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*Plasmodium Para*-Aminobenzoate Synthesis and Salvage Resolve Avoidance of Folate Competition and Adaptation to Host Diet

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Plasmodium Para-Aminobenzoate Synthesis and Salvage Resolve Avoidance of Folate Competition and Adaptation to Host Diet

Graphical Abstract

Highlights

- *Plasmodium* parasites harbor their own pABA biosynthesis pathway
- Combined absence of pABA synthesis and salvage is lethal to parasite blood stages
- Import of pABA, not folate, is physiological for blood-stage parasites
- *Plasmodium* exploits alternative folate acquisition during life cycle progression

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In Brief

Matz et al. present an integrated approach of experimental genetics and nutritional metabolite restriction to demonstrate two complementary folate acquisition strategies of the malaria parasite in the blood: endogenous synthesis and salvage of the folate precursor pABA. Uptake of pABA, and not folate, is physiological, facilitating avoidance of host-parasite competition.
**Plasmodium Para-Aminobenzoate Synthesis and Salvage Resolve Avoidance of Folate Competition and Adaptation to Host Diet**

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**SUMMARY**

Folate metabolism is essential for DNA synthesis and a validated drug target in fast-growing cell populations, including tumors and malaria parasites. Genome data suggest that *Plasmodium* has retained its capacity to generate folates *de novo*. However, the metabolic plasticity of folate uptake and biosynthesis by the malaria parasite remains unresolved. Here, we demonstrate that *Plasmodium* uses an aminodeoxychorismate synthase and an aminodeoxychorismate lyase to promote the biogenesis of the central folate precursor para-aminobenzoate (pABA) in the cytoplasm. We show that the parasite depends on *de novo* folate synthesis only when dietary intake of pABA by the mammalian host is restricted and that only pABA, rather than fully formed folate, is taken up efficiently. This adaptation, which readily adjusts infection to highly variable pABA levels in the mammalian diet, is specific to blood stages and may have evolved to avoid folate competition between the parasite and its host.

**INTRODUCTION**

Malaria parasites replicate at high rates in the mammalian bloodstream. Consequently, they require large quantities of folate and folate precursors, which they scavenge from the serum or synthesize *de novo* (Krungkrai et al., 1989; Müller and Hyde, 2013). In contrast, the mammalian host strictly depends on the dietary uptake of preformed folates. Folates promote essential functions during one-carbon transfer reactions and consist of three major moieties: para-aminobenzoate (pABA), pterin, and glutamate. The growth-inhibitory effects of antifolates targeting the enzymes dihydropteroate synthase (DHPS) and dihydrofolic reductase-thymidylate synthase (DHFR-TS) have been exploited for antimalarial therapy (Nzila, 2006). Due to the emergence of antifolate resistance, these enzymes have received considerable attention (Gregson and Plowe, 2005; Heinberg and Kirkman, 2015). For therapeutic approaches, however, the enzymes promoting biogenesis of the central folate precursor pABA have been neglected.

It is believed that chorismate, which is produced via the shikimate pathway (Roberts et al., 1998), is converted to pABA in a two-step reaction (Figure 1A). A potential aminodeoxychorismate synthase (ADCS, PBANKA_0823300) and an aminodeoxychorismate lyase (ADCL, PBANKA_1322100) are predicted by *Plasmodium* genome sequences (Magnani et al., 2013; Saucedo-Sora and Ward, 2013; Triglia and Cowman, 1999). However, their roles in *Plasmodium* folate metabolism during infection remain unexplored.

Here, we investigate the function of ADCS and ADCL in folate biogenesis. Using a combination of reverse genetics and defined host nutrition, we provide evidence for two complementary pathways: *de novo* biosynthesis and scavenging of pABA. Facultative auxotrophy of pABA during blood infection thus exemplifies the metabolic flexibility of the malaria parasite *in vivo*.

