Apicomplexan parasites are responsible for high impact human diseases such as malaria, toxoplasmosis, and cryptosporidiosis. These obligate intracellular pathogens are dependent on both de novo lipid biosynthesis as well as the uptake of host lipids for biogenesis of parasite membranes. Genome annotations and biochemical studies indicate that apicomplexan parasites can synthesize fatty acids via a number of different biosynthetic pathways that are differentially compartmentalized. However, the relative contribution of each of these biosynthetic pathways to total fatty acid composition of intracellular parasite stages remains poorly defined. Here, we use a combination of genetic, biochemical, and metabolomic approaches to delineate the contribution of fatty acid biosynthetic pathways in Toxoplasma gondii. Metabolic labeling studies with \([13C]\)glucose showed that intracellular tachyzoites synthesized a range of long and very long chain fatty acids (C14:0–26:1). Genetic disruption of the apicoplast-localized type II fatty-acid synthase resulted in greatly reduced synthesis of saturated fatty acids up to 18 carbons long. Ablation of type II fatty-acid synthase activity resulted in reduced intracellular growth that was partially restored by addition of long chain fatty acids. In contrast, synthesis of very long chain fatty acids was primarily dependent on a fatty acid elongation system comprising three elongases, two reductases, and a dehydratase that were localized to the endoplasmic reticulum. The function of these enzymes was confirmed by heterologous expression in yeast. This elongase pathway appears to have a unique role in generating very long unsaturated fatty acids (C26:1) that cannot be salvaged from the host.

Apicomplexans are a phylum of single-celled eukaryotic parasites that includes the causative agents for important human diseases such as malaria, toxoplasmosis, and cryptosporidiosis as well as numerous veterinary pathogens. Control of these diseases is challenging, in no small part due to a rapid emergence of drug resistance that requires a continuous pipeline of new drugs with novel targets and/or modes of action. Apicomplexans are obligate intracellular pathogens and deploy complex mechanisms of synthesis and salvage to meet their essential nutritional requirements. The acquisition of lipids and fatty acids has emerged as a particularly important aspect of the metabolism of intracellular pathogens (1–3). Fatty acids are the main building blocks of membranes and also play a significant role in the post-translational modification of numerous proteins.

Apicomplexans were believed to be incapable of synthesizing their own fatty acids and to rely completely on host-derived lipids. However, the discovery of the apicoplast, a plastid-like organelle challenged this view. As found for the chloroplasts of plants and algae, the apicoplast contains a type II fatty-acid synthesis (FASII) pathway (4). Humans lack plastids and the

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### Background

Parasite fatty acid synthesis is an attractive drug target but complex and poorly understood.

### Results

We delineate the molecular activity of two pathways in *Toxoplasma* combining metabolomic and genetic analyses.

### Conclusion

The apicoplast is a significant source of fatty acids, and its products are further modified in the parasite endoplasmic reticulum.

### Significance

We define the metabolic host-parasite relationship with molecular resolution in intracellular parasites.
**Toxoplasma Fatty Acid Metabolism**

associated and instead rely on type I fatty acid synthesis for the production of bulk long chain fatty acids. A FASII-type synthase is present in human mitochondria, but there are structural differences between its components and those of bacterial or apicomplexan FASII (5). Furthermore, apicoplast FASII also differs from the mammalian FAS pathway, thereby making it a potential target for the treatment of apicomplexan infections (6). Initial pharmacological studies on *Plasmodium falciparum* and *Toxoplasma gondii* supported this idea (7, 8). Subsequent genetic studies found the essentiality of this pathway to be more complicated. We constructed a conditional mutant for the apicoplast acyl carrier protein (ACP) in *T. gondii*. ACP is a key molecule of the FASII pathway; fatty-acyl intermediates are covalently bound to ACP as the enzymes of the pathway act on them. Loss of ACP in this conditional mutant affects apicoplast biogenesis and results in the death of the parasite. Moreover, mice infected with a lethal dose of *T. gondii* can be cured by suppression of the FASII pathway, suggesting its importance in the growth and survival of the parasite (9). In contrast, this pathway is not essential for the bloodstream and mosquito stages of the malarial parasite *Plasmodium*, although it is essential for the liver stage that is required for establishment of infection in the mammalian host (10, 11). Other apicomplexan parasites, such as *Cryptosporidium*, *Babesia*, and *Theileria* appear to completely lack the FASI biosynthetic machinery suggesting that they are dependent on fatty acid salvage from the host (1). Although both *T. gondii* and *P. falciparum* have been shown to take up radiolabeled fatty acids from the culture medium (12, 13), these parasites are predicted to contain a number of other fatty acid biosynthetic pathways that could partly compensate for the loss of FASII (1, 14). These alternative mechanisms include a FASI pathway (in *T. gondii, Eimeria*, and *Cryptosporidium*) and a putative fatty acid elongation pathway (in most apicomplexans with the exception of *Theileria* and *Babesia*). FASI in apicomplexans so far has only been studied in *Cryptosporidium parvum* (15, 16).

