1 **Critical Role for Isoprenoids in Apicoplast Biogenesis by Malaria Parasites**
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ABSTRACT

Isopentenyl pyrophosphate (IPP) is an essential metabolic output of the apicoplast organelle in *Plasmodium falciparum* malaria parasites and is required for prenylation-dependent vesicular trafficking and other cellular processes. We have elucidated a critical and previously uncharacterized role for IPP in apicoplast biogenesis. Inhibiting IPP synthesis blocks apicoplast elongation and inheritance by daughter merozoites, and apicoplast biogenesis is rescued by exogenous IPP and polyprenols. Knockout of the only known isoprenoid-dependent apicoplast pathway, tRNA prenylation by MiaA, has no effect on blood-stage parasites and thus cannot explain apicoplast reliance on IPP. However, we have localized an annotated polyprenyl synthase (PPS) to the apicoplast. PPS knockdown is lethal to parasites, rescued by IPP and long- (C₅₀) but not short-chain ($\leq$ C₂₀) prenyl alcohols, and blocks apicoplast biogenesis, thus explaining apicoplast dependence on isoprenoid synthesis. We hypothesize that PPS synthesizes long-chain polyprenols critical for apicoplast membrane fluidity and biogenesis. This work critically expands the paradigm for isoprenoid utilization in malaria parasites and identifies a novel essential branch of apicoplast metabolism suitable for therapeutic targeting.

INTRODUCTION: *Plasmodium falciparum* malaria parasites are single-celled eukaryotes that harbor a non-photosynthetic plastid organelle called the apicoplast which houses core metabolic pathways and is essential for parasite viability.¹ Because human cells lack this organelle and many of its constituent enzymes, the apicoplast has been viewed as a potentially rich source of new parasite-specific drug targets. However, cashing in on this potential has proved challenging since many apicoplast pathways, including heme^{2, 3} and fatty acid synthesis^{4, 5}, are dispensable during parasite infection of erythrocytes when all malaria symptoms arise. Multiple antibiotics, including doxycycline and clindamycin, block apicoplast biogenesis and inheritance and kill parasites, but their slow activity over several lifecycles has been a fundamental limitation to broad clinical application.⁶

A key, essential function of the apicoplast is biosynthesis and export of the isomeric isoprenoid precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), via the non-mevalonate/methylerythritol phosphate (MEP) pathway. IPP and DMAPP, which can be interconverted by an IPP isomerase, are critical for diverse cellular processes that include prenylation of proteins involved in vesicular trafficking, dolichol-mediated protein glycosylation, and biosynthesis of mitochondrial ubiquinone and heme A.⁷⁻¹⁰ Indeed, exogenous IPP is able to rescue parasites from lethal apicoplast dysfunction or disruption, highlighting the essential requirement for this isoprenoid precursor outside the apicoplast in blood-stage parasites.¹¹ Consistent with these critical cellular roles for IPP, the MEP pathway inhibitor fosmidomycin (FOS) kills parasites in the first lifecycle of treatment.^{8, 11, 12} This first-cycle FOS activity contrasts with the delayed, second-cycle death observed for *Plasmodium* parasites treated with antibiotics such as doxycycline and clindamycin that are thought to block translation of the 35-kb apicoplast genome and the predominantly organelle-

94 maintenance pathways it encodes. These contrasting kinetics have led to a prevailing view in the
95 literature that essential apicoplast functions can be segregated into two general categories: (1)
96 anabolic pathways that produce metabolites required outside the apicoplast and whose inhibition
97 causes first-cycle parasite death or (2) housekeeping pathways that are only required for
98 organelle maintenance and whose inhibition causes delayed, second-cycle defects.¹³⁻¹⁵ Although
99 this simple paradigm has been useful for conceptualizing general apicoplast functions,
100 exceptions to this model have been reported^{12, 16-18} and thus its general validity remains
101 uncertain.

102 Since exogenous IPP rescues parasites from lethal apicoplast disruption,¹¹ isoprenoid
103 biosynthesis has been thought to only serve essential roles outside this organelle.^{7, 12, 15, 16, 19-21}
104 Indeed, *P. falciparum* expresses an essential polyprenyl synthase (PF3D7_1128400) that appears
105 to localize to the cytoplasm and other cellular foci outside the apicoplast and mitochondrion.²²
106 The dual farnesyl/geranylgeranyl pyrophosphate synthase (FPPS/GGPPS) activity of this protein
107 is critical for condensing isoprenoid precursors into longer polyprenyl-PP groups required for
108 diverse cellular processes such as protein prenylation and dolichol synthesis.^{19, 22, 23} In addition,
109 known prenyltransferases, which attach prenyl groups such as FPP and GGPP to client proteins,
110 are also cytoplasmic.²⁰

111 In contrast to this prevailing paradigm, we have unraveled a novel essential arm of
112 isoprenoid metabolism and utilization within the apicoplast and provide direct evidence that IPP
113 and its condensation into downstream linear isoprenoids are required for apicoplast branching
114 and inheritance by daughter merozoites. Genetic knockout of MiaA-dependent tRNA
115 prenylation, the only previously predicted isoprenoid-dependent pathway in the apicoplast,¹ had
116 no effect on blood-stage parasites, and thus MiaA cannot account for apicoplast dependence on

IPP. However, we have localized a previously annotated polyprenyl synthase (PPS, PF3D7_0202700)²⁴ to the apicoplast and show that its conditional knockdown is lethal to parasites, can be rescued by IPP and long- but not short-chain polyprenols, and blocks apicoplast inheritance. We posit that this apicoplast PPS functions downstream of IPP synthesis to produce longer-chain isoprenoids essential for apicoplast membrane fluidity during organelle biogenesis. This discovery critically expands the paradigm for isoprenoid utilization in *P. falciparum*, identifies a potential new apicoplast drug target, and uncovers an organelle maintenance pathway whose inhibition causes first-cycle defects in apicoplast inheritance in contrast to delayed death-inducing antibiotics.

RESULTS

Apicoplast elongation and branching require isoprenoid precursor synthesis. The *P. falciparum* literature has focused almost exclusively on the essential roles of isoprenoid metabolism outside the apicoplast.^{7, 11, 14, 15, 19, 20} Nevertheless, several prior studies reported that MEP-pathway inhibitors such as FOS and MMV008138 blocked apicoplast elongation in lethally treated parasites, suggesting a possible role for IPP in apicoplast biogenesis.²⁵⁻²⁷ These prior studies, however, could not rule out that defects in apicoplast development caused by MEP-pathway inhibitors were due to non-specific effects from the pleiotropic cellular dysfunctions inherent to parasite death.¹⁹ We revisited FOS inhibition of apicoplast biogenesis to further test and distinguish specific versus non-specific effects on organelle development.

We first tested the effect of 10 μ M FOS (10x EC₅₀) on apicoplast elongation in synchronized cultures of two different parasite strains: D10 parasites expressing the apicoplast-targeted acyl carrier protein (ACP) leader sequence fused to GFP (ACP_L-GFP)²⁸ and a recently

published NF54 parasite line (PfMev) that expresses ACP_L-GFP as well as heterologous enzymes that enable cytoplasmic synthesis of IPP from exogenous mevalonate precursor, independent of the apicoplast MEP pathway.²⁹ Consistent with prior reports,^{25-27, 30} we observed that synchronized ring-stage parasites treated with FOS developed into multi-nuclear schizonts but failed to elongate the apicoplast, which retained a focal, unbranched morphology in PfMev (Figure 1A and Figure 1- figure supplement 1) and D10 parasites (Figure 1- figure supplements 2-3). Although MEP pathway activity is detectable in ring-stage parasites,^{31, 32} identical inhibition of apicoplast elongation in schizonts was observed if FOS was added to trophozoites 12 hours after synchronization (Figure 1A and Figure 1- figure supplements 1-3), suggesting continued reliance on de novo synthesis. In contrast to FOS treatment, parasites treated with lethal doses (10-100x EC₅₀) of drugs that target processes outside the apicoplast, including DSM1 (mitochondrial dihydroorotate dehydrogenase inhibitor),³³ atovaquone (ATV, mitochondrial cytochrome b inhibitor),³⁴ blasticidin-S (Blast-S, cytoplasmic translation inhibitor),³⁵ or WR99210 (WR, cytoplasmic dihydrofolate reductase inhibitor),³⁶ exhibited normal apicoplast biogenesis as they developed into schizonts, very similar to untreated parasites (Figure 1, B and C, and Figure 1- figure supplements 2-3). These observations strongly suggest that defects in apicoplast elongation observed with FOS treatment are due to specific inhibition of MEP pathway activity rather than non-specific, secondary effects of parasite death.

Multiple studies have reported that FOS-treated parasites grow normally in the presence of exogenous IPP and do not show evidence of apicoplast loss,^{11, 12, 21, 29} suggesting that IPP rescues apicoplast biogenesis from FOS-induced defects. To directly test this conclusion, we simultaneously treated synchronized rings with 10 μ M FOS and either 200 μ M IPP or 50 μ M mevalonate (for PfMev parasites) and observed normal apicoplast elongation and branching in

schizonts (Figure 1, A and C, and Figure 1- figure supplements 1-3), consistent with a prior report.²⁶ These observations directly support the conclusion that apicoplast elongation requires isoprenoid synthesis.

To further test this conclusion via genetic disruption rather than pharmacological inhibition, we utilized a previously reported line of PfMev parasites in which the apicoplast-targeted deoxyxylulose-5-phosphate synthase (DXS), the first enzyme in the MEP isoprenoid synthesis pathway, had been genetically deleted (Δ DXS).³⁷ These parasites require exogenous mevalonate to support cytoplasmic IPP synthesis, since they lack a functional apicoplast MEP pathway. In the presence of 50 μ M mevalonate, Δ DXS parasites displayed normal apicoplast elongation and branching in schizonts. However, washing out mevalonate from ring-stage Δ DXS parasites to ablate IPP synthesis resulted in multinuclear schizonts with focal, unbranched apicoplast morphologies identical to those observed in the presence of FOS (Figure 1, D and E, and Figure 1- figure supplement 4). These results strongly support the conclusion that apicoplast elongation and branching require IPP synthesis.

Figure 1. Inhibition of isoprenoid precursor biosynthesis specifically blocks apicoplast elongation and branching. Bright field (BF) and fluorescent microscopy images of live NF54 PfMev parasites that were (A) untreated or treated with 10 μ M fosmidomycin (FOS) in the absence or presence of 50 μ M DL-mevalonate (Mev), or (B) treated with 6 μ M blasticidin-S (Blast-S), 2 μ M DSM1, 100 nM atovaquone (ATV), or 5 nM WR99210. (C) Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panels A and B from two independent experiments. Apicoplast morphologies were scored as punctate (focal), elongated, or disrupted (dispersed); counted; and plotted by histogram as the fractional population with the indicated morphology (UT = untreated). Error bars represent standard deviations from replicate experiments. (D) Live-cell imaging of Δ DXS PfMev parasites in the presence or absence of 50 μ M Mev. (E) Population analysis of parasites imaged in panel D and performed as in panel C. In all experiments, synchronized ring-stage parasites were incubated with the indicated treatments for 36 hours prior to live-cell imaging. Parasite nuclei were visualized using 1 μ g/ml Hoechst 33342. The parasite apicoplast was visualized using the ACP_L-GFP encoded by the PfMev line. Absolute parasite counts for microscopy experiments are shown in Figure 1- Source Data 1.

Figure 1- Figure supplement 1. Additional epifluorescence images of PfMev NF54 parasites treated with FOS and other drugs.

Figure 1- Figure supplement 2. Epifluorescence images and analysis of D10 ACP_L-GFP parasites treated with FOS and other drugs.

Figure 1- Figure supplement 3. Additional epifluorescence images of D10 parasites treated with FOS and other drugs.

Figure 1- Figure supplement 4. Additional epifluorescence images of Δ DXS PfMev parasites cultured $\pm$ Mev.

Figure 1- Source data 1. Absolute parasite counts for microscopy experiments.

Inhibition of isoprenoid synthesis prevents apicoplast inheritance by daughter parasites. To stringently test that IPP synthesis is required for apicoplast biogenesis, we next asked if FOS treatment prevented daughter parasites from inheriting the apicoplast, as predicted to occur if the apicoplast fails to elongate and divide in schizonts and as commonly observed for antibiotic inhibitors of apicoplast housekeeping pathways.^{12, 38} Simultaneous treatment of ring-stage parasites with both FOS and IPP rescued growth defects and resulted in normal apicoplast elongation and division (Figure 1, A and C, and Figure 1- figure supplements 1-3), as expected since IPP is the direct anabolic product of the MEP pathway specifically inhibited by FOS. Thus, concomitant treatment with IPP and FOS cannot distinguish whether MEP pathway inhibition prevents apicoplast inheritance by daughter parasites. To bypass this fundamental limitation, we devised the following alternative strategy.

The apicoplast begins to elongate near the onset of schizogony before branching and then dividing in late, segmenting schizonts.^{28, 39} Despite manifesting defects in apicoplast elongation in early schizogony, FOS-treated parasites continue to divide nuclear DNA and transition into mature schizonts before stalling prior to segmentation into merozoites (Figure 1A and Figure 1- figure supplements 1-3).³⁰ This observation suggested that defects in apicoplast biogenesis were

not the immediate cause of parasite death in the current cell cycle and that such defects preceded a broader essential requirement for IPP outside the apicoplast in mature schizonts. Recent works suggest this broader essentiality to be IPP-dependent protein prenylation.^{15, 30} We therefore reasoned that if IPP supplementation were delayed until mid-schizogony, after the onset of apicoplast-elongation defects but before broader cellular death, it might be possible to rescue parasite viability without rescuing apicoplast biogenesis and thereby produce viable parasite progeny that lacked the intact apicoplast.

Synchronized ring-stage PfMev parasites were treated with 10 μ M FOS for 48 hours, with 50 μ M mevalonate added at 0, 30, 34, or 38 hours after synchronization. (Figure 2A). Parasites were allowed to expand for three subsequent cycles in 50 μ M mevalonate, with growth monitored by flow cytometry. We observed a hierarchy of growth rescue by mevalonate, with full rescue (relative to no FOS treatment) of parasites supplemented with mevalonate at 0 hours post-synchronization and decreasing rescue for increasingly delayed supplementation at 30, 34, or 38 hours (Figure 2B), presumably due to fewer viable parasites surviving the initial cycle.

To assess and quantify apicoplast status in rescued parasites, we cloned out individual parasites at 60 hours post-synchronization in the second growth cycle. Apicoplast status in the resulting clones was determined by live parasite microscopy of organelle morphology, apicoplast genome PCR, and growth $\pm$ mevalonate. Although FOS-treated parasites supplemented simultaneously with mevalonate showed no evidence for apicoplast loss in clonal progeny, a fraction of clonal parasites derived from delayed mevalonate rescue showed clear signs of apicoplast loss, including a dispersed apicoplast ACP_L-GFP signal, loss of the apicoplast genome, and growth dependence on exogenous mevalonate (Figure 2C and Figure 2- figure supplements 1–4). The fraction of clonal parasites with a disrupted apicoplast increased from

10% in parasites supplemented with mevalonate at 30 hours to over 80% in parasites supplemented at 38 hours (Figure 2D). These results provide direct evidence that inhibiting IPP synthesis alone is sufficient to block apicoplast biogenesis and prevent organelle inheritance by daughter parasites.

Figure 2. Inhibiting isoprenoid precursor biosynthesis prevents apicoplast inheritance by daughter parasites. **(A)** Schematic summary of delayed mevalonate rescue of fosmidomycin (FOS) treatment. PfMev parasites were synchronized with 5% D-sorbitol and cultured in 10 μ M FOS (washed out after 48 hours in 2nd-cycle rings) without or with addition of 50 μ M DL-mevalonate (Mev) at 0, 30, 34, or 38 hours after synchronization. Clonal parasites from all growth conditions were isolated at 60 hours post-synchronization by limiting dilution and growth in 50 μ M Mev. **(B)** Parasite growth was monitored for 6 days by flow cytometry using acridine orange staining (FOS = treated only with FOS, UT = untreated, 0h – 38h time delay of Mev addition after synchronization and initiation of FOS treatment). **(C)** Bright-field (BF) and fluorescence microscopy images of representative live clonal parasites with disrupted apicoplast (if observed) isolated after 60 hours of growth under the conditions described in panel A. Images of all clones are shown in Figure 2- figure supplements 1-4. Parasite nuclei were visualized using 1 μ g/ml Hoechst 33342. To the right of each clonal image panel is a gel image showing the result of PCR analysis to amplify a (Nu) nuclear (PPS, PF3D7_0202700) and (Api) apicoplast (SufB, PF3D7_API04700) gene and a growth assay to monitor the ability of each indicated clone to grow in the presence or absence of 50 μ M Mev. Data points are the average $\pm$ SD of three biological replicates. **(D)** Graphical representation of the number of clones isolated under each growth condition and the clonal percentage with an intact or disrupted apicoplast (determined by microscope analysis of ACP_L-GFP signal and genomic PCR).

Figure 2- Figure supplement 1. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 0 hours after synchronization.

Figure 2- Figure supplement 2. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 30 hours after synchronization.

Figure 2- Figure supplement 3. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 34 hours after synchronization.

Figure 2- Figure supplement 4. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 38 hours after synchronization.

Figure 2- Source Data 1. Uncropped gel images of clonal PCR analyses.

The MiaA pathway for apicoplast tRNA prenylation is dispensable for blood-stage parasites. Why do apicoplast elongation and branching require IPP synthesis? Currently, the

only predicted isoprenoid-dependent metabolic pathway in the apicoplast is tRNA prenylation by MiaA,^{1, 20} which catalyzes the attachment of a dimethylallyl group to the N⁶ moiety of adenine at position 37 of certain tRNAs.⁴⁰ DMAPP is produced in tandem with IPP in the terminal enzymatic step of the MEP pathway and can be interconverted with IPP by an IPP/DMAPP isomerase.^{7, 11} Prenylation of A37 is often accompanied by methylthiolation by the radical SAM enzyme, MiaB.⁴¹ Although genes encoding MiaA (PF3D7_1207600) and MiaB (PF3D7_0622200) are annotated in the *P. falciparum* genome and MiaA protein has been detected by mass spectrometry in the apicoplast-specific proteome,⁴² neither protein has been studied biochemically in parasites. Nevertheless, both proteins are predicted to be non-essential for blood-stage *Plasmodium* based on genome-wide knockout (KO) studies in *P. berghei*⁴³ and *P. falciparum*.⁴⁴

To directly test whether MiaA function is essential for *P. falciparum* parasites and can account for apicoplast dependence on isoprenoid synthesis, we used CRISPR/Cas9 to target MiaA for gene disruption by double-crossover homologous recombination (Figure 3- figure supplement 1). PfMev parasites were transfected and selected in the presence of 50 μ M mevalonate to ensure that parasites would remain viable even if deletion of MiaA resulted in apicoplast disruption. Parasites that had integrated the knock-out plasmid returned from transfection, and loss of the MiaA gene was confirmed by genomic PCR (Figure 3- figure supplement 1). The Δ MiaA parasites grew equally well in the presence or absence of mevalonate and grew indistinguishably from the parental PfMev parasites (Figure 3A). The presence of an intact apicoplast was confirmed by genomic PCR analysis and live parasite microscopy (Figure 3B and Figure 3- figure supplement 1). These results indicate that MiaA is dispensable for blood-stage parasites and that deletion of this gene does not affect apicoplast biogenesis. Therefore,

loss of function of MiaA, the only predicted isoprenoid-dependent pathway in the apicoplast, cannot account for apicoplast dependence on IPP synthesis, suggesting an alternative role for IPP in organelle elongation.