**RESULTS AND DISCUSSION**

**Experimental Genetics of the pABA Synthesis Pathway**

We initiated our analysis by assessing ADCS and ADCL expression throughout the parasite life cycle, using the murine malaria model parasite *P. berghei*. Transgenic parasite lines expressing the endogenous genes fused to mCherry-3xMyc were generated (Figures S1A and S1B) and analyzed by live fluorescence microscopy. Co-localization with a cytosolic GFP marker revealed that both enzymes localize to the parasite cytoplasm (Figure 1B). ADCS expression was restricted to blood stage trophozoites and liver stages, but remained below the detection limit in rings, in schizonts, and throughout mosquito infection. This finding indicates distinct roles for ADCS in the mammalian host only. In contrast, ADCL was ubiquitously expressed during life cycle progression (Figure 1B).

We next assessed the importance of both enzymes by targeted gene disruption and were successful in generating mutants lacking either ADCS or ADCL, as indicated by diagnostic PCR and Southern blot analysis (Figures 1C, S1C, and S1D).
**Figure 1. Experimental Genetics of the Plasmodium pABA Biosynthesis Pathway**

(A) pABA biosynthesis by the malaria parasite. Conversion of chorismate into aminodeoxychorismate (ADC) is catalyzed by ADC synthase (ADCS). ADC is converted into para-aminobenzoate (pABA) by ADC lyase (ADCL).

(B) ADCS and ADCL are cytoplasmic proteins. Transgenic *P. berghei* parasites expressing mCherry-3xMyc-tagged ADCS or ADCL and a cytoplasmic GFP marker (Kooij et al., 2012) were passed through the life cycle and imaged live during asexual blood stages, gametogenesis (exflagellating male, egressed female in inset), the mosquito oocyst stage, and the liver stage of infection. Shown are the signals of the tagged proteins (red, left), the cytoplasmic GFP marker (green, center left), a merge of both signals (center right), and a merge of differential interference contrast images (DIC) with Hoechst 33342 nuclear dye (DNA, blue, right). The DNA of male gametocytes was not stained due to interference of the dye with exflagellation. Bars, 5 μm.

(C) Targeted gene deletion of *ADCS* and *ADCL*. Diagnostic PCRs of the WT locus (left) and of the drug-selected and isolated parasites (right) using primer combinations indicated in Figure S1C and Table S1. Green frames indicate successful gene deletion.
Plasmodium Blood Stages Depend on Host-Derived Nutritional pABA Only upon Loss of Endogenous Synthesis

Blood propagation of $adcs^-\text{ parasites was compared to wild-type (WT)}$ in mice fed with conventional rodent diet using an intravitral competition assay (Matz et al., 2013). Parasite growth remained unaltered in the absence of ADCS (Figure 2A), suggestive of efficient salvage of folate or folate precursors, such as $pABA$. For this reason, we restricted $pABA$ levels by providing the host with a diet based on spray-dried whole-milk powder. Metabolite quantification by ultra-performance liquid chromatography/Orbitrap-mass spectrometry (UPLC/Orbitrap-MS) confirmed that $pABA$ is abundant in conventional mouse feed and absent from milk (Figures 2B and S2B). Moreover, conventional diet is fortified with high levels of the artificial folate analog folic acid (FA), which is absent from milk. In contrast to conventional feed, milk
diet contained considerable levels of the naturally occurring 5-methyltetrahydrofolate (5-MTHF; Figures 2B and S2B). In the liver, pABA is predominantly converted to three derivatives by either acetylation or glycine conjugation (Figure S2A; Song and Hsu, 1996). We measured the levels of two pABA metabolites, para-acetamidobenzoate (pACBA) and para-aminohippurate (pAHA), in the mouse serum. Although pAHA remained undetectable, pACBA levels were high only in mice fed with conventional diet (Figure S2C).

