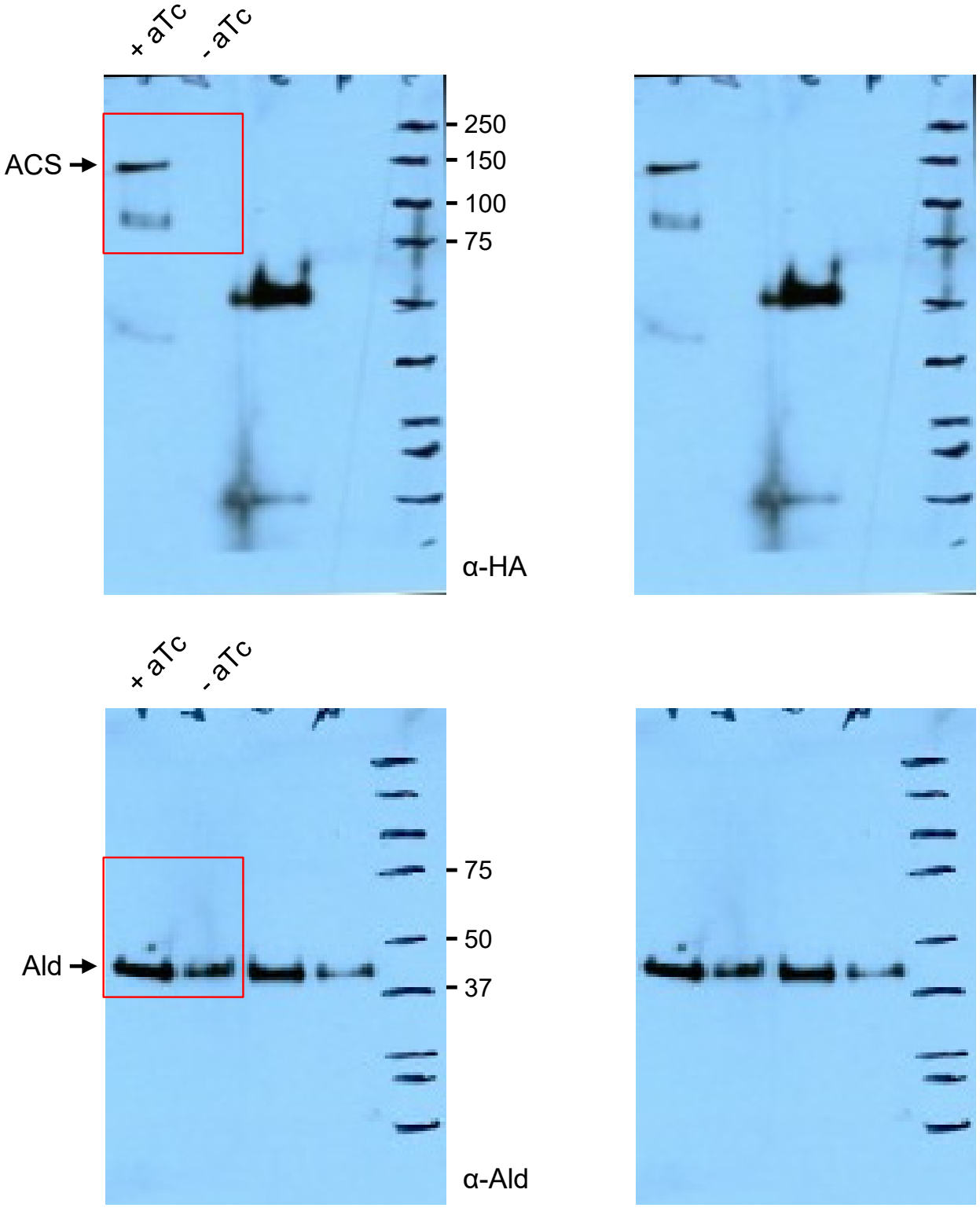

Figure S11

Figure 7-source data. Western blot showing that ACS levels can be regulated with anhydrotetracycline (aTc) in the ↓*acs*/Δ*lipl2* parasite line. After four days of aTc deprivation (-aTc), ACS-HA levels were below the limit of detection. Aldolase (Ald) served as a loading control. The red boxes correspond to the cropped images in **Figure 7D**. The location of Precision Plus Protein Standards (Bio-Rad, #1610373) are marked on each blot image.