Figure 3. Genetic disruption of MiaA has no effect on parasite growth or apicoplast biogenesis. **(A)** Growth analysis indicates that parental PfMev NF54 parasites and Δ MiaA parasites cultured in the absence or presence of 50 μ M Mev grow indistinguishably with identical rate constants (k_{obs}) for asynchronous culture expansion. Parasitemia values for each sample are the average $\pm$ SD of three biological replicates and were normalized to the parasitemia on day 4 and fit with an exponential growth model. **(B)** Live-parasite imaging and genomic PCR analysis indicate normal apicoplast morphology and retention of the apicoplast genome in parental PfMev and Δ MiaA parasites. BF = brightfield, Nu = nuclear gene (LDH, PF3D7_1324900), and Api = apicoplast gene (SufB, PF3D7_API04700).

Figure 3- Figure supplement 1. PCR genotyping of PfMev Δ MiaA parasites and additional epifluorescence images of apicoplast morphology.

Figure 3- Source Data 1. Uncropped gel images of PCR analyses of nuclear and apicoplast genomes.

Figure 3- Source Data 2. Uncropped gel images of PCR analysis to confirm disruption of MiaA gene.

Apicoplast biogenesis requires polyprenyl isoprenoid synthesis. Except for MiaA-catalyzed tRNA prenylation, all proposed roles for isoprenoids in *Plasmodium* parasites require head-to-tail condensation of DMAPP (5 carbons) and one or more IPP subunits (5 carbons) to form longer-chain isoprenoids, starting with formation of geranyl pyrophosphate (GPP, 10 carbons), farnesyl pyrophosphate (FPP, 15 carbons), and geranylgeranyl pyrophosphate (GGPP, 20 carbons).^{7, 20} Recent studies reported that 5 μ M geranylgeraniol (GGOH, the alcohol precursor of GGPP) can provide short-term (~1 cycle) rescue of parasite death due to treatment with FOS or indolmycin, an apicoplast tryptophan tRNA synthetase inhibitor.^{15, 30} Based on these reports, we hypothesized that the dependence of apicoplast biogenesis on IPP might reflect a requirement for

longer-chain isoprenoids such that farnesol (FOH), GGOH, and/or longer-chain polyprenols might rescue the apicoplast branching defects caused by 10 μ M FOS.

We treated synchronized NF54 and D10 parasites with both 10 μ M FOS and 5 μ M of either FOH or GGOH. Consistent with prior reports, 5 μ M GGOH but not FOH partially rescued parasite growth from inhibition by FOS and enabled culture expansion into a second growth cycle (Figure 4- figure supplement 1). Nevertheless, both GGOH and FOH rescued apicoplast elongation and branching defects in schizonts when added simultaneously with FOS to synchronized rings (Figure 4 and Figure 4- figure supplements 2 and 3). We extended these rescue experiments to include 5 μ M decaprenol (50 carbons) and also observed rescue of apicoplast branching from FOS-induced defects. However, 5 μ M β -carotene, which is a nonlinear carotenoid hydrocarbon derived from 8 prenyl groups (40 carbons), did not rescue apicoplast biogenesis from inhibition by FOS (Figure 4 and Figure 4- figure supplements 2 and 3). Although it is possible that β -carotene is not taken up efficiently into the apicoplast, rescue by decaprenol, which is similar in size and hydrophobicity to β -carotene, suggests that apicoplast biogenesis specifically requires synthesis of linear polyprenols containing three or more prenyl groups. This hypothesis is further supported by additional results described in the next two sections.

Figure 4. Apicoplast biogenesis requires linear polyprenyl isoprenoid synthesis. **(A)** 5 μ M farnesol (FOH), geranylgeraniol (GGOH), or decaprenol (C₅₀-OH), but not β -carotene rescues apicoplast biogenesis from inhibition by 10 μ M FOS in PfMev parasites. Synchronized ring-stage parasites were incubated with the indicated treatments for 36 hours and imaged by brightfield (BF) or fluorescence microscopy, with visualization of parasite nuclei by Hoechst staining and the apicoplast by ACP_L-GFP signal. **(B)** Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panel A from two independent experiments. Apicoplast morphologies were scored as punctate (focal), elongated, or disrupted (dispersed); counted; and plotted by histogram as the fractional population with the indicated morphology (UT = untreated). Error bars represent standard deviations from replicate experiments.

Figure 4- Figure supplement 1. 5 μ M GGOH but not FOH partially rescues parasite growth from inhibition by 10 μ M FOS in continuous-growth assays with PfMev parasites.

Figure 4- Figure supplement 2. Additional epifluorescence microscopy images of PfMev parasites treated with FOS and FOH, GGOH, C₅₀-OH, or β -carotene.

Figure 4- Figure supplement 3. Epifluorescence microscopy images of D10 ACP_L-GFP parasites treated with FOS and FOH, GGOH, C₅₀-OH, or β -carotene.

Figure 4- Source data 1. Absolute parasite counts for microscopy experiments.

Localization of an annotated polyprenyl synthase to the apicoplast. Iterative condensation of DMAPP with IPP subunits to form FPP, GGPP, and longer polyprenyl-PPs requires the function of a polyprenyl synthase. This family of enzymes uses a conserved dyad of DDXXD residues positioned near the protein surface of the active site binding pocket to coordinate Mg²⁺ ions that bind the pyrophosphate headgroup of DMAPP, GPP, or FPP and position its allylic head relative to the vinyl tail of the IPP subunit.⁴⁵ Condensation of the two substrates via electrophilic alkylation elongates the nascent isoprenoid chain into the protein interior. Two amino acids just upstream of the first DDXXD motif determine the length of the resulting prenyl chain by forming a hydrophobic “floor” that gates the depth of the protein interior. Indeed, dedicated FPPS enzymes feature an amino acid floor comprised of sequential Phe-Phe residues just upstream of the first DDXXD motif that sterically block synthesis of products longer than FPP.⁴⁶ ⁴⁷ Sequence variations that replace just the more N-terminal Phe or both Phe-Phe groups with smaller residues (e.g., Ala or Ser) open up and extend the binding pocket and enable synthesis of GGPP or longer polyprenyl-PPs up to 14 isoprene units, respectively.⁴⁸

A BLAST search of the *P. falciparum* genome with the sequence of the well-studied chicken FPP synthase (Uniprot P08836) revealed two parasite orthologs (PF3D7_1128400 and PF3D7_0202700) that retain the DDXXD dyads and other conserved sequence features expected of a polyprenyl synthase (Figure 5A and Figure 5- figure supplement 1). The best studied of

these synthases is the dual-functional enzyme, PF3D7_1128400, which shares 34% sequence identity with avian FPPS and has been reported to catalyze formation of both FPP and GGPP.^{22, 49, 50} Consistent with its ability to synthesize GGPP as the terminal product, PF3D7_1128400 has sequential Ser-Phe residues just upstream of the first DDXXD motif (Figure 5A).^{49, 50} This enzyme is reported to be essential based on inhibitor^{19, 23} and gene-disruption studies in *P. berghei*⁴³ and *P. falciparum*⁴⁴ and is thought to synthesize the FPP and GGPP required for broad parasite isoprenoid metabolism, including protein prenylation and synthesis of dolichols, ubiquinone, and heme A.^{7, 20} This proposed function is consistent with its localization to the cytoplasm and other cellular foci outside the apicoplast and mitochondrion.²²

We first considered the model that this FPPS/GGPPS might have an essential role in producing GGPP required for apicoplast biogenesis. A recent study, however, identified a specific inhibitor (MMV019313) of PF3D7_1128400 that is lethal to parasites but does not impact apicoplast biogenesis.¹⁹ We independently confirmed that lethal treatment with MMV019313 did not affect apicoplast branching in the PfMev line (Figure 5- figure supplement 2). These observations strongly suggest that the cytosolic FPPS/GGPPS is not the origin of the polyprenyl synthase activity required for apicoplast biogenesis. Therefore, we turned our attention to the second isoprenoid synthase homolog in *P. falciparum*, PF3D7_0202700, which shares 23% sequence identity with avian FPPS.

Like the FPP/GGPP synthase, PF3D7_0202700 retains the DDXXD sequence dyad expected for a polyprenyl pyrophosphate synthase. In addition, the amino acid floor of PF3D7_0202700 features a sterically smaller Gly-Ser dyad upstream of the first DDXXD (Figure 5A and figure 5- figure supplement 1) that suggests an ability to synthesize longer-chain isoprenoids greater than four isoprene units. Consistent with these features, sequence similarity

searches via NCBI BLAST⁵¹ and MPI HHpred⁵² identify polyprenyl synthase homologs from bacteria, algae, and plants that share ~30% sequence identity with PF3D7_0202700 and have annotated functions in synthesizing polyprenyl isoprenoids of 4 – 10 units (Figure 5- figure supplement 3). Using *E. coli* octaprenyl pyrophosphate synthase (PDB 3WJK, 28% identity) as template, we generated a homology model of PF3D7_0202700 to visualize the possible structure of its active site (Figure 5B).

A prior in vitro study of PF3D7_0202700 function, using truncated recombinant protein expressed in *E. coli* or impure parasite extracts, reported an ability to synthesize polyprenyl-PP products of 8 – 11 isoprene units.²⁴ Based on the authors' description, this truncated recombinant protein appears to have lacked one of the DDXXD motifs. Because of this difference from the native protein and the impurity of the parasite-derived protein, it remains possible that the native, pure protein has a distinct product spectrum than previously reported. Nevertheless, this in vitro activity and the general sequence features of PF3D7_0202700 support its function as a long-chain polyprenyl synthase (PPS).

Prior immunofluorescence studies of this PPS, using a polyclonal antibody raised against the truncated recombinant protein, were unable to localize PF3D7_0202700 to a specific subcellular compartment.⁵³ Analysis of the protein sequence with MitoProt II or PlasmoAP suggested the presence of a subcellular-targeting leader sequence,^{54, 55} but ambiguous sequence features prevented a high-confidence targeting prediction to the mitochondrion or apicoplast. MitoProt II predicted a 55% probability of mitochondrial targeting. PlasmoAP strongly predicted an apicoplast-targeting transit peptide, but SignalP did not recognize the N-terminal sequence as a canonical signal peptide. To localize PPS within parasites, we engineered Dd2 *P. falciparum* lines to episomally express full-length PPS fused to either C-terminal GFP or RFP. In live

parasites, focal PPS-GFP fluorescence was detected in a tubular compartment proximal to but distinct from the mitochondrion, as expected for apicoplast localization. Immunofluorescence analysis of the PPS-RFP line revealed strong co-localization between PPS-RFP and the apicoplast acyl carrier protein (ACP) (Figure 5C and Figure 5- figure supplement 4).

To further confirm apicoplast targeting of PPS, we stably disrupted the apicoplast in the PPS-GFP Dd2 line by culturing these parasites in 2 μ M doxycycline and 200 μ M IPP for one week.^{11, 56} As expected for an apicoplast-targeted protein, the PPS-GFP signal in these parasites displayed a constellation of dispersed fluorescent foci, rather than the concentrated signal observed in untreated parasites (Figure 5D and Figure 5- figure supplement 4). Western blot analysis of the PPS-RFP parasites revealed two bands at the expected sizes for precursor protein and a smaller, N-terminally processed mature form, as expected for import into the apicoplast (Figure 5E).²⁸ In the apicoplast-disrupted parasites, however, only a single PPS-RFP band for the precursor protein was detected, consistent with loss of apicoplast import and lack of transit peptide removal.^{11, 38} On the basis of these observations, we conclude that PF3D7_0202700 is an apicoplast-targeted PPS that is imported into the organelle and N-terminally processed to a mature form. This localization, the predicted ability of this enzyme to synthesize polyprenyl PPs longer than 4 isoprenes, and our observation that decaprenol rescued FOS-induced defects in apicoplast biogenesis all suggested a critical role for this protein in apicoplast maintenance.

Figure 5. Sequence alignment and localization of the polyprenyl synthase (PPS) PF3D7_0202700 to the apicoplast. **(A)** Focal sequence alignment of avian farnesyl pyrophosphate synthase (FPPS, Uniprot P08836) with its two *P. falciparum* homologs reveals the presence of conserved metal-binding DDXXD motifs (red) expected for polyprenyl synthase activity and chain-length determination residues (yellow) upstream of the first DDXXD. **(B)** Homology model of PF3D7_0202700 using *E. coli* octaprenyl pyrophosphate synthase (PDB 3WJK) as a structural template. The inset box is an enlargement of the active-site pocket showing the conserved Asp residues, bound FPP substrate, and product length-determining

residues just upstream of the first DDXXD motif. (C) Brightfield (BF), fluorescence images (top) of live parasites episomally expressing PPS-GFP and stained with 10 nM Mitotracker Red and (bottom) immunofluorescence analysis (IFA) images of fixed parasites episomally expressing PPS-RFP stained with anti-RFP and anti-apicoplast ACP antibodies. (D) IFA images of fixed parasites expressing PPS-GFP that had been treated for >7 days with 2 μ M doxycycline (Dox) and 200 μ M IPP (to stably induce apicoplast loss) and stained with anti-GFP antibody to visualize PPS distribution. (E) Western blot analysis of untreated (UT) or Dox/IPP-treated parasites episomally expressing PPS-RFP. PPS-RFP expression was visualized using an anti-RFP antibody. The full western blot image is included in Figure 5- Source Data 1.

Figure 5- Figure supplement 1. Full sequence alignment of PF3D7_0202700, PF3D7_1128400, and avian FPPS (Uniprot P08836).

Figure 5- Figure supplement 2. Epifluorescence microscopy images and statistical analysis of PfMev and D10 parasites treated with 10 μ M MMV091313.

Figure 5- Figure supplement 3. Results of sequence-similarity searches for PF3D7_0202700 using NCBI BLAST and MPI HHpred.

Figure 5- Figure supplement 4. Additional epifluorescence microscopy images of Dd2 parasite episomally expressing PPS-GFP or PPS-RFP.

Figure 5- Source data 1. Uncropped western blot image detecting PPS-RFP expression in parasites.

PPS is essential for parasite viability and apicoplast biogenesis. The genomic locus for PF3D7_0202700 was reported to be refractory to disruption in recent genome-wide KO studies in *P. berghiei*⁴³ and *P. falciparum*,⁴⁴ suggesting an essential function. To directly test its functional essentiality in *P. falciparum*, we used CRISPR/Cas9⁵⁷ to tag the endogenous gene in Dd2 parasites to encode a C-terminal hemagglutinin (HA)-FLAG epitope fusion and the aptamer/TetR-DOZI system⁵⁸ that enables ligand-dependent regulation of protein expression using the non-toxic small molecule, anhydrotetracycline (aTc). In this system, normal protein expression occurs +aTc and translational repression is induced upon aTc washout (Figure 6A).

We first confirmed correct integration into the genomic locus with the expected genotype in both polyclonal and clonal parasites by Southern blot (Figure 6- figure supplement 1). We then used immunofluorescence microscopy to confirm co-localization of the endogenous PPS

with apicoplast ACP (Figure 6B and Figure 6- figure supplement 2). Expression of the ~60 kDa HA-FLAG-tagged, endogenous mature protein was detected by anti-HA-tag western blot in +aTc conditions, and its signal relative to the loading control was reduced nearly three-fold in parasites grown in -aTc +IPP conditions (Figure 6C). Our detection of only the mature form of endogenous PPS contrasts with our detection of both the precursor and mature forms of episomally expressed PPS-RFP (Figure 5E), suggesting that the precursor form may preferentially accumulate when PPS is over-expressed. We also quantified PPS transcript levels by RT-qPCR and observed robust knockdown of PPS mRNA levels by the second intraerythrocytic cycle of parasite growth in -aTc conditions (Figure 6D). The fate of target mRNA in the aptamer/TetR-DOZI system has not been characterized in depth. Our data are consistent with a prior report⁵⁹ and suggest that TetR-DOZI binding after aTc washout leads to mRNA transcript degradation, possibly within stress granules targeted by DOZI-bound transcripts.⁵⁸

To test PPS essentiality for blood-stage parasite growth, we synchronized PPS knockdown (KD) parasites and monitored their growth $\pm$ aTc over multiple intraerythrocytic lifecycles. In the presence of aTc, culture parasitemia expanded in a step-wise fashion over the ~10 days of the growth assay such that the culture needed to be split multiple times to avoid over-growth (Figure 6E). Without aTc, however, the culture grew normally over the first 3 intraerythrocytic cycles but showed a major growth defect in the fourth cycle consistent with extensive parasite death observed by blood smear (Figure 6E and Figure 6- figure supplement 3). Parasite growth under -aTc conditions was strongly rescued in the presence of 200 μ M exogenous IPP (Figure 6E), indicating an essential PPS function specific to the apicoplast.

To test a role for PPS in synthesizing polyprenyl PP groups, we attempted to rescue parasite growth in -aTc conditions by adding 5 μ M FOH, GGOH, or decaprenol. We observed that only decaprenol, but not FOH or GGOH, rescued parasite growth in -aTc conditions, and the magnitude of rescue by decaprenol was comparable to IPP (Figure 6E). These observations strongly suggest that PPS has an essential function downstream of IPP synthesis in converting isoprenoid precursors into longer-chain linear polyprenyl-PPs containing at least 5-10 isoprene units.

Figure 6. PPS (PF3D7_0202700) is essential for parasite viability and apicoplast function. **(A)** Schematic depiction of the aptamer/TetR-DOZI system for ligand-dependent protein expression. **(B)** Immunofluorescence analysis of fixed parasites endogenously expressing PPS-HA-FLAG and stained with anti-ACP and anti-HA-tag antibodies. The intensity plot displays the overlap in pixel intensity for ACP and HA signals as a function of distance along the white line in the merged image. **(C)** Western blot of endogenously tagged PPS-HA/FLAG showing detection of tagged PPS at the expected size for mature PPS of ~60 kDa for growth in +aTc conditions but diminished signal for parasites grown -aTc +IPP for five days. Densitometry of the PPS signal relative to the EF1 α loading control indicated a 3-fold signal reduction in +aTc versus -aTc/+IPP conditions. **(D)** RT-qPCR analysis of PPS transcript levels (normalized to the average of two nuclear control genes) in biological replicate samples of synchronous parasites cultured for 72 hours $\pm$ aTc or -aTc/+IPP. **(E)** Synchronous growth assay of Dd2 parasites tagged at the PPS locus with the aptamer/TetR-DOZI system and grown $\pm$ aTc and $\pm$ 200 μ M IPP or 5 μ M farnesol (FOH), geranylgeraniol (GGOH), or decaprenol (C₅₀-OH). Parasitemia values for each condition are the average $\pm$ SD of three biological replicates.