In milk-fed mice adcs− parasite propagation was severely impaired, whereas WT parasites displayed normal growth (Figure 2C). This suggests that Plasmodium can compensate for the absence of dietary pABA by ADCS-dependent de novo biosynthesis and that parasite propagation is only arrested in the combined absence of pABA salvage and endogenous synthesis. This phenotype was recapitulated by an independent adcs− parasite line lacking the drug-selectable cassette, thus ruling out interference of the DHFR resistance marker (Figures S1C and S1E–S1G). Upon pABA supplementation via the drinking water, adcs− growth was fully restored, and mutant parasites grew indistinguishable from WT in milk-fed mice (Figure 2D). Moreover, blood propagation of WT parasites was largely independent of dietary pABA, which is indicative of sufficient rates of de novo pABA synthesis (Figure 2E). Our findings are congruent with previous observations that pABA antagonizes the efficacy of antifolate drugs, including sulfadoxine, a structural pABA analog and competitive inhibitor of DHPS (Milhous et al., 1985; Watkins et al., 1985).

We also compared blood infection of transgenic parasites lacking the second pABA synthesis enzyme, ADCL, in mice receiving different diets. adcl− parasites also depend on nutritional pABA, demonstrating that both enzymes, ADCS and ADCL, promote pABA biogenesis during blood infection (Figure 2F). The ubiquitous expression of ADCL suggests additional functions, especially during mosquito stages (Figure 1B). A previous study demonstrated a substantial secondary amino-transferase activity of Plasmodium falciparum ADCL, which was suggested as contributing to the detoxification of exogenous o-amino acids (Magnani et al., 2013).

We also quantified tagged ADCS by live fluorescence microscopy to test for a potential regulation of expression in the absence of pABA (Figure S3). We demonstrated that steady-state levels of ADCS remain constant irrespective of dietary pABA availability.

Continued pABA restriction led to the retraction of adcs− parasitemia from day 12 onward, which was immediately reversed upon addition of pABA on day 18 (Figure 2G). During more extended periods of pABA deficiency, adcs− parasitemia dropped below the detection threshold, followed by alternating periods of re-emergence and retraction, indicative of latent malaria infection (Figure 2H). We propose that adcs− survival is due to the inefficient utilization of alternate folate sources or to parasite hibernation, as described previously for P. falciparum under amino acid-depleted conditions (Babbitt et al., 2012). Upon pABA resupply 2 months later, adcs− parasites continued their cycles of emergence and retraction, which is indicative of parasite tolerance rather than sterile protection (Figure 2H).

The persistence of latent infection precludes the combined use of ADCS or ADCL targeting and pABA-free diet as a viable pharmacological strategy or for experimental blood stage immunizations. Our observations also warrant a cautionary note for the development of attenuated blood stage vaccine strains. Although the murine immune system was exposed to large parasite quantities for >80 days, and despite eliciting robust anti-parasitic antibodies (Figure 2I), infection with adcs− parasites was not resolved, indicating no sterile protection against asexual P. berghei blood stages.

**Metabolite Profiling Identifies pABA Dependency of Plasmodium Blood Stages**

pABA supplementation correlated with elevated pACBA levels in the serum, which is indicative of efficient ingestion, resorption, and biocconversion of pABA by the animals (Figures S2C and S2D). Phenotypic rescue of adcs− parasites in milk-fed animals was also observed upon supplementation with 5-MTHF (Figure 3A). However, 5-MTHF was rapidly degraded to glutamated pABA (para-aminobenzoylglutamate [pABG]) in the drinking water, suggesting that restored adcs− growth was due to the uptake of pABA-related degradation products (Figure 3B). Indeed, 5-MTHF supplementation also led to a significant increase in serum pACBA levels (Figures S2C and S2D). Furthermore, it was previously demonstrated that P. falciparum can form pteroylglutamate from pABG, which is suggestive of efficient uptake and utilization by the parasite (Krugkrai et al., 1989).