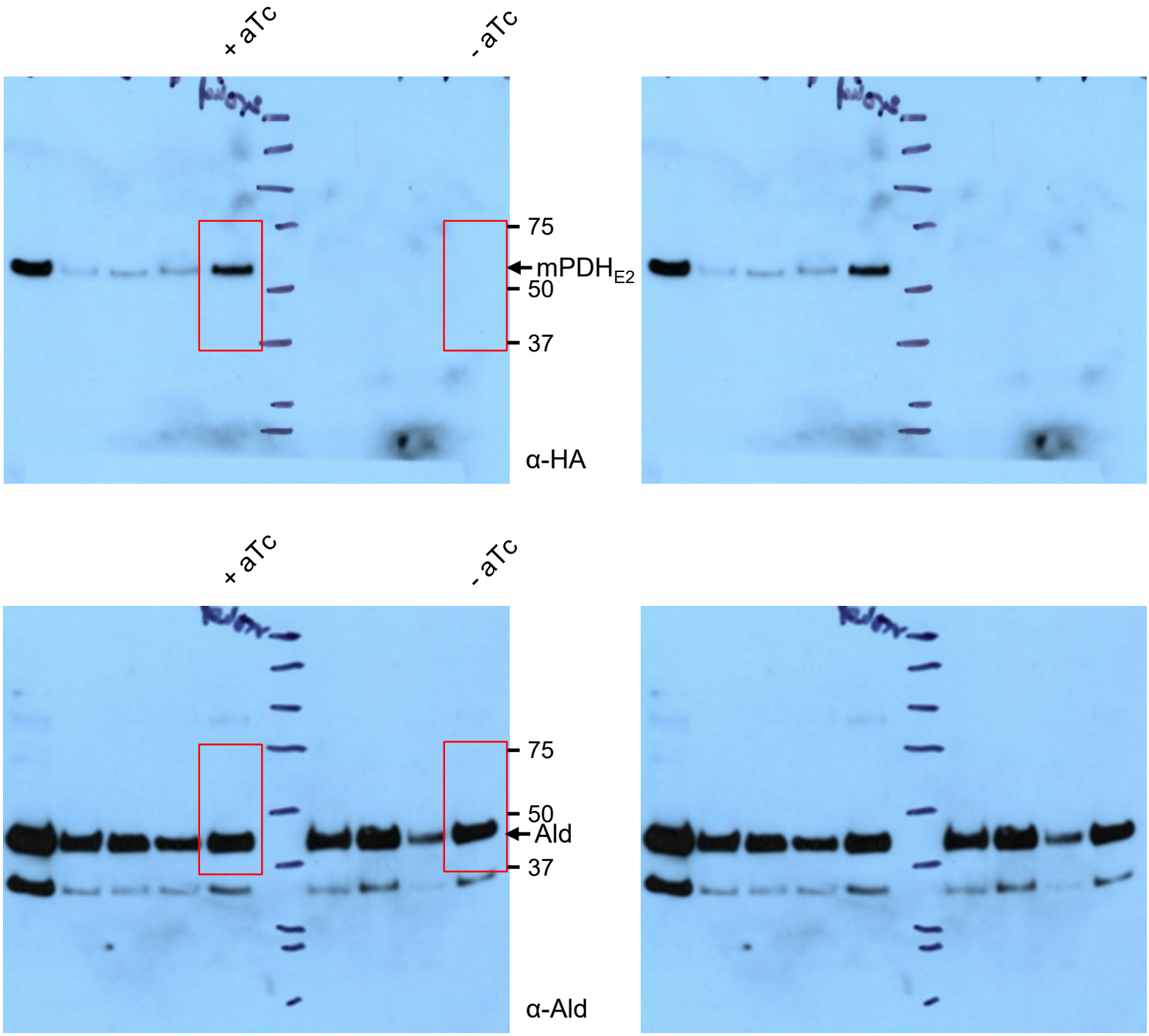

Figure S12

Figure 8-source data. Western blot showing that mPDH$_{E2}$ levels can be regulated with aTc in the ↓p_{E2}/Δk_{E2} line. After four days of aTc deprivation (-aTc), mPDH$_{E2}$-HA levels were below the limit of detection. Aldolase (Ald) served as a loading control. The red boxes correspond to the cropped images in **Figure 8C**. The location of Precision Plus Protein Standards (Bio-Rad, #1610373) are marked on each blot image.