Figure 6- Figure supplement 1. Scheme for modification of the PPS genomic locus to integrate the aptamer/TetR-DOZI system and Southern blot confirming correct integration.

Figure 6- Figure supplement 2. Additional immunofluorescence microscopy images showing co-localization of endogenous PPS and apicoplast ACP.

Figure 6- Figure supplement 3. Blood-smear images of Dd2 parasites tagged at the PPS locus with the aptamer/TetR-DOZI system and grown $\pm$ aTc for 8 days.

Figure 6- Source data 1. Uncropped western blot image detecting endogenous PPS-HA/FLAG in parasites.

Figure 6- Source data 2. Uncropped Southern blot image to probe editing of PPS gene.

To test if PPS function is required for apicoplast biogenesis, we cultured PPS KD parasites in +aTc or -aTc/+IPP conditions for 12 days and then assessed apicoplast morphology in fixed parasites by α ACP immunofluorescence. We reasoned that IPP would rescue parasite viability upon PPS KD but not interfere with assessing any defect in apicoplast biogenesis if IPP synthesis were upstream of PPS function. Immunofluorescence analysis (IFA) revealed that ~30% of parasites cultured in -aTc conditions had a dispersed ACP signal indicative of apicoplast disruption (Figure 7- figure supplement 1). Although this observation supports a critical role for PPS in apicoplast biogenesis, we wondered why PPS KD did not result in a higher fraction of parasites with disrupted apicoplast. We hypothesized that residual PPS expression resulting from incomplete KD combined with high IPP levels due to culture supplementation and endogenous MEP pathway activity might enable sufficient synthesis of polyprenols to attenuate the impact of PPS KD on apicoplast biogenesis.

To test this hypothesis and the contribution of MEP pathway activity to the observed phenotype, we synchronized parasites to the ring stage and cultured them in $\pm$ aTc conditions for 96 hours (two 48-hour growth cycles) to knock down PPS expression before adding FOS and IPP at the start of the third growth cycle (Figure 7A). In this experiment, FOS was expected to inhibit endogenous MEP pathway activity without impacting apicoplast biogenesis since it was added concurrently with IPP, which fully rescues parasites from growth and apicoplast defects induced by FOS (Figure 1).^{11, 21} We first used IFA to assess apicoplast morphology in schizonts at the end of the third growth cycle (38 hours after adding FOS and IPP). We observed normal apicoplast elongation in +aTc parasites but focal, unbranched apicoplast morphology in the vast majority (>80%) of -aTc parasites (Figure 7, B and C, and Figure 7- figure supplement 2). Substitution of IPP with FOH or GGOH resulted in a nearly identical apicoplast elongation

defect in -aTc parasites. In contrast, substituting IPP with decaprenol resulted in normal apicoplast elongation in both +aTc and -aTc parasites (Figure 7, B and C, and Figure 7- figure supplement 2). The selective ability of decaprenol to rescue apicoplast-branching defects in -aTc conditions strongly supports an essential role for PPS in synthesizing long-chain polyprenyl isoprenoids required for apicoplast biogenesis.

To further test this conclusion, we maintained parasites in $\pm$ aTc conditions with FOS and IPP for two additional growth cycles (total of five 48-hour cycles, Figure 7A). Parasites cultured +aTc displayed normal elongated apicoplast morphology. In contrast, the -aTc (+FOS and IPP) culture predominantly contained parasites with a dispersed ACP signal indicative of apicoplast loss (Figure 7, D and E, and Figure 7- figure supplement 3). These -aTc parasites also contained a strongly reduced qPCR signal for apicoplast genomic DNA, relative to +aTc parasites (Figure 7F). These results indicate that PPS is essential for apicoplast maintenance and inheritance by daughter parasites such that loss of PPS function (with IPP supplementation) results in parasite progeny lacking the intact organelle. This essential PPS function downstream of IPP synthesis by the MEP pathway is sufficient to explain our observation that blocking pathway activity by FOS or Δ DXS inhibits apicoplast biogenesis (Figure 1).

Figure 7. PPS is required for apicoplast biogenesis. (A) Scheme summarizing growth of synchronized PPS knockdown parasites cultured $\pm$ aTc, re-synchronized and treated with 10 μ M FOS $\pm$ 200 μ M IPP or 5 μ M farnesol (FOH), geranylgeraniol (GGOH), or decaprenol (C₅₀-OH) at 96 hours after initial synchronization, and imaged at 134 and 182 hours after initial synchronization. **(B)** Immunofluorescence analysis (IFA) of PPS knockdown parasites cultured as described in panel A and imaged at 134 hours (day 6) after initial synchronization to assess apicoplast morphology $\pm$ aTc. **(C)** Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panel A from two independent experiments. Apicoplast morphologies were scored as punctate (focal), elongated, or disrupted (dispersed); counted; and plotted by histogram as the fractional population with the indicated morphology (UT = untreated). Error bars represent standard deviations from replicate experiments. **(D)** Immunofluorescence analysis of PPS knockdown parasites cultured $\pm$ aTc +FOS +IPP and

imaged at 182 hours (day 8) after initial synchronization to assess apicoplast morphology $\pm$ aTc. (E) Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panel D and analyzed as in panel C. (F) Quantitative PCR analysis of the apicoplast:nuclear (Api:Nu) genome ratio for parasites cultured $\pm$ aTc and imaged in panel D, based on amplification of apicoplast TufA (PF3D7_API02900) or nuclear ADSL (PF3D7_0206700) relative to nuclear I5P (PF3D7_0802500) genes. Indicated qPCR ratios were normalized to +aTc in each case and are the average $\pm$ SD of three biological replicates. Significance of $\pm$ aTc differences was analyzed by two-tailed unpaired t-test to determine the stated P value. All parasite samples collected for IFA were imaged by brightfield (BF) and epifluorescence microscopy, with visualization of parasite nuclei by DAPI staining and apicoplast by an anti-apicoplast ACP antibody.

Figure 7- Figure supplement 1. IFA images and analysis of apicoplast morphology in PPS knockdown parasites grown +aTc or -aTc/+IPP (200 μ M) for 12 days.

Figure 7- Figure supplement 2. Additional IFA images of PPS knockdown parasites treated as in Figure 7B.

Figure 7- Figure supplement 3. Additional IFA images of PPS knockdown parasites treated as in Figure 7D.

Figure 7- Source data 1. Absolute parasite counts for microscopy experiments.

No evidence for PPS function in carotenoid synthesis. Despite its strong sequence similarity to known polyprenyl synthases that catalyze the head-to-tail condensation of isoprenoid precursors, PF3D7_0202700 has also been proposed to catalyze the biochemically distinct head-to-head condensation of 20-carbon GGPP groups into 40-carbon phytoene and thus function as a phytoene synthase (PSY) within a broader pathway of carotenoid biosynthesis proposed to exist in *Plasmodium* parasites (Figure 8A).^{53, 60} Polyprenyl synthases and phytoene synthases are mechanistically distinct enzymes that lack significant sequence similarity but are thought to share a common isoprenoid-related protein fold that reflects their ancient divergence from a common ancestral enzyme.^{47, 61} Given the mechanistic differences between head-to-tail and head-to-head condensation of isoprenoids (Figure 8A), which involve distinct positioning of substrate pyrophosphate groups within each active site, there is no known enzyme that is capable of catalyzing both reactions.⁶¹ Thus, the proposal of dual PPS and PSY functions for

PF3D7_0202700 is without biochemical precedent. Nevertheless, we considered whether this protein might also have PSY function and evaluated whether existing observations supported or contradicted a proposed role for this protein in carotenoid biosynthesis.

As noted previously, untargeted sequence similarity searches via NCBI BLAST⁵¹ and MPI HHpred⁵² with PF3D7_0202700 as the query sequence only identify polyprenyl synthase homologs from bacteria, algae, and plants (Figure 5- figure supplement 3) and fail to identify PSY homologs. Furthermore, targeted pairwise alignments show no evidence of significant sequence homology between PF3D7_0202700 and confirmed eukaryotic or prokaryotic PSY sequences from *Arabidopsis thaliana* (Uniprot P37271, chloroplast-targeted)⁶² or *Erwinia herbicola* (*Pantoea agglomerans*, Uniprot D5KXJ0),⁶³ respectively. Finally, the prior proposal of PSY activity by PF3D7_0202700 was based in part on its sequence similarity to an annotated PSY from *Rubrivivax gelatinosus* bacteria (NCBI accession BAA94032) that also appeared to contain sequence features expected of a head-to-tail polyprenyl synthase.⁵³ We noted that the functional annotation of this bacterial protein was subsequently revised to a geranylgeranyl-PP synthase (Uniprot I0HUM5),⁶⁴ thus explaining its sequence similarity to PF3D7_0202700 and the homology of both proteins to known polyprenyl synthases. On the basis of these sequence analyses, we considered it unlikely that PF3D7_0202700 had dual activity as a PSY.

The prior work studied the antiparasitic effects of the squalene synthase inhibitor, zaragozic acid (ZA, also called squalestatin), that inhibited blood-stage *P. falciparum* growth (EC₅₀ ~5 μ M) and was proposed to specifically target PF3D7_0202700 based on observation of a ~6-fold increase in EC₅₀ for parasites episomally expressing a second copy of this protein.⁶⁰ We repeated these experiments in Dd2 parasites and observed a similar EC₅₀ of ~10 μ M for ZA that increased 5-fold to ~50 μ M in Dd2 parasites episomally expressing PPS-RFP (Figure 8- figure

supplement 1). However, in contrast to PPS knockdown (Figure 6D), lethal growth inhibition by ZA was not rescued by exogenous IPP (Figure 8- figure supplement 1) and did not affect apicoplast elongation (Figure 8- figure supplement 2). These contrasting phenotypes strongly suggest that PPS, and more broadly the apicoplast, are not uniquely targeted by ZA. The basis for why PPS over-expression reduces parasite sensitivity to ZA is unclear but may reflect drug interactions with broader isoprenoid metabolism outside the apicoplast that are rescued, directly or indirectly, by reaction products of PPS.

β -carotene, a 40-carbon carotenoid derived from phytoene, was previously detected by mass spectrometry in extracts of *P. falciparum*-infected erythrocytes and suggested to be biosynthesized by parasites based on the lack of detection in extracts of uninfected erythrocytes.⁵³ Using our PPS knockdown line, we tested whether translational repression of PF3D7_0202700 impacted detectable levels of β -carotene in parasites, as predicted to occur if PPS also functioned as a PSY. After synchronization, PPS knockdown parasites were grown $\pm$ aTc for 120 hours and harvested at the end of the third intraerythrocytic growth cycle, which immediately precedes the growth defect observed in Figure 6E. Saponin pellets of these parasites were extracted in acetone and analyzed by liquid chromatography/tandem mass spectrometry for β -carotene (Figure 8- figure supplement 3). We observed indistinguishable low levels of β -carotene in both samples (Figure 8B), providing no evidence that PPS plays a role in carotenoid biosynthesis.

Although uninfected erythrocytes washed in AlbuMAX-free RPMI lacked detectable β -carotene, we observed that extracts of uninfected erythrocytes incubated in complete RPMI medium containing AlbuMAX I had β -carotene levels that were nearly identical to extracts of parasite-infected erythrocytes (Figure 8B). Analysis of AlbuMAX (ThermoFisher catalog

#11020021) by mass spectrometry revealed modest levels of β -carotene (Figure 8- figure supplement 4), consistent with the bovine origin of AlbuMAX (lipid-rich bovine serum albumin) and the plant-based diet of these animals expected to contain β -carotene. These results are sufficient to explain the presence of carotenoids like β -carotene in parasite extracts. In summary, we find no evidence that PPS function contributes to β -carotene levels in *P. falciparum*-infected erythrocytes, which we suggest non-specifically take up exogenous plant-derived β -carotene associated with AlbuMAX in the culture medium.

Parasite-infected erythrocytes were previously reported to incorporate ^3H -labeled GGPP into biosynthetic products that had reverse-phase HPLC retention times similar to all-trans-lutein or β -carotene standards, suggesting de novo synthesis of these isoprenoid products.⁵³ Because the extracted products were radioactive, their identity could not be directly confirmed by tandem mass spectrometry. To directly test if blood-stage *P. falciparum* parasites incorporate isoprenoid precursors into β -carotene, as predicted for active biosynthesis, we cultured the PfMev parasites in 50 μM of 2- ^{13}C -mevalonate in the presence of 10 μM FOS. This strategy was chosen to inhibit MEP pathway activity, ensure full ^{13}C -labeling of the endogenous IPP and DMAPP precursor pool produced by the cytoplasmic bypass enzymes, and result in a distinguishable 8 Da mass increase for any β -carotene derived from de novo synthesis. We previously showed that this strategy results in complete ^{13}C -labeling of endogenous IPP and FPP.²⁹ Parasites were expanded to high parasitemia over several days under ^{13}C -labeling conditions before extraction and analysis by mass spectrometry. Although we readily detected unlabeled β -carotene (m/z 536.4), which we attribute to culture medium AlbuMAX, we were unable to detect ^{13}C -labeled β -carotene (m/z 544.4) (Figure 8C). In contrast to the parasite analysis, we readily detected fully ^{13}C -labeled β -carotene (m/z 576.6) produced by *E. coli* bacteria engineered to biosynthesize β -

carotene⁶⁵ and grown in minimal M9 medium with uniformly labeled ¹³C-glucose as the sole carbon source (Figure 8D). In summary, we find no evidence for PSY function by PF3D7_0202700 or for de novo carotenoid synthesis by blood-stage *P. falciparum* parasites. Collectively, our data strongly support the conclusion that PF3D7_0202700 functions exclusively as a polyprenyl synthase required for apicoplast biogenesis.

Figure 8. No evidence that PPS contributes to carotenoid synthesis by *P. falciparum*. **(A)** Schematic depiction of head-to-tail arrangement of prenyl groups during polyprenyl-PP synthesis versus head-to-head arrangement of geranylgeranyl-PP groups during phytoene synthesis. **(B)** Mass spectrometry determination of unlabeled β -carotene levels in PPS knockdown parasites grown for 6 days $\pm$ aTc or in uninfected red blood cells incubated in complete media containing Albumax. Measured β -carotene levels are the average $\pm$ SD of three biological replicates, whose differences were analyzed by two-tailed unpaired t-test for significance (P values given relative to +aTc sample). **(C)** Intensity versus retention time plot for liquid chromatography-mass spectrometry determination of unlabeled and ¹³C-labeled β -carotene in NF54 PfMev parasites cultured for 6 days in 50 μ M 2-¹³C-mevalonate and 10 μ M fosmidomycin. Below: schematic depiction of the 8 carbon atoms in β -carotene expected to be labeled with ¹³C for synthesis from IPP derived from 2-¹³C-mevalonate in PfMev parasites. **(D)** Intensity versus retention time plot for liquid chromatography-mass spectrometry determination of unlabeled and ¹³C-labeled β -carotene in pAC-BETAipi *E. coli* grown in unlabeled or fully ¹³C-labeled glucose as the sole carbon source in M9 minimal media. The two peaks reflect the presence of an isomeric mix of all-trans and cis β -carotene produced by the pAC-BETAipi *E. coli*, as previously reported.⁶⁵

Figure 8- Figure supplement 1. 48-hour growth inhibition curves for treatment of Dd2 parasites with zaragozic acid without or with episomal expression of PPS-RFP or 200 μ M IPP.

Figure 8- Figure supplement 2. Epifluorescence microscopy images of D10 parasites treated with 160 μ M zaragozic acid as synchronized rings and imaged for ACP_L-GFP and Hoescht 36 hours later as multinuclear schizonts.

Figure 8- Figure supplement 3. Fragment ion spectrum for unlabeled beta-carotene determined by tandem mass spectrometry of β -carotene commercial standard.

Figure 8- Figure supplement 4. Intensity versus retention time plot for liquid chromatography-mass spectrometry determination of unlabeled β -carotene in Albumax I.

DISCUSSION

Biosynthesis of the isoprenoid precursors, IPP and DMAPP, is a well-established essential function of the *Plasmodium* apicoplast, but prior work has focused nearly exclusively on the critical roles of isoprenoids for diverse cellular processes outside this organelle.^{7, 11, 15, 20} We have elucidated a novel arm of isoprenoid metabolism within the apicoplast that is required for biogenesis of this critical organelle (Figure 9). This discovery expands the paradigm for isoprenoid utilization by malaria parasites, uncovers a novel essential feature of apicoplast biology, and identifies a key enzyme in this pathway suitable for development as a therapeutic target.

Implications for general understanding of apicoplast functions. Our study, which was inspired by prior hints in the literature,²⁵⁻²⁷ firmly establishes a novel essential role for MEP pathway activity in supporting apicoplast biogenesis, in addition to its recognized role producing IPP required outside this organelle. Prior work has clearly established that apicoplast maintenance pathways are required to support IPP synthesis and export for essential cellular use,^{11, 15, 21, 38} but the reverse dependence of apicoplast biogenesis on IPP was not previously recognized. Thus, IPP synthesis by the MEP pathway requires apicoplast maintenance, which in turn depends on IPP synthesis. The two processes are convoluted and interdependent. This reciprocal dependence between organelle maintenance and metabolic outputs may extend to other apicoplast pathways in mosquito- and liver-stage *P. falciparum* as well as *Toxoplasma gondii*, where additional apicoplast outputs, such as fatty acids, contribute to parasite fitness and may support apicoplast maintenance.^{4, 5, 66-68}

Our results also support the emerging paradigm^{12, 16-18} that inhibition of apicoplast maintenance pathways can kill parasites with first-cycle kinetics that defy the delayed-death

phenotype commonly observed for translation-blocking antibiotics such as doxycycline that target organelle housekeeping.^{6, 12} Indeed, blocking IPP synthesis causes same-cycle defects in apicoplast biogenesis, which are expected to produce non-viable parasite progeny independent of lethal dysfunctions in isoprenoid-dependent metabolism outside the organelle. Analysis of the timing of FOS-induced defects in apicoplast branching also provides an unexpected and incisive window into the differential compartmentalization of IPP essentiality in parasites. We observed that FOS-treated parasites display apicoplast-elongation defects in early schizogony but continue to divide nuclear DNA and transition into mature schizonts before stalling prior to segmentation (Figure 1A). Thus, the critical role of IPP for apicoplast biogenesis precedes the broader cellular need for IPP outside the organelle in mature schizonts, suggested by recent works to predominantly reflect essential roles for IPP-dependent protein prenylation.^{15, 30} Although MEP pathway activity begins in ring-stage parasites,^{31, 32} we observed identical inhibition of apicoplast elongation in schizonts independent of whether FOS was added to rings concomitant with synchronization or to trophozoites 12 hours after synchronization (Figure 1A). This observation suggests that IPP utilization in the apicoplast depends on de novo synthesis rather than a pre-existing metabolite pool, possibly because IPP does not accumulate in the apicoplast and/or that IPP synthesis within the organelle is differentially partitioned for export and internal utilization.