To prevent folate degradation, we used high concentrations of the stable folate analog FA (Figure 3B). adcs− growth was not rescued by supplementation with 1.3 mg/kg FA (Figure 3C). Excessive FA concentrations, as found in fortified mouse feed (10 mg/kg), improved blood stage development to a certain degree, but did not fully restore WT-like growth, indicating highly inefficient uptake of folate by the parasite (Figure 3D). This finding is in good agreement with the considerable levels of 5-MTHF naturally occurring in milk diet, which could not sustain efficient adcs− parasite growth (Figure 2). Supplementation with FA also led to elevated pACBA levels in the serum (Figures S2C and S2D), indicative of folate catabolism. Since exogenous FA did not restore adcs− growth, it is clear that pACBA is not an important substrate for Plasmodium. Serum pACBA levels must therefore be regarded as a proxy for efficient metabolite conversion in vivo, while the physiological substrate for the parasite remains to be determined. Our metabolic data indicate that pABA and its metabolites rather than folate are salvaged from the host.

We show that de novo synthesis and salvage from the serum are complementary strategies, which can each fully satisfy the pABA requirements of the parasite, thus indicating challenges for the therapeutic exploitation of parasite pABA acquisition. We provide evidence that the uptake of folate, as opposed to folate precursors, is of negligible importance for parasite development in vivo. Inefficient folate salvage by the parasite is a fundamental prerequisite for the efficacy of antifolate drugs and provides a possible explanation for the inconsistent reports about antifolate treatment failure upon food fortification with FA (Kupka, 2015).

Previous studies report large effects of pABA-free diet on the growth and survival of different non-human primate and rodent
malaria parasites, including *P. berghei*, suggestive of a hard-wired metabolic reliance on pABA salvage (Hawking, 1954; Jacobs, 1964; Kicska et al., 2003; Maegraith et al., 1952; Nowell, 1970). In marked contrast, our own results show that WT growth is largely independent of dietary pABA, in good agreement with our experimental genetics data. Growth attenuation was only detected in adcs−-infected mice when fed on milk diet. We note that we validated dietary pABA restriction by metabolite quantification of the milk diet and the respective mouse sera (Figures 2B and S2B–S2D). We cannot formally exclude that differences in pABA dependency may exist between different *Plasmodium* species, but the discrepancies between our own and previous work with the same murine model parasite *P. berghei* remain puzzling. It was previously noted that the WT growth phenotype in the absence of dietary pABA is rather variable in *P. berghei* mouse infections and may depend on the parasite strain (Nowell, 1970). Contrasting phenotypes could also be attributed to the circumstances of animal husbandry, which are known to influence the composition of the gut microbiome (Franklin et al., 2017; Rausch et al., 2016). The bacterial community of the digestive tract of the mouse can shape the outcome of malarial infections via a modulation of the animal’s inflammatory responses (Villarino et al., 2016). Presumably, previous breeding and rearing conditions differed significantly from today’s standards and may thus have affected the microbiota and in consequence the metabolic homeostasis, immune status, and susceptibility of the host toward malaria infection.

Based on our data, we propose that the notion of reduced risk and severity of malaria in breast-fed infants (Brazeau et al., 2016; Murray et al., 1978) cannot be attributed to a pABA-deficient diet but to additional factors, such as slower parasite replication in erythrocytes harboring fetal hemoglobin and the presence of maternal antibodies.

### Plasmodium Exploits Alternative Folate Acquisition during Mosquito and Liver Infection

Next, we investigated the contributions of pABA synthesis and salvage during life cycle progression. WT and adcs− parasites were co-injected into normally fed mice, allowing for efficient blood stage growth and gametocytogenesis, and were then transmitted to *Anopheles stephensi* mosquitoes in competition. Oocyst and sporozoite numbers remained unaltered in the absence of ADCS, independent of pABA supplementation via the mosquito feeding solution (Figures 4A and 4B). We further restricted the carryover of pABA metabolites during the blood meal with the use of milk-fed donor mice. This strategy inevitably led to very low adcs− oocyst densities, but it had no effect on growth or sporulation (Figures 4C–4E), suggesting that other folate-related metabolites from the blood meal promote mosquito stage development. Our findings are in perfect agreement with a previous study showing that *P. falciparum* oocyst infection rates and densities are independent of pABA supplementation in *A. stephensi* and *A. gambiae* (Beier et al., 1994). An attractive hypothesis is that folate requirements may be less stringent during the slow replication in the mosquito vector, as opposed to rapid proliferation in the mammalian host.