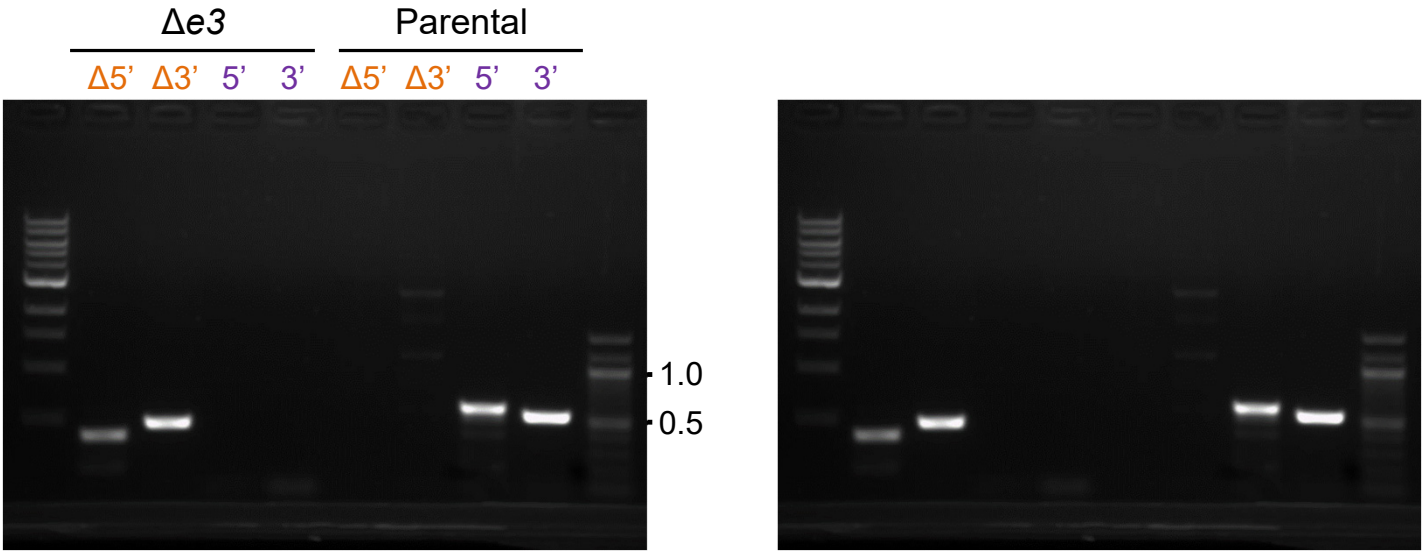

Figure S13

Figure S1-source data. Genotyping PCR reactions confirming the deletion of the *e3* dehydrogenase gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