Localization of PPS to the apicoplast. Our studies identified PF3D7_0202700 as an apicoplast-targeted PPS based on its co-localization with apicoplast ACP, dispersed localization upon apicoplast disruption, and N-terminal processing in an apicoplast-dependent manner. Nevertheless, the sequence features that target PPS to the apicoplast are somewhat obscure. Imported apicoplast proteins canonically contain N-terminal signal and transit peptides that are

789 proteolytically removed in the endoplasmic reticulum (ER) and apicoplast, respectively.^{28, 69} The
790 N-terminus of PPS contains a transit peptide recognized by PlasmoAP⁵⁴ but is not recognized by
791 SignalP⁷⁰ to contain a canonical signal peptide. This targeting ambiguity is not unique to PPS.
792 We are aware of multiple apicoplast-targeted and imported proteins, including several identified
793 in the prior proximity-biotinylation study of the apicoplast proteome,⁴² that contain a
794 recognizable transit peptide but lack an identifiable signal peptide by SignalP. These proteins
795 include the key MEP pathway enzymes DXR (PF3D7_1467300) and IspD (PF3D7_0106900),
796 holo ACP synthase (PF3D7_0420200), FabB/F (PF3D7_0626300) and the E1 subunit of
797 pyruvate dehydrogenase (PF3D7_1446400). These observations suggest that protein N-termini in
798 *Plasmodium* can have sequence properties compatible with apicoplast targeting that are broader
799 and more heterogenous than ER-targeting sequences from other eukaryotes that comprise the
800 training sets upon which SignalP is currently based. It remains a future challenge to understand
801 these N-terminal properties and their relation to apicoplast targeting and import in *Plasmodium*.

802 N-terminal processing of apicoplast-targeted proteins is an exclusive property of known
803 proteins imported into the organelle. Although multiple proteins have been shown or suggested
804 to associate with the apicoplast exterior or target the outer apicoplast membrane, none of these
805 proteins is N-terminally processed (e.g., ATG8, oTPT, and FTSH1).^{16, 71, 72} N-terminal
806 processing of PPS is therefore strongly suggestive of import into the apicoplast. Such processing,
807 however, does not specify the sub-compartment within the apicoplast to which PPS is localized,
808 which could include the matrix and/or multiple membranes or intermembrane spaces. Indeed, N-
809 terminal processing has been reported for both matrix and membrane-targeted proteins imported
810 into the apicoplast.⁷¹ The central conclusions of our study are not affected by this ambiguity. For
811 simplicity, we have depicted PPS localization within the apicoplast matrix (Figure 9), but more

studies and higher resolution (e.g., electron microscopy with immunogold labeling) will be required to specify sub-compartmental targeting of PPS within the apicoplast. PPS lacks obvious membrane-targeting features but required overnight extraction in 2% SDS or LDS for detection by western blot. These features, together with low expression, may explain why PPS has not been detected in prior studies of the apicoplast proteome that used milder extraction conditions.

Implications for other apicomplexan parasites. Polyprenyl synthases are diverse enzymes that perform a variety of cellular functions, whose specific roles can differ between organisms. The reliance of apicoplast biogenesis on isoprenoid synthesis in *Plasmodium* may differ from other apicomplexan parasites. *Toxoplasma gondii* appears to express two polyprenyl synthase homologs, TGME49_224490 and TGME49_269430, that are ~30% identical (in homologous regions) to PF3D7_1128400 (FPPS/GGPPS) and PF3D7_0202700 (PPS), respectively. TGME49_224490 appears to be targeted to the mitochondrion in *T. gondii* (based on MitoProt and HyperLOPIT analyses),^{55, 73} in contrast to FPPS/GGPPS, its closest *P. falciparum* homolog, which localizes to the cytoplasm and other cellular foci outside the mitochondrion. TGME49_269430 does not appear to target the apicoplast in *T. gondii* (based on HyperLOPIT data), which contrasts with our determination of apicoplast targeting for PPS, the closest *P. falciparum* homolog. These observations suggest distinct cellular roles for enzyme homologs in the two parasites. Furthermore, a recent study⁷⁴ showed that loss of MEP pathway activity in *T. gondii* (due to loss of apicoplast ferredoxin) does not impact apicoplast biogenesis, in contrast to our observations in *P. falciparum* based on FOS treatment, DXS deletion, and PPS knockdown. Finally, *Cryptosporidium parvum* lacks an apicoplast but retains two polyprenyl synthase homologs, CPATCC_003578 and CPATCC_001801, whose cellular targeting has not been

reported. These differing enzyme localizations and phenotypes suggest differences in cellular isoprenoid utilization and metabolism between *T. gondii*, *P. falciparum*, and broader apicomplexan organisms that remain to be understood.

Why does *Plasmodium* apicoplast biogenesis depend on IPP synthesis? The essential function of PPS in apicoplast maintenance is sufficient to explain the apicoplast reliance on IPP synthesis unveiled by FOS treatment of parasites. Although the dominant polyprenyl-PP product of apicoplast PPS in parasites remains uncertain, sequence features, prior in vitro enzymology, and the ability of exogenous decaprenol but not GGOH or FOH to rescue PPS knockdown indicate that linear polyprenyl-PP products longer than 4 and as long as 10 isoprene units are critical for apicoplast maintenance.²⁴ Prior work suggested a dual function for PF3D7_0202700 as a phytoene synthase (PSY) that also condenses isoprenoid precursors,^{53, 60} but we found no evidence to support this proposed PSY function or carotenoid biosynthesis more broadly. Synthesis of octaprenyl-PP by PF3D7_0202700 was previously proposed to be critical for ubiquinone biosynthesis in the parasite mitochondrion.²⁴ Localization of this protein to the apicoplast (Figures 5 and 6) and observation that exogenous IPP rescues the growth defects of its knockdown (Figure 6E) strongly suggest that its activity is not required for mitochondrial ubiquinone biosynthesis and that its essential function is specific to the apicoplast.

Plant chloroplasts synthesize linear polyprenyl isoprenoids to serve a wide variety of functions that are only partially understood but include key roles in light harvesting and photosynthesis, oxidative stress protection, and as precursors of signaling and defense molecules that function outside the chloroplast (e.g., abscisic acid, gibberellins, and terpenes).⁷⁵⁻⁷⁷ The *Plasmodium* apicoplast has lost photosynthesis capabilities and has uncertain carotenoid and

858 terpene synthesis capacity. Volatile terpenes⁷⁸ and carotenoids⁵³ have been detected in *P.*
859 *falciparum*-infected erythrocytes, but the parasite genome lacks enzyme homologs of the
860 relevant synthases required for terpene and carotenoid biosynthesis.^{1, 7} Furthermore, we found
861 no evidence of de novo β -carotene synthesis by parasites, and results herein as well as recent
862 studies^{79, 80} indicate that these metabolites can derive from erythrocyte and/or culture medium
863 sources rather than parasite-specific synthesis. Thus, these known functions in chloroplasts seem
864 uncertain or unlikely to explain apicoplast reliance on longer-chain polyprenyl synthase activity
865 in malaria parasites. Longer-chain polyprenyl-PPs and related dolichols serve as membrane-
866 bound glycan carriers for protein glycosylation, but these activities in *Plasmodium* appear to
867 occur in the endoplasmic reticulum as they do in other organisms.^{20, 81, 82}

868 Linear polyprenyl alcohols have been found to be important components of plant
869 membranes, especially chloroplast membranes, where they are proposed to modulate membrane
870 structure, fluidity, and dynamics.^{75, 77, 83, 84} In the absence of other known roles for longer-chain
871 polyprenyl-PPs in the apicoplast, we hypothesize that linear polyprenols or polyprenyl
872 phosphates may serve as critical components of the apicoplast membranes and be required for
873 maintaining membrane fluidity during organelle biogenesis (Figure 9). A prior mass
874 spectrometry-based lipidomics study of isolated apicoplasts focused primarily on the fatty acid
875 and phospholipid composition of this organelle and did not characterize isoprenoid
876 components.⁸⁵ Selective isotopic labeling of parasite-synthesized isoprenoids by 2-¹³C-
877 mevalonate in the PfMev line, combined with apicoplast isolation and our PPS knockdown line,
878 can potentially identify specific apicoplast isoprenoids whose synthesis depends on PPS activity
879 and thus clarify why apicoplast biogenesis requires longer-chain polyprenyl synthase activity.

Independent of its specific role in apicoplast biogenesis, PPS function is critical for parasite survival and thus constitutes a new essential arm of isoprenoid metabolism in the apicoplast suitable for development as a therapeutic target. BLAST analysis of the human genome using the PPS protein sequence as query reveals a variety of polyprenyl synthase homologs with modest 20-30% sequence identity to 25-50% of the PPS sequence. The substantial sequence differences with human orthologs will facilitate selective targeting of PPS by chemical inhibitors. Identification of PPS as an apicoplast-targeted enzyme indicates that new metabolic pathways and functions remain to be discovered and/or localized to the apicoplast. These novel functions, which are predicted to be required for organelle maintenance, will enhance our understanding of fundamental apicoplast biology and provide new candidate drug targets for antimalarial therapies.

Figure 9. Schematic diagram of apicoplast isoprenoid metabolism in blood-stage *P. falciparum* parasites. IPPI = IPP isomerase, PEP = phosphoenolpyruvate, DHAP = dihydroxyacetone phosphate, PYR = pyruvate, Gly3P = glyceraldehyde-3-phosphate. Question marks indicate uncertainty in the identity of the proposed IPP isomerase and in the role of polyprenyl isoprenoid products of PPS in apicoplast biogenesis. For simplicity, we have depicted PPS targeting to the apicoplast matrix. However, further experiments will be needed to test and specify sub-organellar targeting of PPS to the apicoplast matrix and/or intermembrane spaces.

MATERIALS AND METHODS

Key Resources Table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
Cell line (<i>Plasmodium falciparum</i>)	D10 ACPL-GFP	PMID: 10775264		
Cell line	NF54-PfMev	PMID: 32059044		

<i>(Plasmodium falciparum)</i>	ACP _L -GFP			
Cell line <i>(Plasmodium falciparum)</i>	NF54-PfMev ΔDXS	PMID: 32815516		
Cell line <i>(Plasmodium falciparum)</i>	Dd2 PPS-RFP (pTyEOE)	This study		Described in Materials and Methods. Can be obtained from Sigala lab.
Cell line <i>(Plasmodium falciparum)</i>	Dd2 PPS-GFP (pTEOE)	This study		Described in Materials and Methods. Can be obtained from Sigala lab.
Cell line <i>(Plasmodium falciparum)</i>	Dd2 PPS-HA/FLAG 9xAptamer/ TetR-DOZI	This study		Described in Materials and Methods. Can be obtained from Sigala lab.
Cell line <i>(Plasmodium falciparum)</i>	NF54-PfMev MiaA-KO	This study		Described in Materials and Methods. Can be obtained from Prigge lab.
Cell line <i>(Escherichia coli)</i>	Top10 pAC-BETAipi	PMID: 15659105		
Software, algorithm	Prism	GraphPad	RRID: SCR_002798	
Chemical compound, drug	Doxycycline	Sigma-Aldrich	Cat. No. D3447	
Chemical compound, drug	Fosmidomycin	Invitrogen Life Technologies	Cat. No. F23103	
Chemical compound, drug	Isopentenyl pyrophosphate	Isoprenoids	Cat. No. IPP001	
Chemical compound, drug	Farnesol	Sigma-Aldrich	Cat. No. F203	
Chemical compound, drug	Geranylgeraniol	Sigma-Aldrich	Cat. No. G3278	
Chemical compound, drug	Decaprenol	Isoprenoids	Cat. No. polyprenol C50	

Chemical compound, drug	DL-mevalonolactone	Cayman Chemicals	Cat. No. 20348	
Chemical compound, drug	Zaragozic acid	Cayman Chemicals	Cat. No.17452	
Chemical compound, drug	MMV019313	ChemDiv	Cat. No. C498-0579	
Chemical compound, drug	Beta-carotene	Sigma-Aldrich	Cat. No. F203	
Antibody	Anti-EF1 α (rabbit polyclonal)	PMID: 11251817		(1:1000)
Antibody	Anti-ACP (rabbit polyclonal)	PMID: 19768685		(1:1000)
Antibody	Anti-GFP (3E6) (mouse, monoclonal)	Invitrogen Life Technologies	Cat. No. A11120	(1:1000)
Antibody	Anti-RFP (RF5R) (mouse monoclonal)	Invitrogen Life Technologies	Cat. No. MA5-15257	(1:1000)
Antibody	Anti-GFP (goat, polyclonal)	Abcam	Cat. No. Ab5450	(1:1000)
Antibody	Anti-HA (3F10) (rat, monoclonal)	Roche	Cat. No. 11 867 423 001	(1:1000)

903
904

905 **Materials:** All reagents were of the highest purity commercially available. The vendor and
906 catalog number are given for individual compounds when first mentioned. Primary antibodies
907 were generally used at 1:1000 dilution and secondary antibodies at 1:10,000 dilution unless
908 specified otherwise.

909

910 **Fluorescence Microscopy:** For live-cell experiments, parasite samples were collected at 38
911 hours after synchronization with 5% D-sorbitol (Sigma S7900). Parasite nuclei were visualized
912 by incubating samples with 1-2 μ g/ml Hoechst 33342 (Thermo Scientific Pierce 62249) for 10-
913 20 minutes at room temperature. The parasite apicoplast was visualized in D10²⁸ or NF54
914 mevalonate-bypass²⁹ cells using the ACP_L-GFP expressed by both lines. The parasite

mitochondrion was visualized by incubating parasites with 10 nM MitoTracker Red CMXRos (Invitrogen Life Technologies M7512) for 15 minutes prior to wash-out and imaging. For immunofluorescence assay (IFA) experiments, parasites were fixed, stained, and mounted as previously described.⁸⁶ For IFA images, the parasite apicoplast was visualized using a polyclonal rabbit anti-ACP antibody⁸⁷ and a goat anti-rabbit fluorescent 2° antibody (Invitrogen R37117) and the nucleus was stained with ProLong Gold Antifade Mountant with DAPI (Invitrogen Life Technologies P36931). PPS-RFP was visualized with a mouse monoclonal anti-RFP antibody (ThermoFisher MA5-15257) and goat anti-mouse fluorescent 2° antibody (Invitrogen A11001). PPS-GFP was detected with a goat anti-GFP antibody (Abcam ab5450) and anti-goat fluorescent 2° antibody. For IFA images of parasites expressing endogenous PPS with C-terminal HA-FLAG tags, apicoplast ACP was visualized with a polyclonal rabbit anti-ACP antibody as above and PPS was visualized using polyclonal rat anti-HA-tag 1° antibody (Roche 3F10) and goat anti-rat (Invitrogen A11006) and donkey anti-goat (Invitrogen A11055) fluorescent 2° antibodies. Images were taken on DIC/brightfield, DAPI, GFP, and RFP channels using either a Zeiss Axio Imager or an EVOS M5000 imaging system. Fiji/ImageJ was used to process and analyze images, and intensity plots were generated by the “plot profile” function using a shared region of interest (identified by white line) on each channel. All image adjustments, including contrast and brightness, were made on a linear scale. For phenotypic analyses, apicoplast morphologies for each experimental condition were assessed for 25 parasites in each of two biological replicate experiments (50 parasites total per condition). Apicoplast morphologies were scored as elongated, focal, or dispersed; counted; and plotted by histogram as the fractional population with the indicated morphology.

Inhibition and Rescue of Apicoplast Biogenesis: ACP_L-GFP D10 and NF54 PfMev parasites were synchronized with 5% (w/v) D-sorbitol for 10 minutes at room temperature and returned to culture in 10 μ M fosmidomycin (Invitrogen Life Technologies F23103), 100 nM atovaquone (Cayman Chemicals 23802), 2 μ M DSM1,⁸⁸ 6 μ M blasticidin-S (Invitrogen Life Technologies R21001), 5 nM WR99210 (Jacobus Pharmaceuticals), 160 μ M zaragozic acid/squalestatin (Cayman Chemicals 17452), or 2 μ M MMV019313 (ChemDiv C498-0579). For FOS experiments, parasites were left in FOS only or supplemented with 5 μ M farnesol (Sigma F203), 5 μ M geranylgeraniol (Sigma G3278), 5 μ M decaprenol (Isoprenoids polyprenol C50), 5 μ M β -carotene (Sigma C9750), 50 μ M DL-mevalonolactone (Cayman Chemicals 20348), or 200 μ M IPP (NH₄⁺ salt, Isoprenoids IPP001). All parasites were cultured for 36 hours after synchronization and then imaged by live-cell fluorescence microscopy to monitor apicoplast status. All concentrations reflect the final concentration in culture medium.

Parasite synchronization: Parasites were synchronized to the ring stage either by treatment with 5% D-sorbitol (Sigma S7900) or by first magnet-purifying schizonts and then incubating them with uninfected erythrocytes for 5 hr followed by treatment with sorbitol. Results from growth assays and microscopy analyses using either of these synchronization methods were indistinguishable within error, and 5% sorbitol was used unless stated otherwise.

Delayed Mevalonate-Rescue Assay: NF54 PfMev parasites were synchronized with 5% (w/v) D-sorbitol for 10 minutes at room temperature and returned to culture in 10 μ M fosmidomycin. 50 μ M DL-mevalonate was added to cultures immediately or after 30, 34, or 38 hours post synchronization. Parasitemia was measured by flow cytometry every 24 hours. After 60 hours

post-synchronization, parasites from each mevalonate time point were cloned out by limiting dilution. Apicoplast status of all isolated clones was evaluated by live-cell ACP_L-GFP fluorescence. ACP_L-GFP signal was observed for the presence of distinct branching morphology (apicoplast intact) or the presence of scattered punctate signals throughout the cytosol (apicoplast disruption). A total of 9, 17, 18, and 5 clones from the 0, 30, 34, and 38-hour rescue time-points, respectively, were evaluated by microscopy (only 5 clones returned from the 38-hour rescue time point). Apicoplast (SufB: Pf3D7_API04700) and nuclear (PPS: Pf3D7_0202700) genome PCR (primers 4/5 and 1/2) and mevalonate-dependence growth assays were done on 2 clones from each time point to confirm apicoplast status.

Parasite Culturing and Transfection: All experiments were performed using *Plasmodium falciparum* Dd2⁸⁹, ACP_L-GFP D10²⁸, or ACP_L-GFP NF54 PfMev²⁹ parasite strains. Parasite strain identities were confirmed on the basis of expected drug resistance and were *Mycoplasma*-free by PCR test. Parasite culturing was performed in Roswell Park Memorial Institute medium (RPMI-1640, Thermo Fisher 23400021) supplemented with 2.5 g/L Albumax I Lipid-Rich BSA (Thermo Fisher 11020039), 15 mg/L hypoxanthine (Sigma H9636), 110 mg/L sodium pyruvate (Sigma P5280), 1.19 g/L HEPES (Sigma H4034), 2.52 g/L sodium bicarbonate (Sigma S5761), 2 g/L glucose (Sigma G7021), and 10 mg/L gentamicin (Invitrogen Life Technologies 15750060). Cultures were generally maintained at 2% hematocrit in human erythrocytes obtained from the University of Utah Hospital blood bank, at 37 °C, and at 5% O₂, 5% CO₂, 90% N₂. Parasite-infected erythrocytes were transfected in 1X cytomix containing 50-100 µg midi-prep DNA by electroporation in 0.2 cm cuvettes using a Bio-Rad Gene Pulser Xcell system (0.31 kV, 925 µF). Transgenic parasites were selected on the basis of plasmid resistance cassettes encoding human

DHFR³⁶, yeast DHOD⁸⁸, or blasticidin-S deaminase (BSD)³⁵ and cultured in 5 nM WR99210, 2 μ M DSM1, or 6 μ M blasticidin-S, respectively. Gene-edited Dd2 parasites that contained PPS (PF3D7_0202700) tagged with the aptamer/TetR-DOZI cassette⁵⁸ were maintained in 0.5-1 μ M anhydrotetracycline (Cayman Chemicals 10009542). Genetically modified parasites were genotyped by PCR and/or Southern blot, as previously described.⁹⁰ For western blot and IFA studies of PPS-GFP in apicoplast-disrupted Dd2 parasites, transgenic parasites were cultured >7 days in 5 nM WR99210, 1 μ M doxycycline (Sigma D9891), and 200 μ M IPP to induce stable apicoplast loss prior to parasite harvest.