Next, we inoculated hepatocytes with WT and adcs− sporozoites and assessed liver stage development *in vitro*. Cells were grown in pABA-free medium containing dialyzed fetal bovine serum and FA for hepatocyte survival. Parasite morphology, size, and quantity remained unaltered in the absence of ADCS and did not improve upon pABA supplementation (Figures 4F and S4). We then assessed intrahepatic growth *in vivo* by injecting sporozoites into milk-fed mice in the absence or presence of dietary pABA. The duration of liver stage development was independent of ADCS and pABA, as determined by the time of blood stage patentcy (Figure 4G). However, quantification of first-generation
Figure 4. Plasmodium Life Cycle Progression Remains Unaltered in the Absence of ADCS

(A and B) Normally fed mice were co-injected with equal numbers of mCherry-fluorescent Berred WT and GFP-fluorescent adcs−/− blood stage parasites and fed to Anopheles stephensi mosquitoes. + and −, presence or absence of pABA in the mosquito feeding solution.

(A) Quantification of oocysts and sporozoites. Shown is the mean percentage of all single-fluorescent parasites ± SDs. Parasites displaying dual mCherry and GFP fluorescence as a result of cross-fertilization were excluded from analysis. n.s., non-significant; WT versus adcs−/−, paired t test; + versus −, Student’s t test; n = 3.

(B) Infected mosquito midguts 10 days after the blood meal. Shown is a merge of mCherry (WT, red), GFP (adcs−/−, green), and differential interference contrast (DIC) images (DIC). Bar, 100 μm.

(C–E) Normal oocyst maturation in the absence of ADCS and pABA. Parasites were transmitted from milk-fed mice to restrict pABA carryover during the blood meal.

(C) Oocyst size on day 11 after the blood meal. Lines, mean values. n.s., non-significant; one-way ANOVA, Tukey’s multiple comparison test; n = 50 oocysts from 5 mosquitoes.

(D) Morphology of 11-day-old oocysts. Shown is a merge, as described above. Bar, 10 μm.

(E) Oocyst sporulation on day 14. Mean values ± SDs are shown. n.s., non-significant; one-way ANOVA, Tukey’s multiple comparison test; n = 10 infected midguts.

(F) Quantification of liver stage parasites in vitro. Hepatoma cells were co-infected with WT and adcs−/− sporozoites and imaged 24, 48, and 65 hr after infection. + and −, presence or absence of pABA in the culture medium. Shown is the mean percentage of all parasites ± SDs. n.s., non-significant; WT versus adcs−/−, paired t test; + versus −, Student’s t test; n = 3. See also Figure S4.

(G) Time to development of patent blood infection. Milk-fed mice were injected with equal numbers of WT and adcs−/− sporozoites. Peripheral blood was monitored daily by flow cytometry. n.s., non-significant; Mantel-Cox test; n = 7 mice from 3 independent experiments.

(H and I) Beneficial effect of dietary pABA on the first obligate parasite expansion phase in the liver. + and −, presence or absence of pABA in the mouse drinking water.

(legend continued on next page)
blood stage parasitemia and of parasite transcript abundance in infected livers suggests a 3- to 5-fold fitness cost in the absence of pABA, which is indicative of a growth-stimulatory function during liver stage development in vivo. The lack of ADCS expression caused no significant defects during this stage (Figures 4H and 4I). Since the liver is a major folate pool (Lin et al., 2004), we hypothesize that liver stage survival may be due to the salvage of preformed folates in the absence of ADCS. Optimized folate concentrations in the culture medium may thus account for the discrepancies in pABA dependency observed in vitro and in vivo.

It remains to be determined whether the pABA requirements of human malaria pathogens during intrahepatic growth are comparable to those of P. berghei, especially due to their significantly longer development times in the liver. If confirmed by experimental data, then differential requirements of folate building blocks in different host tissues may signify opportunities for stage-specific antimalarial intervention strategies.