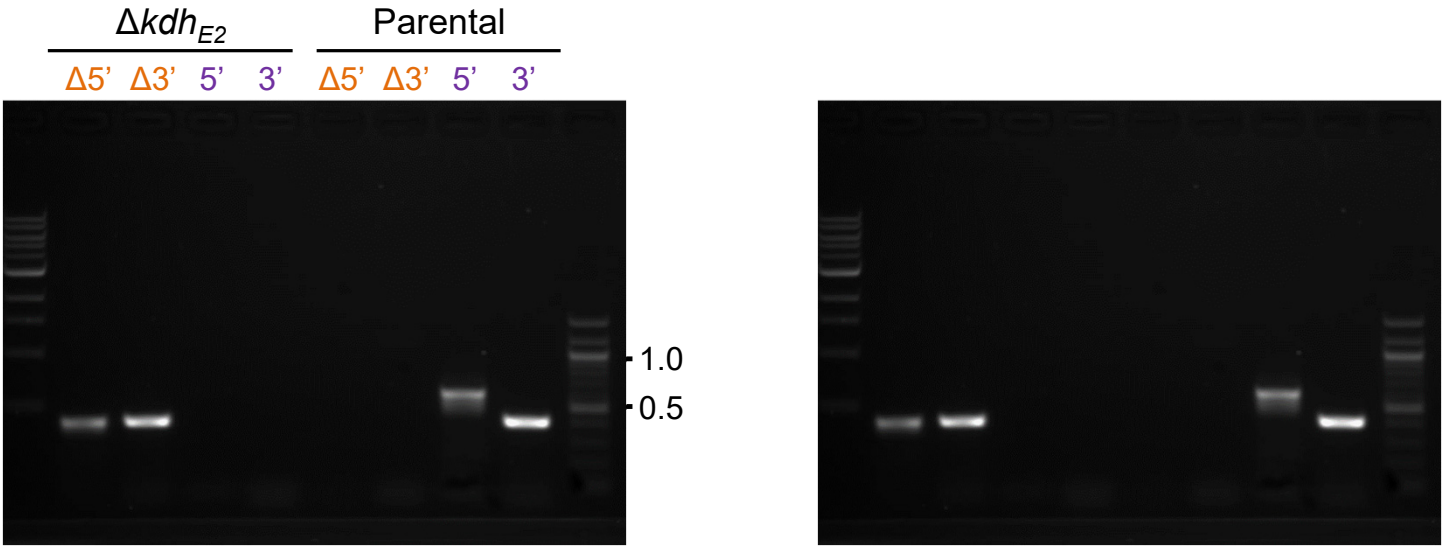

Figure S14

Figure S2-source data. Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

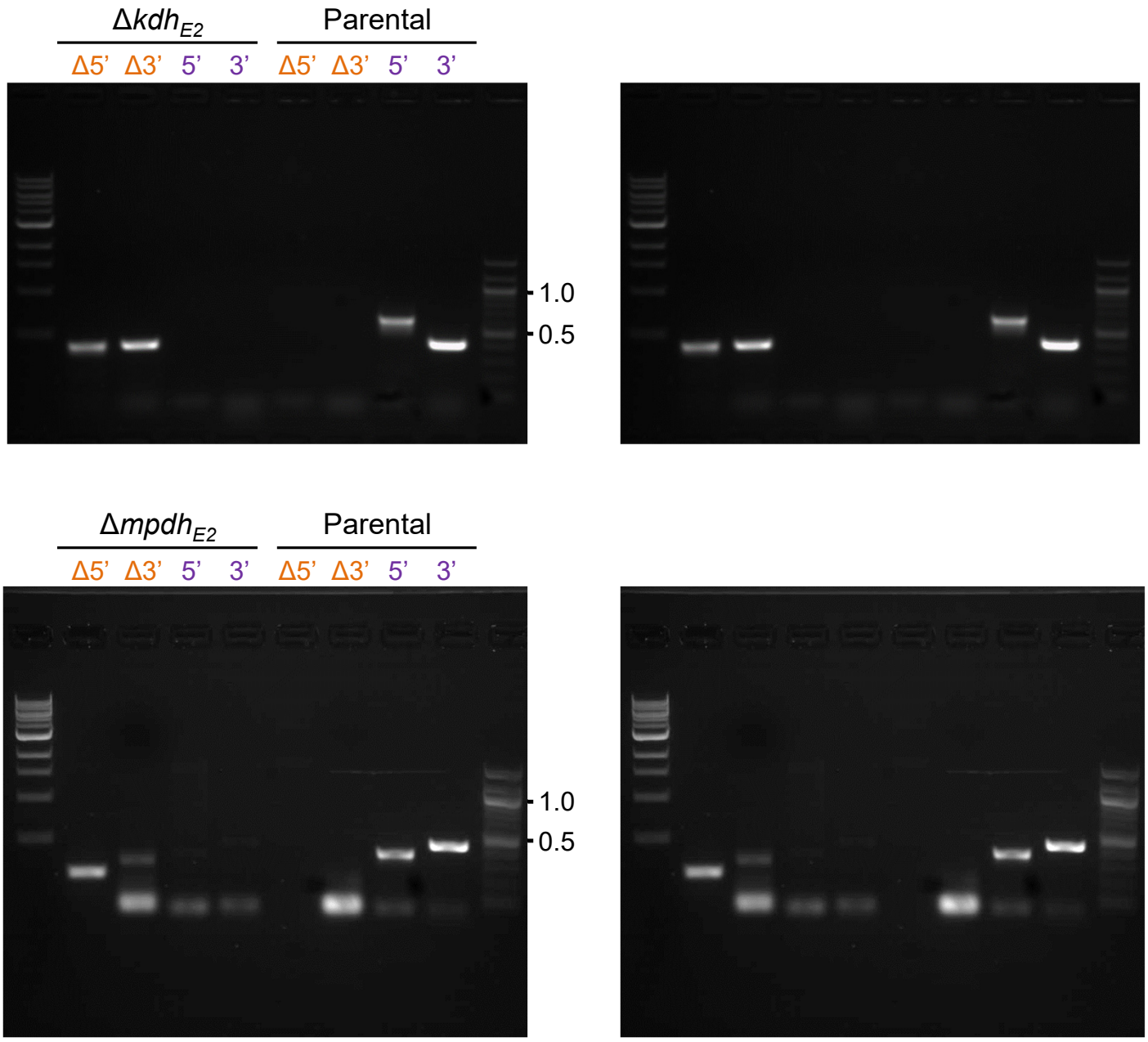

Figure S15

Figure S3-source data. Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ and *mpdh*$_{E2}$ genes. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