Parasite Growth Assays: Parasite growth was monitored by diluting asynchronous or sorbitol-synchronized parasites to ~0.5% parasitemia and allowing culture expansion over several days with daily media changes. Parasitemia was monitored daily by flow cytometry by diluting 10 μ l of each parasite culture well from each of three biological replicate samples into 200 μ l of 1.0 μ g/ml acridine orange (Invitrogen Life Technologies A3568) in phosphate buffered saline (PBS) and analysis on a BD FACSCelesta system monitoring SSC-A, FSC-A, PE-A, FITC-A, and PerCP-Cy5-5-A channels. Daily parasitemia measurements for asynchronous cultures were plotted as function of time and fit to an exponential growth equation using GraphPad Prism 9.0. For EC₅₀ determinations, synchronous ring-stage parasites were diluted to 1% parasitemia and incubated with variable drug concentrations for 48-72 hours without media changes. Parasitemia was determined by flow cytometry in biological triplicate samples for each drug concentration, normalized to the parasitemia in the absence of drug, plotted as a function of the log of the drug concentration (in nM or μ M), and fit to a 4-parameter dose-response model using GraphPad Prism 9.0.

Cloning and Episomal Expression of PPS: The gene encoding PPS (PF3D7_0202700) lacks introns and was cloned by PCR from Dd2 parasite genomic DNA using primers designed for insertion into the XhoI/AvrII sites of pTYEOE (yeast DHOD positive selection cassette)⁹¹ and pTEOE (human DHFR positive selection cassette)⁵⁶ vectors in frame with C-terminal RFP and GFP tags, respectively. These vectors are designed to drive episomal protein expression using the HSP86 promoter and for co-transfection with plasmid pHTH that contains the piggyBac transposase⁹² for integration into the parasite genome. A single forward primer was used for PPS cloning into both vectors (primer 1) while reverse primers were vector-specific (primers 2 and 3). All PCR primer sequences are shown in Supplementary file 1. Cloning was completed using ligation-independent cloning (QuantaBio RepliQa HiFi Assembly Mix). Cloning products were transformed into Top10 chemically competent cells, and bacterial clones were selected for carbenicillin (Sigma C3416) resistance. Correct plasmid sequence in isolated clonal bacteria was confirmed by both AscI/AatII (NEB) restriction digest and Sanger sequencing (University of Utah DNA Sequencing Core). 100 µg of either purified PPS-RFP-TyEOE or PPS-GFP-TEOE in combination with 25 µg of the pHTH transposase plasmid was transfected into Dd2 parasites by electroporation, as described above. Transfected parasites were allowed to expand in the absence of drug for 48 hours before selection with either 2 µM DSM1 or 5 nM WR99210 for PPS-RFP-TyEOE or PPS-GFP-TEOE respectively. Stable, drug-resistant parasites returned from transfection in 3-6 weeks.

PPS Gene-Editing to Enable Ligand-Dependent Regulation of Protein Expression: Crispr/Cas9-stimulated repair by double-crossover homologous recombination was used to tag

the PPS gene (PF3D7_0202700) to encode a C-terminal hemagglutinin (HA)-FLAG epitope tag and the 3' 10X aptamer/TetR-DOZI system⁵⁸ to enable regulated PPS expression using anhydrotetracycline. Guide RNA sequences corresponding to TGATATAAAACAAAGTAGCG, CGTGCTAGTTCTATTTTTGC, and GATGATTCAAATAAAAGAAG (primers 6-11) were cloned into a modified version of the previously published pAIO vector,⁹³ in which the BtgZI site was replaced with a unique HindIII site to facilitate cloning (primers 12 and 13). To tag the PPS gene, a donor pMG75⁵⁸ repair plasmid was prepared by PCR-amplifying 635 bp of the 3' coding sequence and 679 bp of the 3' untranslated region (UTR) as homology flanks to the PPS gene, fusing these fragments together by PCR with an AflII site in between (679 bp 3' UTR-AflII-635 bp 3' coding sequence), and inserting this fused fragment into the AscI and AatII sites of the pMG75 vector (primers 14-17). A shield mutation was introduced to the 3' end of the coding-sequence homology flank corresponding to the gRNA sequence TGATATAAAACAAAGTAGCG. This mutation (introduced using primer 18) ablated the CRISPR PAM sequence AGG that immediately following the gRNA sequence above by mutating it to AAG, resulting in a silent mutation of the Glu523 codon from GAG to GAA. Sanger sequencing confirmed the correct sequence of the homology flanks inserted into the pMG75 vector. PCR analysis of the final pMG75 vector using primers 39-40 revealed that only 9 copies of the aptamer sequence were retained. Before transfection, the pMG74 vector was linearized by AflII digestion performed overnight at 37° C, followed by deactivation with Antarctic Phosphatase (NEB M0289S).

Dd2 parasites were transfected with 50 µg of pAIO Cas9/gRNA vector and 50 µg of the linearized pMG75 donor plasmid, as described above. Parasites were selected on the basis of the BSD resistance cassette encoded by the pMG75 plasmid and returned from transfection after 4-6

weeks. Gene-edited Dd2 parasites resulting from transfection with pAIO Cas9/gRNA-4 (produced with primers 10/11) contained PPS (PF3D7_0202700) tagged with the aptamer/TetR-DOZI cassette⁵⁸ and were maintained in 0.5-1 μ M anhydrotetracycline (Cayman Chemicals 10009542). Genetically modified parasites were genotyped by Southern blot, as previously described.⁹⁰ Briefly, genomic DNA from the polyclonal parasites that returned from transfection was digested with BamHI and SpeI (New England Biolabs) and transferred to membrane (Nytran SuPerCharge) using the TurboBlotter system (VWR 89026-838). A DNA probe consisting of the 5' 750 bp of the PPS gene was produced by PCR (primers 16/17). Probe labeling, hybridization, and visualization was performed using the AlkPhos Direct Labeling and Detection System (VWR 95038-288) and CDP-Star reagent (VWR 95038-292). The Southern blot confirmed complete integration into the PPS locus without evidence for unmodified parasites, and the polyclonal parasites were used for all subsequent experiments.

Analysis of PPS transcript levels: Biological replicate cultures of PPS-aptamer/TetR-DOZI parasites were synchronized in 5% D-sorbitol and grown for 72 hours in +aTc (four biological replicates), -aTc (four biological replicates), or -aTc/+IPP (200 μ M) (two biological replicates) conditions prior to harvest. 4-ml cultures at approximately 10% were harvested by centrifugation (2000 rpm for 3 min.) and stored at -20 °C until use. Total RNA was isolated from frozen parasite-infected blood pellets using a modified Trizol (Invitrogen) extraction protocol. 5 mL Trizol (Invitrogen) was added to thawed pellets on ice, pipetted 20-30 times to resuspend, and pulse-vortexed 20 times for 15 s. 2 mL chloroform was added to each sample and vortexed, incubated on ice for 5 minutes, then spun for 10 minutes at 4°C at 5000rpm without brake. The top, aqueous layer (approximately 3 mL) was transferred to a new tube. 5 mL of isopropanol was

1076 added to each sample, gently mixed, and incubated at -80 °C for 20 minutes or -20 °C overnight.
1077 Samples were spun at 5000 rpm for 30 min, washed with freshly-made solution of 70% ethanol,
1078 then spun again for 10 min. Ethanol was removed and pellets were dried 30 min on ice. RNA
1079 pellets were resuspended in RNase-free water, quantitated, and used immediately or stored at -
1080 80 °C. 1 µg of RNA was DNase-treated and reverse-transcribed using Superscript IV kit
1081 (Invitrogen) with the addition of gene-specific reverse primers 31-38. Subsequent cDNA was
1082 analyzed in technical duplicate through qPCR reactions with SYBR Green fluorescent probe
1083 (Invitrogen) in a Roche Lightcycler. Cp values for PPS (primers 35-36) were normalized to the
1084 average of 2 nuclear-encoded control genes (I5P, PF3D7_0802500; ADSL, PF3D7_0206700;
1085 primers 31-34), then used to calculate relative RNA abundance values for each culture condition,
1086 with the +aTc sample normalized to a value of one. Data is reported as average ±SD of replicates
1087 for each condition.

1088

1089 **Synchronous Growth Assays of PPS Knockdown Parasites:** Dd2 parasites tagged at the
1090 genomic PPS locus with the aptamer/TetR-DOZI system were synchronized by 5% D-sorbitol to
1091 ring-stage parasites and allowed to expand ±aTc in two or three biological replicate samples.
1092 Parasitemia values were measured daily by flow cytometry and plotted as the average ±SD of
1093 replicate samples. For growth-rescue experiments, synchronous parasites were allowed to expand
1094 ±aTc, and -aTc plus 200 µM IPP, 5 µM farnesol (FOH), 5 µM geranylgeraniol (GGOH), or 5
1095 µM decaprenol (C₅₀-OH). For growth-rescue experiments involving fosmidomycin (FOS), PPS
1096 KD parasites were synchronized to rings with 5% D-sorbitol and grown for 4 days (96 hours)
1097 ±aTc. After 96 hours, all culture wells were synchronized again with 5% D-sorbitol and
1098 supplemented with 10 µM FOS and 200 µM IPP, 5 µM FOH, 5 µM GGOH, or 5 µM decaprenol.

1099 Parasites were cultured for another 38 hours before harvest at 134 total hours post-initial
1100 synchronization for IFA analysis of apicoplast morphology. Parasites grown $\pm$ aTc with 10 μ M
1101 FOS and 200 μ M IPP were allowed to expand for an additional 48 hours and harvested at 182
1102 hours post-initial synchronization for analysis by IFA and qPCR for apicoplast morphology and
1103 apicoplast:nuclear genome levels, respectively.

1104

1105 **qPCR Analysis of Apicoplast:Nuclear genomic DNA levels:** Genomic DNA was extracted
1106 from triplicate parasite samples grown $\pm$ aTc with 10 μ M FOS and 200 μ M IPP and harvested at
1107 182 hours post-initial synchronization. DNA extraction was performed using the QIAmp DNA
1108 Blood Mini Kit (Qiagen 51104). Primers for qPCR were designed to amplify a 120-140 bp
1109 region of an apicoplast gene (TufA, PF3D7_API02900, primers 35-36) and each of two nuclear
1110 genes (I5P, PF3D7_0802500; ADSL, PF3D7_0206700; primers 31-34). Approximately 100 ng
1111 of DNA was amplified in each of three biological replicates with PowerUp SYBR Green Master
1112 Mix (ThermoFisher A25741) in a 96-well plate with 20 μ l reaction volume on a Quantstudio3
1113 Real Time PCR system. Specificity of primer amplification was confirmed for every sample by
1114 identifying only one melting temperature for the product of each qPCR reaction. Abundance of
1115 apicoplast relative to nuclear DNA was determined by comparative C_t analysis,⁹⁴ with
1116 amplification of TufA (apicoplast) and I5P (nuclear) and calculation of $2^{\Delta C_t}$, where $\Delta C_t = C_{tTufA}$
1117 $- C_{tI5P}$. As a positive control, abundance of a second nuclear gene (ADSL) relative to I5P was
1118 calculated similarly. The $2^{\Delta C_t}$ value for TufA or ADSL was normalized to +aTc for each gene to
1119 determine a normalized target gene:control gene DNA abundance. Error bars represent the
1120 standard deviation between replicates, and P values were determined by two-tailed unpaired t-
1121 test in GraphPad Prism 9.0.

1122

1123 **MiaA Gene Disruption:** The gene encoding MiaA (PF3D7_1207600) was disrupted in the
1124 NF54 PfMev line using CRISPR/Cas9 and gene deletion by double-crossover homologous
1125 recombination, similar to the recently described disruption of the DXPR gene
1126 (PF3D7_1467300).²⁹ Homology arm regions (411 bp for the 5' arm and 540 bp for the 3' arm)
1127 were PCR-amplified from genomic DNA with primers 19-22 and cloned into the vector pRS²⁹
1128 using ligation-independent cloning (In-Fusion, Clontech). A guide RNA with sequence
1129 AATAACGATATTAAATGTAA was cloned into a modified pAIO vector called pCasG⁹⁵ using
1130 primers 23 and 24. 75 µg of pRS-miaA-KO plasmid was combined with 75 µg of the pCasG
1131 guide RNA plasmid and transfected into NF54 PfMev parasites. Transfected parasites were
1132 allowed to expand for 48 hours in 50 µM mevalonate before selection with 5 nM WR99210 and
1133 50 µM mevalonate. Parasites returning from positive selection were genotyped by PCR using
1134 primers 25-30. Asynchronous growth of Δ MiaA PfMev parasites $\pm$ Mev compared to parental
1135 PfMev parasites was performed on biological duplicate samples. Average parasitemia values
1136 $\pm$ SD were plotted versus time and fit to an exponential growth equation in GraphPad Prism 9.0.
1137 Apicoplast (SufB: PF3D7_API04700) and nuclear (LDH: PF3D7_1324900) genome PCR was
1138 performed to confirm apicoplast status in parental PfMev and Δ MiaA parasites, as previously
1139 reported.²⁹

1140

1141 **Western Blots:** Samples of episomal PPS-GFP Dd2 or endogenously HA-FLAG-tagged PPS
1142 Dd2 parasites were harvested by centrifugation and treated with 0.05% saponin (Sigma 84510) in
1143 PBS for 5 min at room temperature and spun down by centrifuge at 5,000 rpm for 30 minutes at
1144 4°C. 2% SDS or LDS sample buffer (Life Technologies NP0007) was added to saponin pellets,

resuspended by sonication, and incubated overnight at 4°C. 5x Sample buffer containing beta-mercaptoethanol (BME) was added to parasite samples before heating at 95°C for 5 minutes and centrifuging at 13,000 rpm for 5 minutes. Samples were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide gels run at 120 V in the BIO-RAD mini-PROTEAN electrophoresis system. Fractionated proteins were transferred from polyacrylamide gel to a nitrocellulose membrane at 100V for one hour using the BIO-RAD wet transfer system. Membranes were blocked in 1% casein/PBS for one hour at room temperature and then probed with primary antibody overnight at 4°C and secondary antibody at room temperature for 1 hour. Episomal PPS-RFP parasite samples were probed with 1:1000 mouse anti-RFP (Invitrogen Life Technologies MA5-15257) and 1:10,000 donkey anti-mouse DyLight800 (Invitrogen Life Technologies SA5-10172). Endogenous HA-FLAG-tagged PPS parasite samples were probed with Roche rat anti-HA monoclonal 3F10 (Sigma 11867423001) and mouse anti-FLAG (Sigma F1804) primary antibodies and Goat anti-Rat IRDye800CW (Licor 926-32219) and Goat anti-mouse IRDye800CW (Licor 925-32210) secondary antibodies. As a loading control, blots were probed with rabbit anti-EF1 α 1° antibody⁹⁶ and donkey anti-rabbit-IRDye680 2° antibody (Licor 926-68023). Densitometry analysis of band intensities was performed using the Licor Image Studio Lite software and median local background.

Sequence Similarity Analysis and Structural Homology Modeling: Sequence similarity searches for *P. falciparum* homologs to chicken FPPS (Uniprot P08836) were performed by BLASTP analysis as implemented at the Plasmodium Genomics Resource webpage (www.plasmodb.org). Sequence similarity searches using the PPS (PF3D7_0202700) protein sequence as query were carried out using NCBI BLAST⁵¹ (excluding organisms in the phylum

Apicomplexa to which *P. falciparum* belongs) and MPI HHpred⁵². A homology model of PPS was generated by the MPI HHpred software using the X-ray crystallographic structural model of *E. coli* OPPS (PDB 3WJK), which was one of the top 10 homology hits by HHpred analysis, as template. Structural models were visualized using PyMol (Schrödinger).

β-Carotene Extraction and Analysis by Mass Spectrometry: For determination of beta-carotene levels in parasite-infected versus uninfected erythrocytes, 35 ml of 4% hematocrit *P. falciparum* culture infected at 13-15% parasitemia with the Dd2 PPS aptamer/TetR-DOZI knockdown parasites were collected after 6 days of growth in the presence or absence of 1 μM aTc. Uninfected erythrocyte samples were prepared by collecting 20 ml of 4% hematocrit uninfected culture incubated for 6 days in RPMI media that lacked or contained 2.5 g/L AlbuMAX. Samples of infected or uninfected erythrocytes were harvested by centrifugation, lysed by 0.05% saponin, and pelleted by centrifugation. Saponin pellets were washed in PBS and then extracted three times in 1 ml of chilled acetone (pellet was briefly sonicated after addition of the first acetone volume). The supernatant of each extraction was pooled and dried down by vacuum concentration (Speed Vac). Three biological replicates of each sample were prepared. For analysis of AlbuMAX, 85 mg of dry AlbuMAX (equivalent to the AlbuMAX content in 35 ml of complete culture media) was extracted in 3 volumes of cold acetone, and supernatants were combined and dried as above.

For analysis of β-carotene synthesis, NF54 PfMev parasites were cultured and expanded over three intraerythrocytic cycles in media containing 50 μM 2-¹³C-mevalonate and 10 μM fosmidomycin. This strategy was chosen to inhibit MEP pathway activity, ensure full ¹³C-labeling of the endogenous IPP and DMAPP precursor pool within parasites produced by the

1191 cytoplasmic bypass enzymes, and result in a distinguishable 8 Da mass increase for any β -
1192 carotene derived from de novo synthesis. Final parasite samples contained 70 ml of culture at
1193 15% parasitemia and were collected by centrifugation prior to 0.05 % saponin lysis,
1194 centrifugation, and washing the pellet in PBS. The final pellet was extracted in acetone and
1195 dried, as described above.

1196 As a positive control for detecting isotopic incorporation of ^{13}C -labeled precursors into
1197 biosynthesized β -carotene, we turned to studies of *E. coli* bacteria engineered to biosynthesize β -
1198 carotene⁶⁵ and grown in minimal M9 medium with uniformly labeled ^{13}C -glucose as the sole
1199 carbon source. Growth of bacteria in these conditions was expected to lead to a 40-Da mass
1200 increase in detected β -carotene. 5-ml cultures of pAC-BETAipi *E. coli* or untransformed Top10
1201 *E. coli* were allowed to expand over two days at 30° C in the dark. Bacterial cultures were
1202 harvested by centrifugation at 5,000 rpm for 10 min., and bacterial pellets were extracted three
1203 times in cold acetone and dried, as described above.