Our results reveal critical roles of Plasmodium ADCS and ADCL in endogenous folate synthesis during food-mediated pABA scarcity in the mammalian host, but facultative pABA auxotrophy is restricted to blood infection. Thus, by using two complementary pathways, pABA salvage and endogenous biosynthesis, Plasmodium elegantly solves its metabolic dependence on this central folate precursor. We conclude that the uptake of pABA and its derivatives, and not of folate, promotes blood stage growth in vivo. Since pABA is metabolically irrelevant to mammals, its salvage may serve to prevent folate competition between the malaria parasite and its host, thereby significantly contributing to their shared biological fitness.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.12.062.

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AUTHOR CONTRIBUTIONS

This work was conceived by J.M.M. and K.M. J.M.M. and M.F. performed the P. berghei experiments. M.W., T.T., and R.H. performed and analyzed the UPLC/Orbitrap-MS measurements. J.M.M. and K.M. wrote the manuscript with input from all of the authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


# STAR★METHODS

## KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joachim Michael Matz (joachim.michael.matz@hu-berlin.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
This study was carried out in strict accordance with the German ‘Tierschutzgesetz in der Fassung vom 22. Juli 2009’ and the Directive 2010/63/EU of the European Parliament and Council ‘On the protection of animals used for scientific purposes’. The protocol was approved by the ethics committee of the Berlin state authority (‘Landesamt für Gesundheit und Soziales Berlin’, permit number G0294/15).

C57BL/6NCrl mice were used for sporozoite infections. All other parasite infections were conducted with Crl:NMRI(Han) mice. Experimental groups consisted of 8-12 weeks old female litter mates, which were housed together in groups of 3 to 6 animals in filter top cages (Tecniplast, Buguggiate, Italy). The cages were bedded with aspen wood chips (LTE E-002, Abedd, Vienna, Austria) and enriched with plastic tunnels (Ehret, Emmendingen, Germany). Mice were kept at room temperature under a 12 h light / 12 h dark cycle at a constant relative humidity of 45%. For information regarding mouse diet, see Method Details.

Mosquitoes
A. stephensi larvae were reared in water containing 0.1% NaCl and were fed with dry cat food pellets (Brekkies, Affinity Petcare S.A, Barcelona, Spain). 200-400 pupae were collected per mosquito cage. Infected mice were fed to the adult mosquitoes 7-10 days after hatching. For information regarding mosquito feeding solutions, see Method Details.

Parasites
All parasite strains used in this study are derived from transfection of P. berghei ANKA cl15cy1. For more information on parasite infections, see Method Details.
METHOD DETAILS

Transfection Constructs
Constructs for endogenous tagging of ADCS and ADCL with mCherry-3xMyc were designed for single homologous integration (Figure S1A). The sequences directly upstream of the stop codons were amplified from genomic DNA (1,053 and 1,663 bp, respectively) and inserted into the pBAT vector (Kooij et al., 2012) using HpaI in combination with EcoRI or NgoMIV. For the generation of the ADCS deletion construct, the 3’ fragment of the gene was amplified from genomic DNA (1,185 bp) and cloned into the pBAT vector using the XhoI and KpnI restriction sites. Subsequently, the 5’ fragment (508 bp) was amplified and cloned into the intermediate vectors using SacI in combination with HpaI. Prior to transfection, the ADCS knockout vector was linearized with Sall. Deletion of ADCL was performed with the respective NotI-digested PlasmoGEM vector (Schwach et al., 2015). Internal restriction sites were used for linearization of the tagging vectors.

Recombinant Parasite Lines
We used advanced experimental genetic techniques to generate all recombinant parasite lines (Matz and Kooij, 2015). Purified WT schizonts were transfected with 5 µg DNA using the Nucleofector™ 2b Device and the Human T Cell Nucleofector™ Kit (Lonza, Basel, Switzerland; Janse et al., 2006). Transfected parasites were injected intravenously into naïve NMRI mice, and selected in vivo with pyrimethamine-containing drinking water (70 mg/l; MP Biomedicals, Santa Ana, CA, USA). Isogenic ADCS knockout mutants were isolated by fluorescence-activated cell sorting (Kenthirapalan et al., 2012). ADCL-deficient parasites were cloned by limiting dilution.