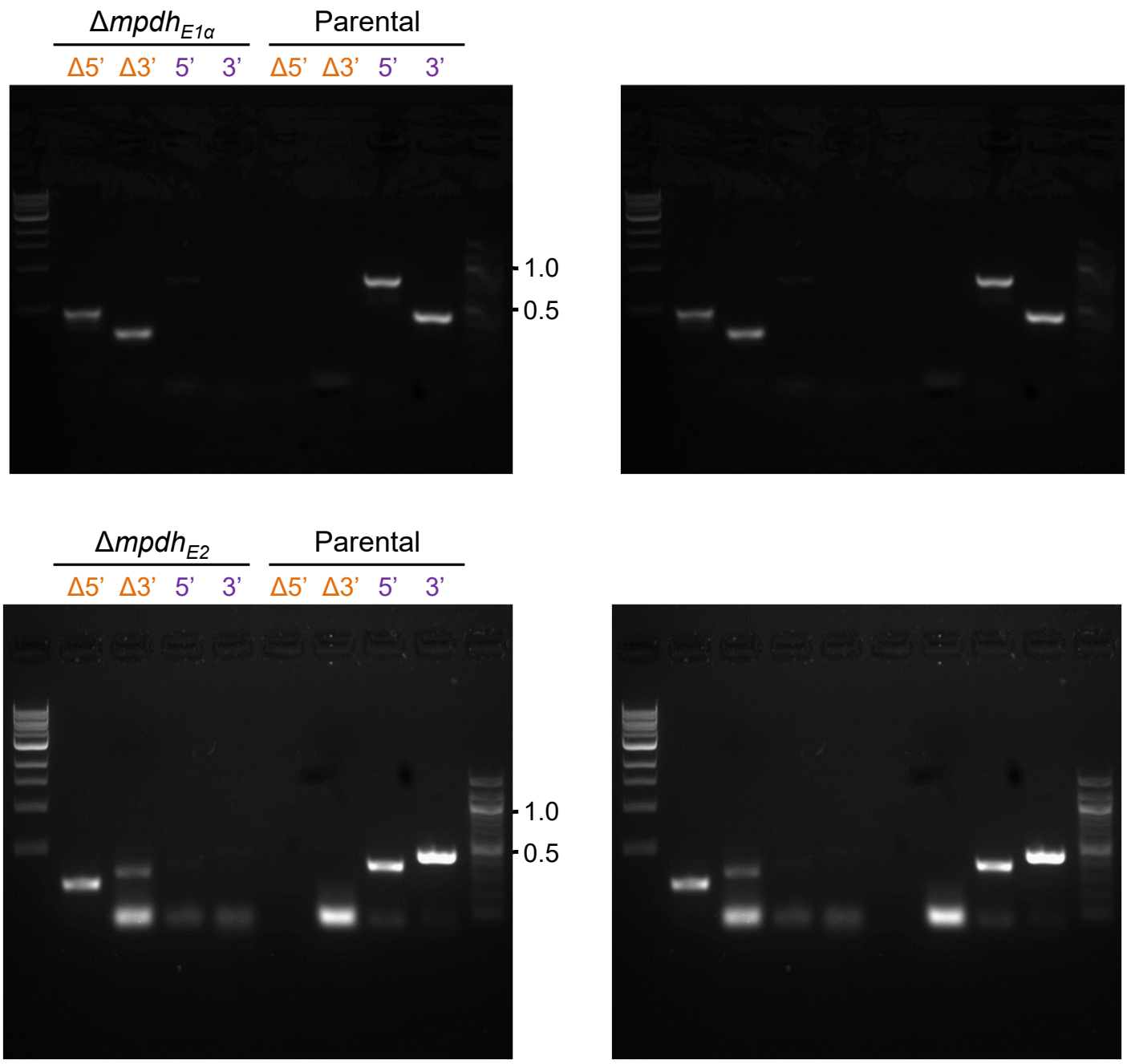

Figure S16

Figure S5-source data. Genotyping PCR reactions confirming the deletion of the *mpdh*$_{E1α}$ and *mpdh*$_{E2}$ genes. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

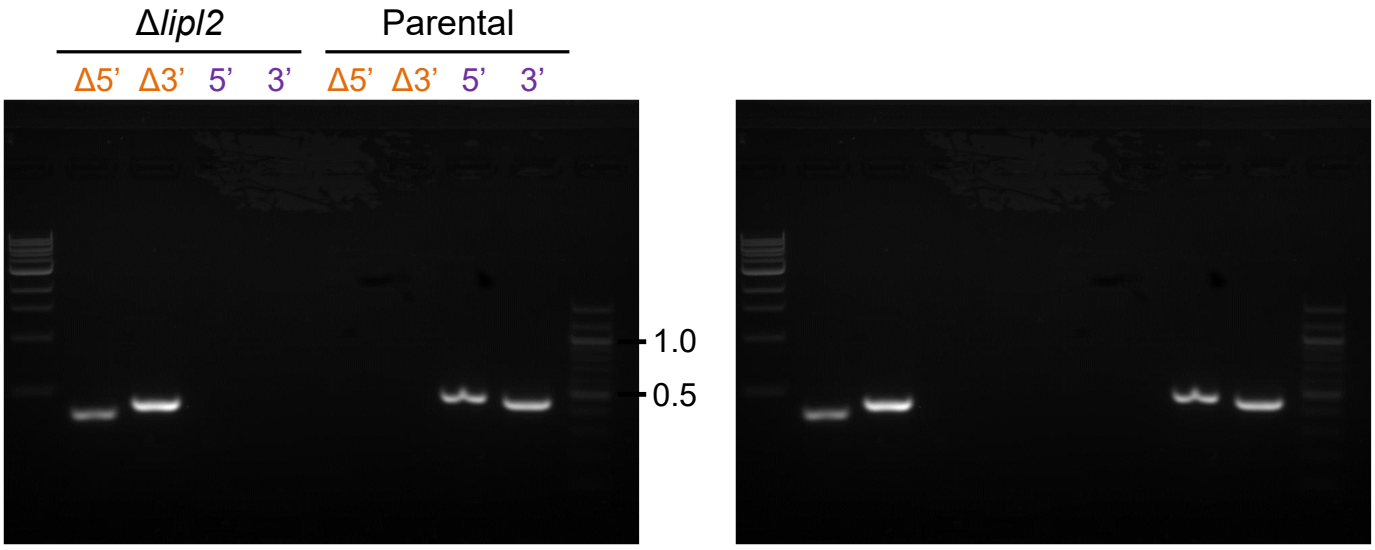

Figure S17

Figure S6-source data. Genotyping PCR reactions confirming the deletion of the *lipl2* gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5’ and Δ3’ loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5’ and 3’ loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**. New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