1204 LC-MS-grade methanol, acetonitrile, isopropyl alcohol, chloroform, and formic acid
1205 were purchased from VWR. Samples were resuspended in 50 μl MeOH/ CHCl_3 (2mM LiI), and a
1206 sample volume of 10 μl was injected onto a Phenomenex Luna 150 x 2.1 mm reverse-phase C8
1207 column maintained at 30 °C and connected to an Agilent HiP 1290 Sampler, Agilent 1290
1208 Infinity pump, and Agilent 6545 Accurate Mass Q-TOF dual AJS-ESI mass spectrometer. The
1209 instrument was operated in positive ion mode, and the source gas temperature was 275 °C with a
1210 drying gas flow of 12 L/min, nebulizer pressure of 35 psig, sheath gas temp of 325 °C and sheath
1211 gas flow of 12 L/min. VCap voltage was set at 3500 V, nozzle voltage 250 V, fragmentor at 90
1212 V, skimmer at 65 V, octopole RF peak at 750 V and a scan range m/z 40 - 900. The mobile
1213 solvent phase A was H_2O with 0.1% formic acid, and mobile phase B was MeOH:ACN:IPA

(2:2:1 v/v) with 0.1% formic acid. The chromatography gradient started at 80% mobile phase B then increased to 100% B over 6 min where it was held until 9.9 min and then returned to the initial conditions and equilibrated for 5 min. The column flow rate was 0.5 mL/min.

Results from LC-MS experiments were collected using Agilent Mass Hunter (MH) Workstation and analyzed using the software packages MH Qual and MH Quant (Agilent Technologies, Inc.). Unlabeled, 2-¹³C-mevalonate-labeled, and uniform ¹³C-glucose-labeled β -carotene were analyzed using the molecular ions of m/z 536.4377, m/z 544.4645, and m/z 576.5718, respectively. Fragmentation profiling of unlabeled β -carotene by MS/MS confirmed the expected product ions at m/z 444 and m/z 119, as previously reported.⁹⁷ For quantitation of unlabeled β -carotene levels in experimental samples and determination of a limit of detection (LOD), a calibration curve was constructed using serial dilutions of commercial β -carotene (Sigma C9750). The concentration LOD for a 10- μ L sample of unlabeled β -carotene in this assay was 2.6 ng/mL. Integrated peak areas for unlabeled β -carotene in experimental samples were converted to concentration values in the 10- μ L sample using this calibration curve.

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1237 **REFERENCES**

- 1238 [1] Ralph, S. A., van Dooren, G. G., Waller, R. F., Crawford, M. J., Fraunholz, M. J., Foth, B. J.,
 1239 Tonkin, C. J., Roos, D. S., and McFadden, G. I. (2004) Metabolic maps and functions of
 1240 the *Plasmodium falciparum* apicoplast, *Nat. Rev. Microbiol.* 2, 203-216. DOI:
 1241 <https://doi.org/10.1038/nrmicro843>
- 1242 [2] Ke, H., Sigala, P. A., Miura, K., Morrissey, J. M., Mather, M. W., Crowley, J. R., Henderson,
 1243 J. P., Goldberg, D. E., Long, C. A., and Vaidya, A. B. (2014) The heme biosynthesis
 1244 pathway is essential for *Plasmodium falciparum* development in mosquito stage but not
 1245 in blood stages, *J. Biol. Chem.* 289, 34827-34837. DOI:
 1246 <https://doi.org/10.1074/jbc.M114.615831>
- 1247 [3] Goldberg, D. E., and Sigala, P. A. (2017) Plasmodium heme biosynthesis: To be or not to be
 1248 essential?, *PLoS Pathog.* 13, e1006511. DOI:
 1249 <https://doi.org/10.1371/journal.ppat.1006511>
- 1250 [4] Yu, M., Kumar, T. R. S., Nkrumah, L. J., Coppi, A., Retzlaff, S., Li, C. D., Kelly, B. J.,
 1251 Moura, P. A., Lakshmanan, V., Freundlich, J. S., Valderramos, J. C., Vilcheze, C.,
 1252 Siedner, M., Tsai, J. H. C., Falkard, B., Sidhu, A. B. S., Purcell, L. A., Gratraud, P.,
 1253 Kremer, L., Waters, A. P., Schiehsler, G., Jacobus, D. P., Janse, C. J., Ager, A., Jacobs,
 1254 W. R., Sacchettini, J. C., Heussler, V., Sinni, P., and Fidock, D. A. (2008) The Fatty Acid
 1255 Biosynthesis Enzyme FabI Plays a Key Role in the Development of Liver-Stage Malarial
 1256 Parasites, *Cell Host Microbe* 4, 567-578. DOI:
 1257 <https://doi.org/10.1016/J.Chom.2008.11.001>
- 1258 [5] Shears, M. J., Botte, C. Y., and McFadden, G. I. (2015) Fatty acid metabolism in the
 1259 *Plasmodium* apicoplast: Drugs, doubts and knockouts, *Mol. Biochem. Parasitol.* 199, 34-
 1260 50. DOI: <https://doi.org/10.1016/j.molbiopara.2015.03.004>
- 1261 [6] Dahl, E. L., and Rosenthal, P. J. (2007) Multiple antibiotics exert delayed effects against the
 1262 *Plasmodium falciparum* apicoplast, *Antimicrob. Agents Chemother.* 51, 3485-3490. DOI:
 1263 <https://doi.org/10.1128/AAC.00527-07>
- 1264 [7] Guggisberg, A. M., Amthor, R. E., and Odom, A. R. (2014) Isoprenoid biosynthesis in
 1265 *Plasmodium falciparum*, *Eukaryot. Cell* 13, 1348-1359. DOI:
 1266 <https://doi.org/10.1128/EC.00160-14>
- 1267 [8] Jomaa, H., Wiesner, J., Sanderbrand, S., Altincicek, B., Weidemeyer, C., Hintz, M.,
 1268 Turbachova, I., Eberl, M., Zeidler, J., Lichtenthaler, H. K., Soldati, D., and Beck, E.
 1269 (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as
 1270 antimalarial drugs, *Science* 285, 1573-1576. DOI:
 1271 <https://doi.org/10.1126/science.285.5433.1573>
- 1272 [9] van Dooren, G. G., Stimmler, L. M., and McFadden, G. I. (2006) Metabolic maps and
 1273 functions of the *Plasmodium* mitochondrion, *FEMS Microbiol. Rev.* 30, 596-630. DOI:
 1274 <https://doi.org/10.1111/j.1574-6976.2006.00027.x>
- 1275 [10] Simao-Gurge, R. M., Wunderlich, G., Cricco, J. A., Cubillos, E. F. G., Domenech-Carbo,
 1276 A., Cebrian-Torrejon, G., Almeida, F. G., Cirulli, B. A., and Katzin, A. M. (2019)
 1277 Biosynthesis of heme O in intraerythrocytic stages of *Plasmodium falciparum* and
 1278 potential inhibitors of this pathway, *Sci. Rep.* 9, 19261. DOI:
 1279 <https://doi.org/10.1038/s41598-019-55506-y>

- [11] Yeh, E., and DeRisi, J. L. (2011) Chemical rescue of malaria parasites lacking an apicoplast defines organelle function in blood-stage *Plasmodium falciparum*, *PLoS Biol.* 9, e1001138. DOI: <https://doi.org/10.1371/journal.pbio.1001138>
- [12] Uddin, T., McFadden, G. I., and Goodman, C. D. (2018) Validation of Putative Apicoplast-Targeting Drugs Using a Chemical Supplementation Assay in Cultured Human Malaria Parasites, *Antimicrob. Agents Chemother.* 62. DOI: <https://doi.org/10.1128/AAC.01161-17>
- [13] Ramya, T. N., Mishra, S., Karmodiya, K., Surolia, N., and Surolia, A. (2007) Inhibitors of nonhousekeeping functions of the apicoplast defy delayed death in *Plasmodium falciparum*, *Antimicrob. Agents Chemother.* 51, 307-316. DOI: <https://doi.org/10.1128/AAC.00808-06>
- [14] Kennedy, K., Crisafulli, E. M., and Ralph, S. A. (2019) Delayed Death by Plastid Inhibition in Apicomplexan Parasites, *Trends Parasitol.* 35, 747-759. DOI: <https://doi.org/10.1016/j.pt.2019.07.010>
- [15] Kennedy, K., Cobbold, S. A., Hanssen, E., Birnbaum, J., Spillman, N. J., McHugh, E., Brown, H., Tilley, L., Spielmann, T., McConville, M. J., and Ralph, S. A. (2019) Delayed death in the malaria parasite *Plasmodium falciparum* is caused by disruption of prenylation-dependent intracellular trafficking, *PLoS Biol.* 17, e3000376. DOI: <https://doi.org/10.1371/journal.pbio.3000376>
- [16] Amberg-Johnson, K., Hari, S. B., Ganesan, S. M., Lorenzi, H. A., Sauer, R. T., Niles, J. C., and Yeh, E. (2017) Small molecule inhibition of apicomplexan FtsH1 disrupts plastid biogenesis in human pathogens, *Elife* 6. DOI: <https://doi.org/10.7554/eLife.29865>
- [17] Boucher, M. J., and Yeh, E. (2019) Disruption of Apicoplast Biogenesis by Chemical Stabilization of an Imported Protein Evades the Delayed-Death Phenotype in Malaria Parasites, *mSphere* 4. DOI: <https://doi.org/10.1128/mSphere.00710-18>
- [18] Okada, M., Guo, P., Nalder, S. A., and Sigala, P. A. (2020) Doxycycline has distinct apicoplast-specific mechanisms of antimalarial activity, *Elife* 9. DOI: <https://doi.org/10.7554/eLife.60246>
- [19] Gisselberg, J. E., Herrera, Z., Orchard, L. M., Llinas, M., and Yeh, E. (2018) Specific Inhibition of the Bifunctional Farnesyl/Geranylgeranyl Diphosphate Synthase in Malaria Parasites via a New Small-Molecule Binding Site, *Cell Chem. Biol.* 25, 185-193 e185. DOI: <https://doi.org/10.1016/j.chembiol.2017.11.010>
- [20] Imlay, L., and Odom, A. R. (2014) Isoprenoid metabolism in apicomplexan parasites, *Curr. Clin. Microbiol. Rep.* 1, 37-50. DOI: <https://doi.org/10.1007/s40588-014-0006-7>
- [21] Gisselberg, J. E., Dellibovi-Ragheb, T. A., Matthews, K. A., Bosch, G., and Prigge, S. T. (2013) The suf iron-sulfur cluster synthesis pathway is required for apicoplast maintenance in malaria parasites, *PLoS Pathog.* 9, e1003655. DOI: <https://doi.org/10.1371/journal.ppat.1003655>
- [22] Gabriel, H. B., de Azevedo, M. F., Palmisano, G., Wunderlich, G., Kimura, E. A., Katzin, A. M., and Alves, J. M. (2015) Single-target high-throughput transcription analyses reveal high levels of alternative splicing present in the FPPS/GGPPS from *Plasmodium falciparum*, *Sci. Rep.* 5, 18429. DOI: <https://doi.org/10.1038/srep18429>
- [23] No, J. H., de Macedo Dossin, F., Zhang, Y., Liu, Y. L., Zhu, W., Feng, X., Yoo, J. A., Lee, E., Wang, K., Hui, R., Freitas-Junior, L. H., and Oldfield, E. (2012) Lipophilic analogs of zoledronate and risedronate inhibit *Plasmodium* geranylgeranyl diphosphate synthase

- (GGPPS) and exhibit potent antimalarial activity, *Proc. Natl. Acad. Sci. U. S. A.* 109, 4058-4063. DOI: <https://doi.org/10.1073/pnas.1118215109>
- [24] Tonhosolo, R., D'Alessandri, F. L., Genta, F. A., Wunderlich, G., Gozzo, F. C., Eberlin, M. N., Peres, V. J., Kimura, E. A., and Katzin, A. M. (2005) Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*, *Biochem. J.* 392, 117-126. DOI: <https://doi.org/10.1042/BJ20050441>
- [25] Nair, S. C., Brooks, C. F., Goodman, C. D., Sturm, A., McFadden, G. I., Sundriyal, S., Anglin, J. L., Song, Y., Moreno, S. N., and Striepen, B. (2011) Apicoplast isoprenoid precursor synthesis and the molecular basis of fosmidomycin resistance in *Toxoplasma gondii*, *J. Exp. Med.* 208, 1547-1559. DOI: <https://doi.org/10.1084/jem.20110039>
- [26] Bowman, J. D., Merino, E. F., Brooks, C. F., Striepen, B., Carlier, P. R., and Cassera, M. B. (2014) Antiapicoplast and gametocytocidal screening to identify the mechanisms of action of compounds within the malaria box, *Antimicrob. Agents Chemother.* 58, 811-819. DOI: <https://doi.org/10.1128/AAC.01500-13>
- [27] Goodman, C. D., and McFadden, G. I. (2014) Ycf93 (Orf105), a small apicoplast-encoded membrane protein in the relict plastid of the malaria parasite *Plasmodium falciparum* that is conserved in Apicomplexa, *PLoS One* 9, e91178. DOI: <https://doi.org/10.1371/journal.pone.0091178>
- [28] Waller, R. F., Reed, M. B., Cowman, A. F., and McFadden, G. I. (2000) Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway, *EMBO J.* 19, 1794-1802. DOI: <https://doi.org/10.1093/emboj/19.8.1794>
- [29] Swift, R. P., Rajaram, K., Liu, H. B., Dziedzic, A., Jedlicka, A. E., Roberts, A. D., Matthews, K. A., Jhun, H., Bumpus, N. N., Tewari, S. G., Wallqvist, A., and Prigge, S. T. (2020) A mevalonate bypass system facilitates elucidation of plastid biology in malaria parasites, *PLoS Pathog.* 16, e1008316. DOI: <https://doi.org/10.1371/journal.ppat.1008316>
- [30] Howe, R., Kelly, M., Jimah, J., Hodge, D., and Odom, A. R. (2013) Isoprenoid biosynthesis inhibition disrupts Rab5 localization and food vacuolar integrity in *Plasmodium falciparum*, *Eukaryot. Cell* 12, 215-223. DOI: <https://doi.org/10.1128/EC.00073-12>
- [31] Zhang, B., Watts, K. M., Hodge, D., Kemp, L. M., Hunstad, D. A., Hicks, L. M., and Odom, A. R. (2011) A second target of the antimalarial and antibacterial agent fosmidomycin revealed by cellular metabolic profiling, *Biochemistry* 50, 3570-3577. DOI: <https://doi.org/10.1021/bi200113y>
- [32] Cassera, M. B., Gozzo, F. C., D'Alessandri, F. L., Merino, E. F., del Portillo, H. A., Peres, V. J., Almeida, I. C., Eberlin, M. N., Wunderlich, G., Wiesner, J., Jomaa, H., Kimura, E. A., and Katzin, A. M. (2004) The methylerythritol phosphate pathway is functionally active in all intraerythrocytic stages of *Plasmodium falciparum*, *J. Biol. Chem.* 279, 51749-51759. DOI: <https://doi.org/10.1074/jbc.M408360200>
- [33] Phillips, M. A., Gujjar, R., Malmquist, N. A., White, J., El Mazouni, F., Baldwin, J., and Rathod, P. K. (2008) Triazolopyrimidine-based dihydroorotate dehydrogenase inhibitors with potent and selective activity against the malaria parasite *Plasmodium falciparum*, *J. Med. Chem.* 51, 3649-3653. DOI: <https://doi.org/10.1021/jm8001026>
- [34] Fry, M., and Pudney, M. (1992) Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80),

- Biochem. Pharmacol.* 43, 1545-1553. DOI: [https://doi.org/10.1016/0006-2952\(92\)90213-3](https://doi.org/10.1016/0006-2952(92)90213-3)
- [35] Mamoun, C. B., Gluzman, I. Y., Goyard, S., Beverley, S. M., and Goldberg, D. E. (1999) A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*, *Proc. Natl. Acad. Sci. U. S. A.* 96, 8716-8720. DOI: <https://doi.org/10.1073/pnas.96.15.8716>
- [36] Fidock, D. A., and Wellems, T. E. (1997) Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil, *Proc. Natl. Acad. Sci. U. S. A.* 94, 10931-10936. DOI: <https://doi.org/10.1073/pnas.94.20.10931>
- [37] Swift, R. P., Rajaram, K., Keutcha, C., Liu, H. B., Kwan, B., Dziedzic, A., Jedlicka, A. E., and Prigge, S. T. (2020) The NTP generating activity of pyruvate kinase II is critical for apicoplast maintenance in *Plasmodium falciparum*, *Elife* 9. DOI: <https://doi.org/10.7554/eLife.50807>
- [38] Dahl, E. L., Shock, J. L., Shenai, B. R., Gut, J., DeRisi, J. L., and Rosenthal, P. J. (2006) Tetracyclines specifically target the apicoplast of the malaria parasite *Plasmodium falciparum*, *Antimicrob. Agents Chemother.* 50, 3124-3131. DOI: <https://doi.org/10.1128/AAC.00394-06>
- [39] van Dooren, G. G., Marti, M., Tonkin, C. J., Stimmler, L. M., Cowman, A. F., and McFadden, G. I. (2005) Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*, *Mol. Microbiol.* 57, 405-419. DOI: <https://doi.org/10.1111/j.1365-2958.2005.04699.x>
- [40] Persson, B. C., Esberg, B., Olafsson, O., and Bjork, G. R. (1994) Synthesis and function of isopentenyl adenosine derivatives in tRNA, *Biochimie* 76, 1152-1160. DOI: [https://doi.org/10.1016/0300-9084\(94\)90044-2](https://doi.org/10.1016/0300-9084(94)90044-2)
- [41] Esberg, B., Leung, H. C., Tsui, H. C., Bjork, G. R., and Winkler, M. E. (1999) Identification of the miaB gene, involved in methylthiolation of isopentenylated A37 derivatives in the tRNA of *Salmonella typhimurium* and *Escherichia coli*, *J. Bacteriol.* 181, 7256-7265. DOI: <https://doi.org/10.1128/JB.181.23.7256-7265.1999>
- [42] Boucher, M. J., Ghosh, S., Zhang, L., Lal, A., Jang, S. W., Ju, A., Zhang, S., Wang, X., Ralph, S. A., Zou, J., Elias, J. E., and Yeh, E. (2018) Integrative proteomics and bioinformatic prediction enable a high-confidence apicoplast proteome in malaria parasites, *PLoS Biol.* 16, e2005895. DOI: <https://doi.org/10.1371/journal.pbio.2005895>
- [43] Bushell, E., Gomes, A. R., Sanderson, T., Anar, B., Girling, G., Herd, C., Metcalf, T., Modrzynska, K., Schwach, F., Martin, R. E., Mather, M. W., McFadden, G. I., Parts, L., Rutledge, G. G., Vaidya, A. B., Wengelnik, K., Rayner, J. C., and Billker, O. (2017) Functional Profiling of a *Plasmodium* Genome Reveals an Abundance of Essential Genes, *Cell* 170, 260-272 e268. DOI: <https://doi.org/10.1016/j.cell.2017.06.030>
- [44] Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Liao, X., Adapa, S. R., Udenze, K., Bronner, I. F., Casandra, D., Mayho, M., Brown, J., Li, S., Swanson, J., Rayner, J. C., Jiang, R. H. Y., and Adams, J. H. (2018) Uncovering the essential genes of the human malaria parasite *Plasmodium falciparum* by saturation mutagenesis, *Science* 360, eaap7847. DOI: <https://doi.org/10.1126/science.aap7847>
- [45] Kellogg, B. A., and Poulter, C. D. (1997) Chain elongation in the isoprenoid biosynthetic pathway, *Curr. Opin. Chem. Biol.* 1, 570-578. DOI: [https://doi.org/10.1016/s1367-5931\(97\)80054-3](https://doi.org/10.1016/s1367-5931(97)80054-3)