To validate correct integration of the transfection vectors and absence of remaining WT parasites, primer combinations were used as indicated in Figures S1A and S1C and Table S1. Southern blot analysis using the PCR DIG Probe Synthesis kit and the DIG Luminescent Detection kit (Roche, Rotkreuz, Switzerland) was performed according to the manufacturer’s instructions to confirm the correct genotype of the adcs− parasite line. The probe was amplified with primers that were used to generate the 5’ integration sequence of the ADCS deletion construct (see Table S1) and hybridized to XmnI restriction-digested genomic DNA.

The adcs− parasites were also subjected to in vivo negative selection with 5-Fluorocytosine-containing drinking water (1 g/l; Sigma-Aldrich, St. Louis, MO, USA) and to subsequent cloning by limiting dilution. Loss of the drug-selectable cassette was confirmed by diagnostic PCR using the primer combinations indicated in Figure S2C.

Live Cell Imaging
In order to assess protein expression, transgenic parasites expressing mCherry-3xMyc-tagged proteins were passed through the parasite life cycle and imaged live. Fluorescence imaging was performed with a Zeiss AxioObserver Z1 epifluorescence microscope equipped with a Zeiss AxioCam MRm camera (Zeiss, Oberkochen, Germany).

For the detection of anti-parasitic antibodies, 0.5 to 1 mL of blood was collected by cardiac puncture and cellular components and serum were separated by centrifugation. Sera of adcs−-infected mice were pooled. Immunofluorescence analysis was performed at room temperature by incubating live Berred WT-infected erythrocytes in 100% mouse serum containing 1:500 Alexa Fluor® 488-coupled goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) for one hour.

During quantitative assessment of ADCS expression, the GFP-fluorescent Bergreen WT reference strain was used as a negative control (Kooij et al., 2012). Microscope settings and exposure times were kept constant to ensure comparability. Signal intensities and parasite sizes were analyzed using FIJI with the appropriate plugins (Schindelin et al., 2012; Schneider et al., 2012).

Metabolite Restriction and Supplementation
Mice were fed ad libitum with conventional fortified rodent diet (V1534-300, ssniff, Soest, Germany), or with a diet based on spray-dried whole milk powder (Saliter, Obergrünzburg, Germany). Milk powder was mixed with water, formed into pellets and dried at 60 to 80°C. Mice readily accepted the diet. For phenotypic rescue experiments, drinking water was supplemented with 20 μg/l pABA (Sigma-Aldrich) or equimolar concentrations of 5-MTHF (Sigma-Aldrich). Due to low water solubility, 1.3 mg/kg or 10 mg/kg FA (Sigma-Aldrich) was added to the milk powder. The concentration of 1.3 mg/kg is in the range of the FA levels used in the United States food fortification program (Cridar et al., 2011) and exceeds the endogenous 5-MTHF levels of the milk diet by eight times (Figure 2B).

For growth analysis, mice were given different diets, starting two days before parasite injection. For metabolite quantification of mouse sera, naive animals were given different diets for seven days.

Throughout mosquito infection, the feeding solution contained 10% sucrose and either none or 0.05% pABA. During liver stage analysis, cells were grown in Dulbecco’s Modified Eagle Medium (DMEM), containing 10% dialyzed fetal bovine serum (Thermo Fisher Scientific, Waltham, USA) with or without 5 mg/l pABA.

Metabolite Quantification
Mouse sera, supplemented drinking water, and diet were stored at −80°C prior to metabolite extraction. 200 μL of frozen mouse serum were homogenized in 800 μL of 100% methanol by using a mixer mill with a zirconia bead for 1 minute at 20 Hz. The extract was sonicated for 10 minutes at 4°C. Afterward, the sample was centrifuged at 20,000 g for 10 minutes at 4°C. Supernatants were
dried overnight in a speed vacuum concentrator. The dried sample was dissolved in 150 µl of 80% methanol followed by centrifugation as described above.