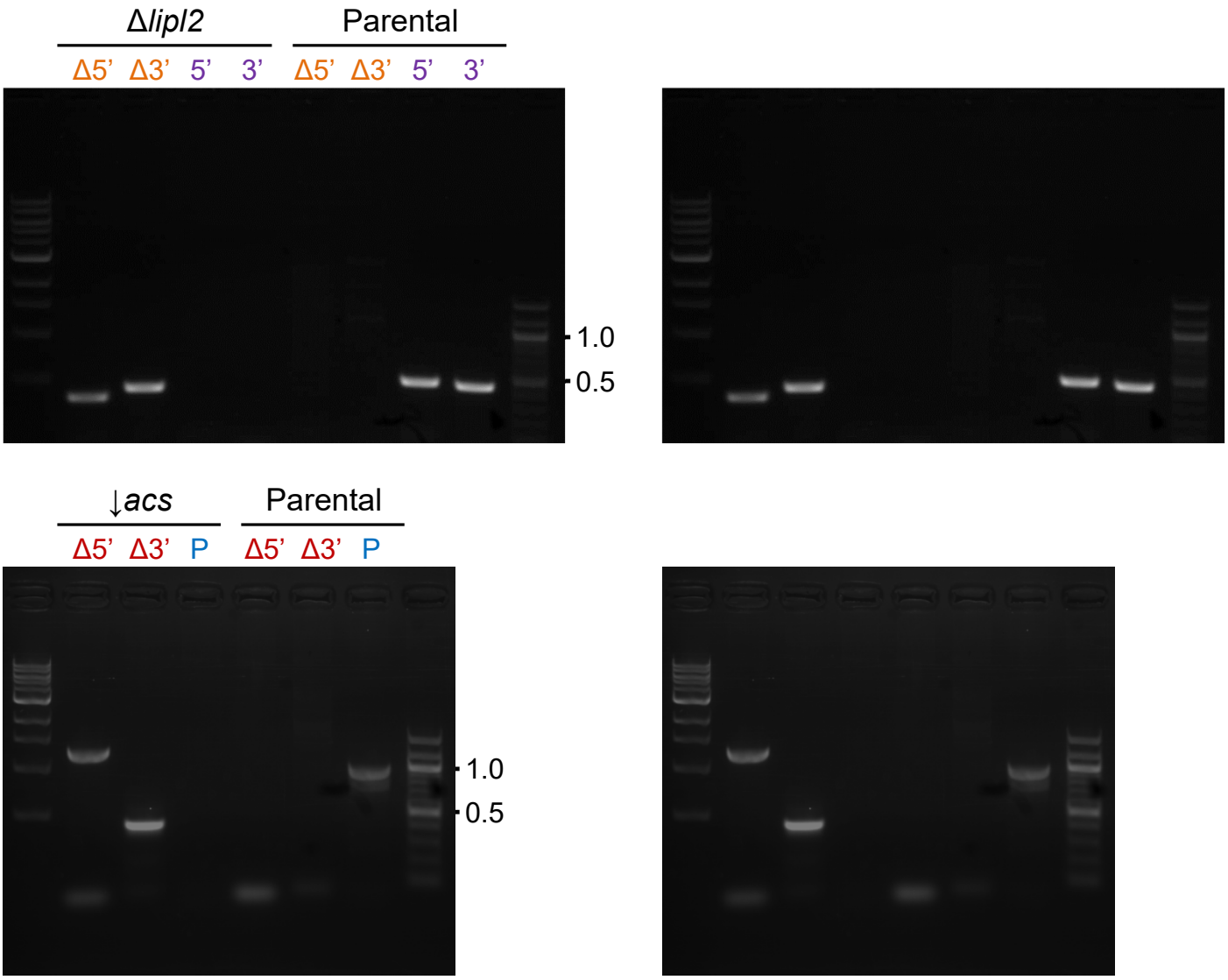

Figure S18

Figure S8-source data. (Top) Genotyping PCR reactions confirming the deletion of the *lipl2* gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**.