- [46] Poulter, C. D. (2006) Farnesyl diphosphate synthase. A paradigm for understanding structure and functional relationships in E-polyprenyl diphosphate synthases, *Phytochem. Rev.* 5, 17-26. DOI: <https://doi.org/10.1007/s11101-005-4887-1>
- [47] Thulasiram, H. V., and Poulter, C. D. (2006) Farnesyl diphosphate synthase: The art of compromise between substrate selectivity and stereoselectivity, *J. Am. Chem. Soc.* 128, 15819-15823. DOI: <https://doi.org/10.1021/ja065573b>
- [48] Tarshis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) Regulation of product chain length by isoprenyl diphosphate synthases, *Proc. Natl. Acad. Sci. U. S. A.* 93, 15018-15023. DOI: <https://doi.org/10.1073/pnas.93.26.15018>
- [49] Jordao, F. M., Gabriel, H. B., Alves, J. M., Angeli, C. B., Bifano, T. D., Breda, A., de Azevedo, M. F., Basso, L. A., Wunderlich, G., Kimura, E. A., and Katzin, A. M. (2013) Cloning and characterization of bifunctional enzyme farnesyl diphosphate/geranylgeranyl diphosphate synthase from *Plasmodium falciparum*, *Malar. J.* 12, 184. DOI: <https://doi.org/10.1186/1475-2875-12-184>
- [50] Artz, J. D., Wernimont, A. K., Dunford, J. E., Schapira, M., Dong, A., Zhao, Y., Lew, J., Russell, R. G., Ebetino, F. H., Oppermann, U., and Hui, R. (2011) Molecular characterization of a novel geranylgeranyl pyrophosphate synthase from *Plasmodium* parasites, *J. Biol. Chem.* 286, 3315-3322. DOI: <https://doi.org/10.1074/jbc.M109.027235>
- [51] Boratyn, G. M., Camacho, C., Cooper, P. S., Coulouris, G., Fong, A., Ma, N., Madden, T. L., Matten, W. T., McGinnis, S. D., Merezuk, Y., Raytselis, Y., Sayers, E. W., Tao, T., Ye, J., and Zaretskaya, I. (2013) BLAST: a more efficient report with usability improvements, *Nucleic Acids Res.* 41, W29-33. DOI: <https://doi.org/10.1093/nar/gkt282>
- [52] Zimmermann, L., Stephens, A., Nam, S. Z., Rau, D., Kubler, J., Lozajic, M., Gabler, F., Soding, J., Lupas, A. N., and Alva, V. (2018) A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core, *J. Mol. Biol.* 430, 2237-2243. DOI: <https://doi.org/10.1016/j.jmb.2017.12.007>
- [53] Tonhosolo, R., D'Alexandri, F. L., de Rosso, V. V., Gazarini, M. L., Matsumura, M. Y., Peres, V. J., Merino, E. F., Carlton, J. M., Wunderlich, G., Mercadante, A. Z., Kimura, E. A., and Katzin, A. M. (2009) Carotenoid biosynthesis in intraerythrocytic stages of *Plasmodium falciparum*, *J. Biol. Chem.* 284, 9974-9985. DOI: <https://doi.org/10.1074/jbc.M807464200>
- [54] Foth, B. J., Ralph, S. A., Tonkin, C. J., Struck, N. S., Fraunholz, M., Roos, D. S., Cowman, A. F., and McFadden, G. I. (2003) Dissecting apicoplast targeting in the malaria parasite *Plasmodium falciparum*, *Science* 299, 705-708. DOI: <https://doi.org/10.1126/science.1078599>
- [55] Claros, M. G., and Vincens, P. (1996) Computational method to predict mitochondrially imported proteins and their targeting sequences, *Eur. J. Biochem.* 241, 779-786. DOI: <https://doi.org/10.1111/j.1432-1033.1996.00779.x>
- [56] Sigala, P. A., Crowley, J. R., Henderson, J. P., and Goldberg, D. E. (2015) Deconvoluting heme biosynthesis to target blood-stage malaria parasites, *Elife* 4. DOI: <https://doi.org/10.7554/eLife.09143>
- [57] Ghorbal, M., Gorman, M., Macpherson, C. R., Martins, R. M., Scherf, A., and Lopez-Rubio, J. J. (2014) Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system, *Nat. Biotechnol.* 32, 819-821. DOI: <https://doi.org/10.1038/nbt.2925>

- [58] Ganesan, S. M., Falla, A., Goldfless, S. J., Nasamu, A. S., and Niles, J. C. (2016) Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites, *Nat. Commun.* 7, 10727. DOI: <https://doi.org/10.1038/ncomms10727>
- [59] Maruthi, M., Ling, L., Zhou, J., and Ke, H. (2020) Dispensable Role of Mitochondrial Fission Protein 1 (Fis1) in the Erythrocytic Development of *Plasmodium falciparum*, *mSphere* 5. DOI: <https://doi.org/10.1128/mSphere.00579-20>
- [60] Gabriel, H. B., Silva, M. F., Kimura, E. A., Wunderlich, G., Katzin, A. M., and Azevedo, M. F. (2015) Squalestatin is an inhibitor of carotenoid biosynthesis in *Plasmodium falciparum*, *Antimicrob. Agents Chemother.* 59, 3180-3188. DOI: <https://doi.org/10.1128/AAC.04500-14>
- [61] Bouvier, F., Rahier, A., and Camara, B. (2005) Biogenesis, molecular regulation and function of plant isoprenoids, *Prog. Lipid Res.* 44, 357-429. DOI: <https://doi.org/10.1016/j.plipres.2005.09.003>
- [62] Zhou, X., Welsch, R., Yang, Y., Alvarez, D., Riediger, M., Yuan, H., Fish, T., Liu, J., Thannhauser, T. W., and Li, L. (2015) Arabidopsis OR proteins are the major posttranscriptional regulators of phytoene synthase in controlling carotenoid biosynthesis, *Proc. Natl. Acad. Sci. U. S. A.* 112, 3558-3563. DOI: <https://doi.org/10.1073/pnas.1420831112>
- [63] Iwata-Reuyl, D., Math, S. K., Desai, S. B., and Poulter, C. D. (2003) Bacterial phytoene synthase: molecular cloning, expression, and characterization of *Erwinia herbicola* phytoene synthase, *Biochemistry* 42, 3359-3365. DOI: <https://doi.org/10.1021/bi0206614>
- [64] Nagashima, S., Kamimura, A., Shimizu, T., Nakamura-Isaki, S., Aono, E., Sakamoto, K., Ichikawa, N., Nakazawa, H., Sekine, M., Yamazaki, S., Fujita, N., Shimada, K., Hanada, S., and Nagashima, K. V. (2012) Complete genome sequence of phototrophic betaproteobacterium *Rubrivivax gelatinosus* IL144, *J. Bacteriol.* 194, 3541-3542. DOI: <https://doi.org/10.1128/JB.00511-12>
- [65] Cunningham, F. X., Jr., and Gantt, E. (2005) A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*, *Plant J.* 41, 478-492. DOI: <https://doi.org/10.1111/j.1365-3113X.2004.02309.x>
- [66] van Schaijk, B. C., Kumar, T. R., Vos, M. W., Richman, A., van Gemert, G. J., Li, T., Eappen, A. G., Williamson, K. C., Morahan, B. J., Fishbaugher, M., Kennedy, M., Camargo, N., Khan, S. M., Janse, C. J., Sim, K. L., Hoffman, S. L., Kappe, S. H., Sauerwein, R. W., Fidock, D. A., and Vaughan, A. M. (2014) Type II fatty acid biosynthesis is essential for *Plasmodium falciparum* sporozoite development in the midgut of *Anopheles* mosquitoes, *Eukaryot. Cell* 13, 550-559. DOI: <https://doi.org/10.1128/EC.00264-13>
- [67] Krishnan, A., Kloehn, J., Lunghi, M., Chiappino-Pepe, A., Waldman, B. S., Nicolas, D., Varesio, E., Hehl, A., Lourido, S., Hatzimanikatis, V., and Soldati-Favre, D. (2020) Functional and Computational Genomics Reveal Unprecedented Flexibility in Stage-Specific *Toxoplasma* Metabolism, *Cell Host Microbe* 27, 290-306 e211. DOI: <https://doi.org/10.1016/j.chom.2020.01.002>
- [68] Mazumdar, J., E, H. W., Masek, K., C, A. H., and Striepen, B. (2006) Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in *Toxoplasma gondii*, *Proc. Natl. Acad. Sci. U. S. A.* 103, 13192-13197. DOI: <https://doi.org/10.1073/pnas.0603391103>

- [69] van Dooren, G. G., Su, V., D'Ombraín, M. C., and McFadden, G. I. (2002) Processing of an apicoplast leader sequence in *Plasmodium falciparum* and the identification of a putative leader cleavage enzyme, *J. Biol. Chem.* 277, 23612-23619. DOI: <https://doi.org/10.1074/jbc.M201748200>
- [70] Almagro Armenteros, J. J., Tsirigos, K. D., Sonderby, C. K., Petersen, T. N., Winther, O., Brunak, S., von Heijne, G., and Nielsen, H. (2019) SignalP 5.0 improves signal peptide predictions using deep neural networks, *Nat. Biotechnol.* 37, 420-423. DOI: <https://doi.org/10.1038/s41587-019-0036-z>
- [71] Mullin, K. A., Lim, L., Ralph, S. A., Spurck, T. P., Handman, E., and McFadden, G. I. (2006) Membrane transporters in the relict plastid of malaria parasites, *Proc. Natl. Acad. Sci. U. S. A.* 103, 9572-9577. DOI: <https://doi.org/10.1073/pnas.0602293103>
- [72] Tomlins, A. M., Ben-Rached, F., Williams, R. A., Proto, W. R., Coppens, I., Ruch, U., Gilberger, T. W., Coombs, G. H., Mottram, J. C., Muller, S., and Langsley, G. (2013) *Plasmodium falciparum* ATG8 implicated in both autophagy and apicoplast formation, *Autophagy* 9, 1540-1552. DOI: <https://doi.org/10.4161/auto.25832>
- [73] Barylyuk, K., Koreny, L., Ke, H., Butterworth, S., Crook, O. M., Lassadi, I., Gupta, V., Tromer, E., Mourier, T., Stevens, T. J., Breckels, L. M., Pain, A., Lilley, K. S., and Waller, R. F. (2020) A Comprehensive Subcellular Atlas of the *Toxoplasma* Proteome via hyperLOPIT Provides Spatial Context for Protein Functions, *Cell Host Microbe* 28, 752-766 e759. DOI: <https://doi.org/10.1016/j.chom.2020.09.011>
- [74] Henkel, S., Frohnecke, N., Maus, D., McConville, M. J., Laue, M., Blume, M., and Seeber, F. (2021) *Toxoplasma gondii* apicoplast-resident ferredoxin is an essential electron transfer protein for the MEP isoprenoid biosynthetic pathway, *J. Biol. Chem.*, 101468. DOI: <https://doi.org/10.1016/j.jbc.2021.101468>
- [75] Akhtar, T. A., Surowiecki, P., Siekierska, H., Kania, M., Van Gelder, K., Rea, K. A., Virta, L. K. A., Vatta, M., Gawarecka, K., Wojcik, J., Danikiewicz, W., Buszewicz, D., Swiezewska, E., and Surmacz, L. (2017) Polyprenols Are Synthesized by a Plastidial cis-Prenyltransferase and Influence Photosynthetic Performance, *Plant Cell* 29, 1709-1725. DOI: <https://doi.org/10.1105/tpc.16.00796>
- [76] Joyard, J., Ferro, M., Masselon, C., Seigneurin-Berny, D., Salvi, D., Garin, J., and Rolland, N. (2009) Chloroplast proteomics and the compartmentation of plastidial isoprenoid biosynthetic pathways, *Mol. Plant* 2, 1154-1180. DOI: <https://doi.org/10.1093/mp/ssp088>
- [77] Van Gelder, K., Rea, K. A., Virta, L. K. A., Whitnell, K. L., Osborn, M., Vatta, M., Khozin, A., Skorupinska-Tudek, K., Surmacz, L., and Akhtar, T. A. (2018) Medium-Chain Polyprenols Influence Chloroplast Membrane Dynamics in *Solanum lycopersicum*, *Plant Cell Physiol.* 59, 2350-2365. DOI: <https://doi.org/10.1093/pcp/pcy157>
- [78] Kelly, M., Su, C. Y., Schaber, C., Crowley, J. R., Hsu, F. F., Carlson, J. R., and Odom, A. R. (2015) Malaria parasites produce volatile mosquito attractants, *mBio* 6. DOI: <https://doi.org/10.1128/mBio.00235-15>
- [79] Emami, S. N., Lindberg, B. G., Hua, S., Hill, S. R., Mozuraitis, R., Lehmann, P., Birgersson, G., Borg-Karlson, A. K., Ignell, R., and Faye, I. (2017) A key malaria metabolite modulates vector blood seeking, feeding, and susceptibility to infection, *Science* 355, 1076-1080. DOI: <https://doi.org/10.1126/science.aah4563>
- [80] Miller, J. J., and Odom John, A. R. (2020) The Malaria Metabolite HMBPP Does Not Trigger Erythrocyte Terpene Release, *ACS Infect. Dis.* 6, 2567-2572. DOI: <https://doi.org/10.1021/acsinfecdis.0c00548>

- [81] Couto, A. S., Kimura, E. A., Peres, V. J., Uhrig, M. L., and Katzin, A. M. (1999) Active isoprenoid pathway in the intra-erythrocytic stages of *Plasmodium falciparum*: presence of dolichols of 11 and 12 isoprene units, *Biochem. J.* 341 (Pt 3), 629-637.
- [82] Zimbres, F. M., Valenciano, A. L., Merino, E. F., Florentin, A., Holderman, N. R., He, G., Gawarecka, K., Skorupinska-Tudek, K., Fernandez-Murga, M. L., Swiezewska, E., Wang, X., Muralidharan, V., and Cassera, M. B. (2020) Metabolomics profiling reveals new aspects of dolichol biosynthesis in *Plasmodium falciparum*, *Sci. Rep.* 10, 13264. DOI: <https://doi.org/10.1038/s41598-020-70246-0>
- [83] Hartley, M. D., and Imperiali, B. (2012) At the membrane frontier: a prospectus on the remarkable evolutionary conservation of polyprenols and polyprenyl-phosphates, *Arch. Biochem. Biophys.* 517, 83-97. DOI: <https://doi.org/10.1016/j.abb.2011.10.018>
- [84] Swiezewska, E., and Danikiewicz, W. (2005) Polyisoprenoids: structure, biosynthesis and function, *Prog. Lipid Res.* 44, 235-258. DOI: <https://doi.org/10.1016/j.plipres.2005.05.002>
- [85] Botte, C. Y., Yamaro-Botte, Y., Rupasinghe, T. W., Mullin, K. A., MacRae, J. I., Spurck, T. P., Kalanon, M., Shears, M. J., Coppel, R. L., Crellin, P. K., Marechal, E., McConville, M. J., and McFadden, G. I. (2013) Atypical lipid composition in the purified relict plastid (apicoplast) of malaria parasites, *Proc. Natl. Acad. Sci. U. S. A.* 110, 7506-7511. DOI: <https://doi.org/10.1073/pnas.1301251110>
- [86] Tonkin, C. J., van Dooren, G. G., Spurck, T. P., Struck, N. S., Good, R. T., Handman, E., Cowman, A. F., and McFadden, G. I. (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method, *Mol. Biochem. Parasitol.* 137, 13-21. DOI: <https://doi.org/10.1016/j.molbiopara.2004.05.009>
- [87] Gallagher, J. R., and Prigge, S. T. (2010) *Plasmodium falciparum* acyl carrier protein crystal structures in disulfide-linked and reduced states and their prevalence during blood stage growth, *Proteins* 78, 575-588. DOI: <https://doi.org/10.1002/prot.22582>
- [88] Ganesan, S. M., Morrissey, J. M., Ke, H., Painter, H. J., Laroia, K., Phillips, M. A., Rathod, P. K., Mather, M. W., and Vaidya, A. B. (2011) Yeast dihydroorotate dehydrogenase as a new selectable marker for *Plasmodium falciparum* transfection, *Mol. Biochem. Parasitol.* 177, 29-34. DOI: <https://doi.org/10.1016/j.molbiopara.2011.01.004>
- [89] Wellems, T. E., Panton, L. J., Gluzman, I. Y., do Rosario, V. E., Gwadz, R. W., Walker-Jonah, A., and Krogstad, D. J. (1990) Chloroquine resistance not linked to *mdr*-like genes in a *Plasmodium falciparum* cross, *Nature* 345, 253-255. DOI: <https://doi.org/10.1038/345253a0>
- [90] Klembe, M., Beatty, W., Gluzman, I., and Goldberg, D. E. (2004) Trafficking of plasmepsin II to the food vacuole of the malaria parasite *Plasmodium falciparum*, *J. Cell Biol.* 164, 47-56. DOI: <https://doi.org/10.1083/jcb200307147>
- [91] Beck, J. R., Muralidharan, V., Oksman, A., and Goldberg, D. E. (2014) PTEX component HSP101 mediates export of diverse malaria effectors into host erythrocytes, *Nature* 511, 592-595. DOI: <https://doi.org/10.1038/nature13574>
- [92] Balu, B., Shoue, D. A., Fraser, M. J., and Adams, J. H. (2005) High-efficiency transformation of *Plasmodium falciparum* by the lepidopteran transposable element piggyBac, *Proc. Natl. Acad. Sci. U. S. A.* 102, 16391-16396. DOI: <https://doi.org/10.1073/Pnas.0504679102>

- [93] Spillman, N. J., Beck, J. R., Ganesan, S. M., Niles, J. C., and Goldberg, D. E. (2017) The chaperonin TRiC forms an oligomeric complex in the malaria parasite cytosol, *Cell Microbiol.* 19. DOI: <https://doi.org/10.1111/cmi.12719>
- [94] Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method, *Nat. Protoc.* 3, 1101-1108. DOI: <https://doi.org/10.1038/nprot.2008.73>
- [95] Rajaram, K., Liu, H. B., and Prigge, S. T. (2020) Redesigned TetR-Aptamer System To Control Gene Expression in *Plasmodium falciparum*, *mSphere* 5. DOI: <https://doi.org/10.1128/mSphere.00457-20>
- [96] Mamoun, C. B., and Goldberg, D. E. (2001) *Plasmodium* protein phosphatase 2C dephosphorylates translation elongation factor 1beta and inhibits its PKC-mediated nucleotide exchange activity in vitro, *Mol. Microbiol.* 39, 973-981. DOI: <https://doi.org/10.1046/j.1365-2958.2001.02289.x>
- [97] Rivera, S. M., Christou, P., and Canela-Garayoa, R. (2014) Identification of carotenoids using mass spectrometry, *Mass Spectrom. Rev.* 33, 353-372. DOI: <https://doi.org/10.1002/mas.21390>

FIGURE SUPPLEMENT LEGENDS

Figure 1- figure supplement 1. Additional epifluorescence images of PfMev ACP_L-GFP NF54 parasites treated with FOS and other drugs at the indicated concentrations. Parasites were synchronized to the ring stage with 5% D-sorbitol and incubated with the indicated treatments for 38 hours prior to live-cell imaging.