35 mg of ground milk pellets and conventional mouse diet were homogenized in 350 µl of 80% methanol followed by centrifugation and extraction as above. Food and serum extracts were spiked with 5 µg/ml isovitexin. Drinking water samples were centrifuged and processed as above.

UPLC/Orbitrap-MS analysis for folate-related metabolites was performed as described previously (Giavalisco et al., 2011). For UPLC separation, 5 µl of the sample were injected into an Acquity UPLC I-Class system equipped with an HSS T3 VanGuard pre-column and an HSS T3 column (Waters, Milford, MA, USA; see also Key Resources Table). Mobile phases were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. Mass spectra were acquired using an Exactive™ Orbitrap mass spectrometer (Thermo Fisher Scientific). pABA, pAHA, pACBA, pABG, 5-MTHF and FA were identified and quantified by high resolution m/z detection using data obtained by co-elution profile of the respective standard compounds (Sigma Aldrich). Data were analyzed using Xcalibur (version 4.1.31.9, Thermo Fisher Scientific).

Blood Stage Development

Blood stage development was assessed using the previously described intravital competition assay (Matz et al., 2013). In short, 500 mCherry-fluorescent Berred WT and 500 GFP-fluorescent adcs™ blood stage parasites were co-injected intravenously into NMRI mice. For single infections with WT, adcs™ or adc™, 1,000 blood stage parasites were injected. Parasitemia was analyzed daily by flow cytometry or by microscopic analysis of Giemsa-stained thin blood films.

Mosquito Infections

To test parasite performance during mosquito infection normally fed NMRI mice were co-injected intravenously with 5x10⁶ Berred WT and 5x10⁶ adcs™ blood stage parasites and fed to A. stephensi mosquitoes three days later. Prior to feeding parasitemias of donor mice were measured using flow cytometry. Oocysts and sporozoites were visualized and quantified by live fluorescence microscopy. In order to restrict the carry-over of pABA-related metabolites from the blood meal, 10⁷ Berred WT or 10⁷ adcs™ blood stage parasites were injected individually into milk-fed animals. For sporozoite production and subsequent liver stage analysis Berred WT and adcs™ parasites were also transmitted individually.

Liver Stage Analysis

Analysis of liver stage development in vitro was performed with human hepatoma cells (HuH-7, not independently authenticated) which were seeded onto µ-slide 8 well glass bottom slides (Ibidi, Munich, Germany) and inoculated with a mixed population of 5,000 Berred WT and 5,000 adcs™ sporozoites at subconfluence. Infected cells were grown at a temperature of 37°C and a CO₂ concentration of 5%. Liver stages were imaged and quantified live 24, 48 and 65 hours after infection.

To assess liver stage development in vivo C57BL/6 mice were injected intravenously with 10⁴ Berred WT or 10⁴ adcs™ sporozoites. For the measurement of first generation parasitemia peripheral blood was subjected to flow cytometry three days after infection. For the quantification of parasite loads livers were harvested 42 hours after infection and total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized from 1 µg of total RNA with the RETROscript kit (Thermo Fisher Scientific) and quantitative PCR was performed with StepOnePlus (Thermo Fisher Scientific) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). The assay was performed in triplicate using primers against mouse GAPDH and parasite 18S rRNA (Table S1; Friesen et al., 2010).

QUANTIFICATION AND STATISTICAL ANALYSIS

Growth curves were analyzed with an ANCOVA. Values > 1% were excluded to restrict analysis to the exponential growth phase. During competitive life cycle progression mutant and WT were compared with a paired t test, comparisons between supplemented and non-supplemented conditions were analyzed with a Student’s t test. For comparison of multiple independent experimental conditions, a one-way ANOVA was used in combination with Tukey’s multiple comparison test. Time to blood stage patency was analyzed with a Mantel-Cox test. All statistical testing was performed with Prism (version 5.03, GraphPad Software, La Jolla, CA, USA). Effects with P values smaller than 0.05 were considered significant. Statistical details can be found in the corresponding Figure legends.