(Bottom) Genotyping PCR reactions confirming the modification of the *acs* gene for knockdown experiments. Based on the scheme shown in **Figure S8A**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify parental (P) locus). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1.**

New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

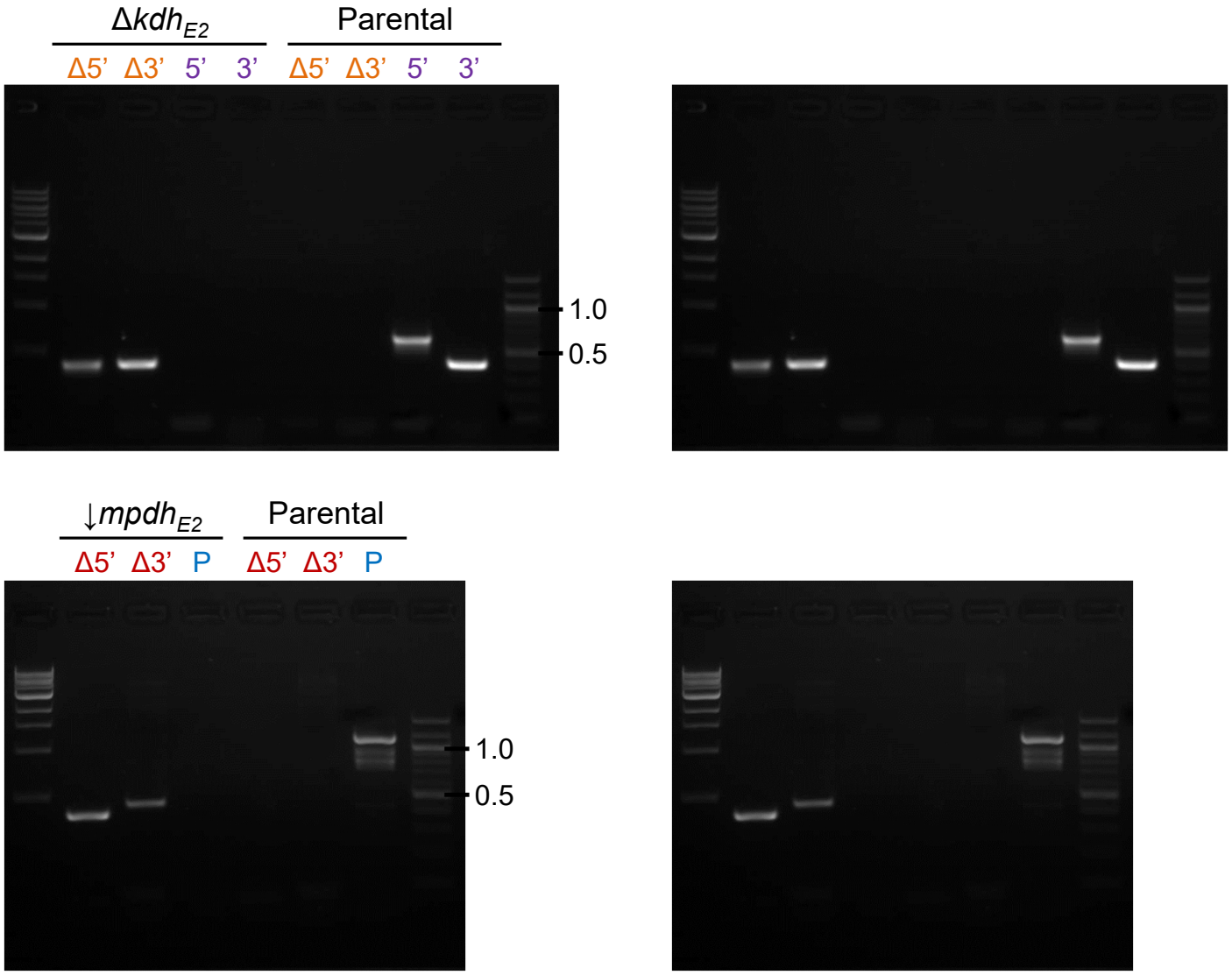

Figure S19

Figure S9-source data. (Top) Genotyping PCR reactions confirming the deletion of the *kdh*$_{E2}$ gene. Based on the scheme shown in **Figure 1C**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify at the wild type 5' and 3' loci). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**.

(Bottom) Genotyping PCR reactions confirming the modification of the *mpdh*$_{E2}$ gene for knockdown experiments. Based on the scheme shown in **Figure S8A**, PCR amplicons demonstrate integration at the Δ5' and Δ3' loci and lack of parental parasites (as indicated by the failure to amplify parental (P) locus). The parental line was used as a control. The primer sequences and amplicon lengths are described in **Dataset S1**.

New England Biolabs 1kb DNA Ladder (#N3232) are shown on the left and NEB 100bp DNA Ladder (#N3231) standards are shown on the right of each gel image.

Table S1. Predicted essentiality of genes based on forward genetic screens.