Figure 1- figure supplement 2. Epifluorescence images and analysis of D10 ACP_L-GFP parasites treated with FOS and other drugs. Bright field (BF) and fluorescent microscopy images of live ACP_L-GFP D10 parasites that were (A) untreated or treated with 10 μM fosmidomycin (FOS) in the absence or presence of 200 μM IPP, or (B) treated with 6 μM blasticidin-S (Blast-S), 2 μM DSM1, or 100 nM atovaquone (ATV). (C) Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panels A and B from two independent experiments. Apicoplast morphologies were scored as punctate (focal), elongated, or disrupted (dispersed); counted; and plotted by histogram as the fractional population with the indicated morphology (UT=untreated). Error bars represent standard deviations from replicate experiments. In all experiments, synchronized ring-stage parasites were incubated with the indicated treatments for 36 hours prior to live-cell imaging. Parasite nuclei were visualized using 1 μg/ml Hoechst 33342. The parasite apicoplast was visualized using the ACP_L-GFP encoded by the D10 line.

Figure 1- figure supplement 3. Additional epifluorescence images of D10 parasites treated with FOS and other drugs.

Figure 1- figure supplement 4. Additional epifluorescence images of ΔDXS PfMev parasites that were synchronized to ring stage with 5% D-sorbitol and incubated for 36 hours ±Mev hours prior to live-cell imaging. Parasite nuclei were visualized using 1 μg/ml Hoechst 33342. The parasite apicoplast was visualized using the ACP_L-GFP encoded by the PfMev line.

Figure 2- figure supplement 1. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 0 hours after synchronization.

Figure 2- figure supplement 2. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 30 hours after synchronization. Clone headers for parasites with a disrupted apicoplast are yellow.

Figure 2- figure supplement 3. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 34 hours after synchronization. Clone headers for parasites with a disrupted apicoplast are yellow.

Figure 2- figure supplement 4. Epifluorescence microscopy images of clonal parasites isolated after FOS treatment and rescue by mevalonate addition at 38 hours after synchronization. Clone headers for parasites with a disrupted apicoplast are yellow.

Figure 3- supplement 1. PCR genotyping of PfMev Δ MiaA parasites and additional epifluorescence images of apicoplast morphology. **(A)** Schematic depiction of the MiaA gene-disruption strategy using CRISPR/Cas9 and positive selection with human DHFR. Colored arrows depict PCR primer pairs used in panel B to test for retention or disruption of the MiaA gene. **(B)** Genomic PCR analysis of parental NF54 PfMev parasites and polyclonal transfected parasite progeny confirmed successful disruption of the MiaA gene. **(C)** Additional images of Δ MiaA parasites.

Figure 4- figure supplement 1. 5 μ M GGOH but not FOH partially rescues parasite growth from inhibition by 10 μ M FOS in continuous-growth assays with PfMev parasites. Data points are the average $\pm$ SD of three biological replicates. Parasites were synchronized to ring stage with 5% D-sorbitol and incubated with the indicated treatments. Daily parasitemia values were determined by flow cytometry.

Figure 4- figure supplement 2. Additional epifluorescence microscopy images of PfMev parasites treated with FOS and FOH, GGOH, C₅₀-OH, or β -carotene. Synchronized ring-stage parasites were incubated with the indicated treatments for 36 hours and imaged by brightfield (BF) or fluorescence microscopy, with visualization of parasite nuclei by Hoechst staining and the apicoplast by ACP_L-GFP signal.

Figure 4- figure supplement 3. Epifluorescence microscopy images of D10 ACP_L-GFP parasites treated with FOS and FOH, GGOH, C₅₀-OH, or β -carotene. **(A)** 5 μ M farnesol (FOH), geranylgeraniol (GGOH), or decaprenol (C₅₀-OH) but not β -carotene, rescues apicoplast biogenesis from inhibition by 10 μ M FOS. **(B)** Statistical analysis (average $\pm$ SD) of apicoplast status in D10 parasites treated with 10 μ M FOS. **(C)** Additional images of D10 parasites treated with FOS and FOH, GGOH, C₅₀OH, or β -carotene.

Figure 5- figure supplement 1. Full sequence alignment of PF3D7_0202700, PF3D7_1128400, and avian FPPS (Uniprot P08836).

Figure 5- figure supplement 2. Epifluorescence microscopy images and statistical analysis of PfMev and D10 parasites treated with 10 μ M MMV091313. **(A)** Synchronized ring-stage parasites were incubated with 10 μ M MMV091313 for 36 hours and imaged by brightfield (BF) or fluorescence microscopy, with visualization of parasite nuclei by Hoechst staining and the apicoplast by ACP_L-GFP signal. **(B)** Population analysis of apicoplast morphology for 50 total parasites imaged for each condition in panel A from two independent experiments (UT=untreated). Apicoplast morphologies were scored as punctate (focal), elongated, or disrupted (dispersed); counted; and plotted by histogram as the fractional population with the indicated morphology. Error bars represent standard deviations from replicate experiments.

Figure 5- figure supplement 3. Results of sequence-similarity searches for PF3D7_0202700 using NCBI BLAST and MPI HHpred.

Figure 5- figure supplement 4. Additional epifluorescence microscopy images of Dd2 parasite episomally expressing PPS-GFP or PPS-RFP. **(A)** Additional images of live parasite expressing PPS-GFP. Additional immunofluorescence images of Dd2 parasites episomally expressing **(B)** PPS-RFP and stained with anti-apicoplast ACP and an anti-RFP antibody and **(C)** PPS-GFP, cultured for 7 days in 2 μ M doxycycline and 200 IPP, and stained with an anti-GFP antibody.

Figure 6- figure supplement 1. Scheme for modification of the PPS genomic locus to integrate the aptamer/TetR-DOZI system and Southern blot confirming correct integration.

Figure 6- figure supplement 2. Additional immunofluorescence microscopy images showing co-localization of endogenous PPS and apicoplast ACP, along with pixel intensity overlap as a function of distance along the white line in the merged images.

Figure 6- figure supplement 3. Blood-smear images of Dd2 parasites tagged at the PPS locus with the aptamer/TetR-DOZI system and grown $\pm$ aTc for 8 days.

Figure 7- figure supplement 1. IFA images and analysis of apicoplast morphology in PPS knockdown parasites grown +aTc or -aTc/+IPP (200 μ M) for 12 days. Approximately 50-70 parasites were imaged and counted for each condition.

Figure 7- figure supplement 2. Additional IFA images of PPS knockdown parasites treated as in Figure 7B.

Figure 7- figure supplement 3. Additional IFA images of PPS knockdown parasites treated as in Figure 7D.

Figure 8- figure supplement 1. 48-hour growth inhibition curves for treatment of Dd2 parasites with zaragozic acid without or with episomal expression of PPS-RFP or 200 μ M IPP. Parasitemia values were determined for biological replicates at each condition, plotted as the average $\pm$ SD, and fit to a four-parameter dose-response curve in GraphPad Prism 9.0.

Figure 8- figure supplement 2. Epifluorescence microscopy images of D10 parasites treated with 160 μ M zaragozic acid as synchronized rings and imaged for ACP_L-GFP and Hoescht 36 hours later as multinuclear schizonts.

Figure 8- figure supplement 3. Fragment ion spectrum for unlabeled beta-carotene determined by tandem mass spectrometry of β -carotene commercial standard.

Figure 8- figure supplement 4. Intensity versus retention time plot for liquid chromatography-mass spectrometry determination of unlabeled β -carotene in Albumax I

A. ACP_L-GFP PfMev (NF54)

B. ACP_L-GFP PfMev (NF54)

C. ACP_L-GFP PfMev (NF54) Apicoplast Status

D. ΔDXS-ACP_L-GFP PfMev (NF54)

E. ΔDXS PfMev Apicoplast Status

A.

ACPL-GFP PfMev (NF54)

B.

ACPL-GFP PfMev (NF54) Apicoplast Status

A.

260	TVLDNQCKIIAASEIIHMG S LLH DD VI D DSNKRRGVIALHKKFGNKISIL	Pf3D7_0202700
82	ESLRCALAVGWCIELFQA F FLVA DD IM D QSLTRRGQLCWYKKEGVGL-DA	FPPS_CHICK
93	NEWKVICIAWCIEILQA S FLVA DD IM D KGETRRNKHCWYLLKDVEIKNA	Pf3D7_1128400
390	ND-TIIQLCFNLGLHIGMAFQLY DD YL D YKIDDNNTNKPILNDLKNNIKTA	Pf3D7_0202700
235	SKEE-HENAKAILLEMGEYFQIQ DD YL D CFGDPALTGKVGTDIQDNKCSW	FPPS_CHICK
244	LDNLLYKKVENIAILMGEYFQVH DD YI D TFGDSKKTGKVGSDIQNNKLTW	Pf3D7_1128400

B. Pf3D7_0200700
E. coli OPPS

C. PPS-KD + 10μM FOS Apicoplast Status

D. 10μM FOS+IPP (Cycle 4)

E. 10μM FOS+IPP (Cycle 4) Apicoplast Status

F. 10μM FOS+IPP (Cycle 4) qPCR

A. **Head to tail** condensation **Head to head** condensation

C. **PfMev *P. falciparum***

D. **pAC-BET *E. coli***

Δ DXS PfMev (NF54) + 50 μ M Mev

BF Hoechst GFP Merge

Δ DXS PfMev (NF54) - Mev

BF Hoechst GFP Merge

A. **B.** **C.** ΔMiaA - Mev PfMev (NF54)

PfMev (NF54) 10 μ M FOS + 5 μ M GGOH

PfMev (NF54) 10 μ M FOS + 5 μ M FOH

PfMev (NF54) 10 μ M FOS + 10 μ M C₅₀OH

PfMev (NF54) 10 μ M FOS + 5 μ M β -carotene

C. ACPL-GFP (D10) 10μM FOS + 5μM FOH

ACPL-GFP (D10) 10μM FOS + 5μM GGOH

ACPL-GFP (D10) 10μM FOS + 5μM C₅₀OH

ACPL-GFP (D10) 10μM FOS + 5μM β-carotene

TR O96130 O96130_PLAF7	MVHLSKRNNIKSFLNYCKAKYLNPLLINKNEDIKETSILKNNNLYSRKESNVFIEILKS	60
SP P08836 FPPS_CHICK	-----	
TR Q8II79 Q8II79_PLAF7	-----	
TR O96130 O96130_PLAF7	SFIKFRGQKINEEINNHNHNIINNSSHNNNNHNIYHDTNKKKKQQYEEKHNVFHTENMHKEV	120
SP P08836 FPPS_CHICK	-MHKFTGVN-----AKFQ-----QPA	15
TR Q8II79 Q8II79_PLAF7	-----	
TR O96130 O96130_PLAF7	LLCMDVLQYEEDKVNRELHLLHSYFNKE-----RTNIDPYTLCESKIKNIDEYIYNI	172
SP P08836 FPPS_CHICK	LRNL-----SPVVVEREREFEVGFPPQIVR---DLTEDGIGHPEVGD-----	54
TR Q8II79 Q8II79_PLAF7	---M-----ENEQNNQDSENGLDYFRSMYDRYRDVFINHINDYVLEDD-----IKII	44
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TR O96130 O96130_PLAF7	IKTNYKNIDEFITYIYLYKGKRFRVILSILLKNILHHIDNVSKIKNFKNRNIQRKFFKS	232
SP P08836 FPPS_CHICK	AVARLKEV---LQYN-APGGKCNRGLTVVAA-----	81
TR Q8II79 Q8II79_PLAF7	ISKYYKLL---FDYN-CLGGKNNRGILVILI-----	71
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TR O96130 O96130_PLAF7	NKLTSNYLSNKLKLYNLKITQKKNICETVLDNQCKIIAASEIIHM GSLLHDDVID DSNK	292
SP P08836 FPPS_CHICK	-----YRE--LSGPGQKDAESLRCALAVGWCIELFQA FFLVA DDIMD QSLT	125
TR Q8II79 Q8II79_PLAF7	-----YEY--VKNRDI-NCNEWKEKVACIAWCIEILQA SFLVA DDIMD KGET	114
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TR O96130 O96130_PLAF7	RRGVIALHKKFGNKISILSGD-YLLARASSIFAGTGSPKICRSFSYVVE-----S	341
SP P08836 FPPS_CHICK	RRGQLCWYKKEGVGL-DAINDSFLLESS--VYRV--LKKYCRQRPYYVHLLFLQTAYQ	180
TR Q8II79 Q8II79_PLAF7	RRNKHWCYLLKDVEIKNAVNDVFLLYNA--IYKL--LDVYLRNDNCYLDLITSFREATLK	170
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TR O96130 O96130_PLAF7	LIKGEFLQRNLK-----FNNV-----EEALKMYLIKS-----HKTA--SLF	376
SP P08836 FPPS_CHICK	TELGQMLDLITA-----PVSKVDLSHFSEERYKAIVKYKTAFYSFY	221
TR Q8II79 Q8II79_PLAF7	TIVGQHLDTNIFSDKYSHIDKDIDVNNINISQENKININMLNFKVYQNIHKTAYYSFF	230
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TR O96130 O96130_PLAF7	SHLFACIAILSFKND-TIIQLCFNLGLHIGMAFQLY DDYL DKIDDNTNKPILNDLKNNI	435
SP P08836 FPPS_CHICK	LPVAAAMYMVGIDSKEE-HENAKAILLEMGEYFQIQ DDYL DCFGDPALTGKVGTDIQDNK	280
TR Q8II79 Q8II79_PLAF7	LPIVCGMQMGGISLDNLLYKKVENIAILMGEYFQVH DDYI DTFGDSKKTGKVGSDIQNNK	290
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TR O96130 O96130_PLAF7	KTAPLLFSYNYNPQVILQLINKNSYTNNDIENILYY-----IQHSNSMKK	480
SP P08836 FPPS_CHICK	CSWLVVQCLQRTPEQRQLLEDN-YGRKEPEKVAKVKELYEAVGMRAAFQQYEESYRRL	339
TR Q8II79 Q8II79_PLAF7	LTWPLIKAFELCSQPEKEDIIRN-YGKDNVTCIKFINDIYEHYNIIRDHYVEYEKKQMKMI	349
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TR O96130 O96130_PLAF7	NELCSLLHIKKASDILYSLISHCNKPSTNKNNTKHDDIKQSSEALINLILNVLNRNVK--	538
SP P08836 FPPS_CHICK	QELIEKHSNR-----LPKEIFLGLAQKIYKR	365
TR Q8II79 Q8II79_PLAF7	LEAINQLHHE-----GIEYVLKYVMDILFTGA-----	376
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TR O96130 O96130_PLAF7	--	
SP P08836 FPPS_CHICK	QK	367
TR Q8II79 Q8II79_PLAF7	--

Top 10 NCBI Blast results for PF3D7_02020700^{*,}**

Description	Species	% Ident	Accession
Geranylgeranyl pyrophosphate synthase	Haematococcus lacustris	30.17%	KFY27494.1
Decaprenyl-diphosphate synthase subunit 1	Fistulifera solaris	29.52%	APX64486.1
Decaprenyl-diphosphate synthase subunit 1	Strongylocentrotus purpuratus	29.38%	XP_030829069.1
Trans-prenyltransferase 4	Haslea ostrearia	29.94%	AYV97144.1
Solanesyl diphosphate synthase 3 isoform X2	Brassica napus	30.54%	XP_006410610.1
Solanesyl diphosphate synthase 1 isoform X1	Asparagus officinalis	28.51%	XP_020277177.1
Solanesyl diphosphate synthase 3 isoform X2	Arabidopsis lyrata	30.24%	XP_02883166.1
Geranyl diphosphate synthase 1	Arabidopsis thaliana	29.94%	NP_850234.1
Solanesyl diphosphate synthase 3	Capsella rubella	30.65%	XP_023639436.1
Solanesyl diphosphate synthase 1	Physcomitrella patens	28.10%	XP_024

*excluding Apicomplexan (taxid:5794)

**excluding predicted and hypothetical proteins

Top 10 HHPred results for PF3D7_02020700^{*}

Description	Species	E-value	HH-Hit
Geranylgeranyl pyrophosphate synthase	Arabidopsis thaliana	5.8e-38	3APZ_A
Geranylgeranyl pyrophosphate synthase	Corynebacterium glutamicum	2.2e-37	3LMD_A
Trans-hexaprenyltransferase	Pseudoalteromonas atlantica	2.8e-37	4JXY_A
Hexaprenyl diphosphate transferase	Micrococcus luteus	3.7e-37	3AQB_D
Decaprenyl diphosphate synthase	Rhodobacter capsulatus	3.7e-37	3MZV_A
Octaprenyl diphosphate synthase	Escherichia coli	4.4e-37	3WJK_B
Polyprenyl synthase	Acinetobacter baumannii	4.4e-37	4LOB_A
Trans-isoprenyl diphosphate synthase	Caulobacter crescentus	7.6e-37	3OYR_A
Geranylgeranyl pyrophosphate synthase	Lactobacillus brevis	1.1e-36	3PKO_A
Farnesyl pyrophosphate synthase	Staphylococcus aureus	1.7e-36	5H9D_A

*excluding hypothetical proteins

DIC

 α -ACP α -HA

Merge

Intensity Plots

PPS 9xAPT gRNA4 Clone 4A4 Day 8
+aTc

-aTc

PPS-KD (Dd2) + aTc

Untreated

10μM FOS + 200μM IPP

10μM FOS + 5μM FOH

10μM FOS + 5μM GGOH

10μM FOS + 5μM C₅₀OH

PPS-KD (Dd2) - aTc

Untreated

10μM FOS + 200μM IPP

10μM FOS + 5μM FOH

10μM FOS + 5μM GGOH

10μM FOS + 5μM C₅₀OH

10 μ M FOS + 200 μ M IPP (Cycle 4)

PfMev (NF54) 160 μ M Zaragozic Acid

BF

Hoechst

GFP

Merge

AlbuMAX sample Beta-carotene m/z 536.44