	P. falciparum PiggyBac[12]			*P. berghei* PlasmoGem[13]		
Gene Name	**Gene ID PF3D7_#**	**Mutagenesis Index Score**	**Mutagenesis Fitness Score**	**Gene ID PBANKA_#**	**Growth Rate**	**Phenotype**
ACS	0627800	**0.17**	-2.84	1126500	0.1	**Essential**
LipL1	1314600	**0.54**	-3.46	1413100	0.03	**Essential**
LipL2	0923600	**0.13**	-3.1	0824500	0.44	**Slow**
KDH_{E1}	0820700	**1**	-1.66	0710100	0.97	**Dispensable**
KDH_{E2}	1320800	**1**	0.4	1419100	1	**Dispensable**
$mPDH_{E1\alpha}$	1312600	**0.2**	-2.74	1411100	0.55	**Slow**
$mPDH_{E1\beta}$	0504600	**1**	-1.66	1104200	0.61	**Slow**
$mPDH_{E2}$	0303700	**0.58**	-3.12	0402300	0.51	**Slow**
E3	1232200	**0.2**	-2.78	1446900	0.83	**Slow**

Green text indicates genes likely to be dispensable and red text indicates genes likely to be essential.

Table S2. Incorporation of ^{13}C-Glucose into protein acetylation sites in NF54 parasites.

^{13}C-Glucose NF54attB	**^{13}C-label detected**			**^{12}C-label detected**		
Experiment	^{13}C-Acetyl Proteins	^{13}C-Acetyl Sites*		^{12}C-Acetyl Proteins	^{12}C-Acetyl Sites*	
1	9	9	(6)	2	2	(2)
2	12	15	(5)	3	3	(2)
3	11	13	(6)	2	2	(2)
Totals	32	37	(17)	7	7	(6)

*Numbers in brackets correspond to acetylation sites that have been previously observed by Cobbold and coworkers.[14]

References

1 Ghorbal, M. *et al.* Genome editing in the human malaria parasite Plasmodium falciparum using the CRISPR-Cas9 system. *Nat Biotechnol* **32**, 819-821, doi:10.1038/nbt.2925 (2014).

2 Rajaram, K., Liu, H. B. & Prigge, S. T. Redesigned TetR-Aptamer System To Control Gene Expression in Plasmodium falciparum. *mSphere* **5**, doi:10.1128/mSphere.00457-20 (2020).

3 Swift, R. P. *et al.* The NTP generating activity of pyruvate kinase II is critical for apicoplast maintenance in Plasmodium falciparum. *Elife* **9**, doi:10.7554/eLife.50807 (2020).

4 Swift, R. P. *et al.* A mevalonate bypass system facilitates elucidation of plastid biology in malaria parasites. *PLoS Pathog* **16**, e1008316, doi:10.1371/journal.ppat.1008316 (2020).

5 Swift, R. P., Rajaram, K., Liu, H. B. & Prigge, S. T. Dephospho-CoA kinase, a nuclear-encoded apicoplast protein, remains active and essential after Plasmodium falciparum apicoplast disruption. *EMBO J* **40**, e107247, doi:10.15252/embj.2020107247 (2021).

6 Ganesan, S. M., Falla, A., Goldfless, S. J., Nasamu, A. S. & Niles, J. C. Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites. *Nat Commun* **7**, 10727, doi:10.1038/ncomms10727 (2016).

7 Keller, A., Nesvizhskii, A. I., Kolker, E. & Aebersold, R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem* **74**, 5383-5392, doi:10.1021/ac025747h (2002).

8 Nesvizhskii, A. I., Keller, A., Kolker, E. & Aebersold, R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem* **75**, 4646-4658, doi:10.1021/ac0341261 (2003).

9 Schalkwijk, J. *et al.* Antimalarial pantothenamide metabolites target acetyl-coenzyme A biosynthesis in Plasmodium falciparum. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aas9917 (2019).

10 Hsiao, J. J., Potter, O. G., Chu, T. W. & Yin, H. Improved LC/MS Methods for the Analysis of Metal-Sensitive Analytes Using Medronic Acid as a Mobile Phase Additive. *Anal Chem* **90**, 9457-9464, doi:10.1021/acs.analchem.8b02100 (2018).

11 Agrawal, S. *et al.* El-MAVEN: A Fast, Robust, and User-Friendly Mass Spectrometry Data Processing Engine for Metabolomics. *Methods Mol Biol* **1978**, 301-321, doi:10.1007/978-1-4939-9236-2_19 (2019).

12 Zhang, M. *et al.* Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. *Science* **360**, doi:10.1126/science.aap7847 (2018).

13 Bushell, E. *et al.* Functional Profiling of a Plasmodium Genome Reveals an Abundance of Essential Genes. *Cell* **170**, 260-272 e268, doi:10.1016/j.cell.2017.06.030 (2017).

14 Cobbold, S. A., Santos, J. M., Ochoa, A., Perlman, D. H. & Llinas, M. Proteome-wide analysis reveals widespread lysine acetylation of major protein complexes in the malaria parasite. *Sci Rep* **6**, 19722, doi:10.1038/srep19722 (2016).