Overall, these studies suggest that apicomplexan fatty acid metabolism is complex and that multiple potentially redundant biosynthetic pathways could contribute to the total cellular fatty acid pool.Identifying the specific biochemical activities of individual components and delineating their sequence and interaction and their relative importance under different growth conditions are critical for understanding host-parasite interaction and identifying potential drug targets. Here, we develop a combined genetic and metabolomic strategy to identify the products of FASII and the elongation pathways for *T. gondii*. De novo fatty acid synthesis was monitored in wild type parasites and in mutant parasite lines in which specific fatty acid biosynthetic enzymes were selectively ablated by labeling parasite-infected host cells with [U-13C]glucose and analysis of parasite lipids by gas chromatography–mass spectrometry (GC-MS). The activity of specific fatty acid elongases was further examined by heterologous expression and biochemical analyses. Our analyses suggest that the FASII and elongase pathways have nonredundant functions in intracellular tachyzoite stages in maintaining the fatty acid composition of bulk lipids and that these de novo pathways cannot be by-passed by fatty acid salavage from the host cell.

**EXPERIMENTAL PROCEDURES**

**Gene Identification and Gene Tagging**—The protein sequences of the yeast fatty acid elongation machinery (ELO1–3, ketoacyl-CoA reductase, dehydratase, and enoyl-CoA reductase; GenBank™ accession numbers NP_012339, NP_009963, NP_13476, AAS56194, NP_012438, NP_010269) were used as query sequences for BLAST searches against the *T. gondii* genome and GenBank™ databases. To evaluate the computational predictions for these genes, we conducted RT-PCR experiments. The predictions for *T. gondii* ELO-A, ELO-B, ELO-C, and DEH were confirmed. However, for ECR and KCR, the beginning and ending of the coding sequences were different and were subsequently established by RT-PCR, subcloning, and sequencing. The experimentally validated *T. gondii* genes described in this study are ELO-A, ELO-B, ELO-C, KCR, DEH, and ECR (see supplemental Table S1 for GenBank™ accession numbers). The full-length coding sequences for all genes were amplified from *T. gondii* cDNA using primers introducing flanking BglII and AvrII restriction sites, subcloned into plasmid pCR2.1 (Invitrogen), and subsequently introduced into the equivalent sites of either plasmid pdt7s4H3 or pdt7s4M3 (17), placing them under the control of a tetracycline-regulatable promoter and fusing a triple HA (pDT7S4H3) or a triple c-Myc epitope tag (pDT7S4M3), respectively, to the 3′-end. All primers used are listed in supplemental Table S1.

**Parasite Culture and Construction of Mutants**—Parasites were cultured and genetically manipulated as described previously (18). Conditional mutants were generated using the previously described two-step strategy (9, 19). Plasmids containing a second copy of the target gene under the control of a regulatable promoter (pdt7s4H3) were stably introduced into a *T. gondii* tetracycline transactivator strain (20). Stable clones for ELO-A, -B, or -C were established in the presence of pyrimethamine. We next engineered a plasmid-based construct to delete the ELO-C locus and cosmid-based targeting vectors for ELO-A and -B. For ELO-C, sequences (~2 kb) upstream and downstream of the ELO-C genomic locus were amplified by PCR and subcloned into the respective BamHI/KpnI and AatII/SpeI restriction sites of the PTCY vector flanking a chloramphenicol resistance cassette (the vector also contains a YFP cassette for negative selection (9)). This construct was linearized with Spel and transfected into the pDT7S4-ELO-C-H3 parasite line. Transgenic parasites that were chloramphenicol-resistant and YFP-negative were isolated. Clones were tested by PCR for integration, and gene disruption was confirmed by Southern blot analyses. (Plasmids used for the probes are listed in supplemental Table S2.) Cosmids (TOXP929/ELO-B or TOXO789/ELO-A) were modified to replace the coding sequence with a chloramphenicol resistance cassette by recombineering (19). Targeting cosmids were transfected into the respective parasite, and transgenic clones were selected in the presence of chloramphenicol. Mutants were identified as described for ELO-C. We conducted plaque assays in the absence or presence of 0.5 μM ATc to evaluate the growth of mutants (18). For chemical complementation assays, myristic and palmitic acids.
were dissolved in equal molar amounts in ethanol, dried into a microcentrifuge tube, and complexed to fatty acid-free BSA in water (Invitrogen, 1:3 molar ratio) with sonication prior to addition to normal growth medium as indicated.

**Microscopy**—Immunofluorescence microscopy was performed as described previously (17). Infected coverslip cultures were fixed with 3% paraformaldehyde in PBS, followed by permeabilization in 0.25% Triton X-100 in PBS. After blocking, rat anti-HA antibodies (Roche Applied Science) were used at a dilution of 1:100, rabbit anti-ACP (a kind gift from Geoff McFadden, University of Melbourne (4)) at 1:1000, mouse anti-GFP (Torrey Pines Bioslabs) at 1:400, and rabbit anti-Myc (Roche Applied Science) at 1:100. Secondary antibodies were used at a dilution of 1:200 and were anti-rat Alexa Fluor 546, anti-rabbit Alexa Fluor 488, and anti-mouse Alexa Fluor 488 (all from Invitrogen). Images were taken using an Applied Precision Delta Vision microscope, and images were deconvoluted and adjusted for contrast using Softworx. Protein localization annotations have been submitted to the ApiLoc data base of protein subcellular localization in Apicomplexa.

**Western Blotting**—Western blot analyses were performed as described previously (17) and used rat anti-HA antibodies (Roche Applied Science) at 1:100 and anti-GRAB antibodies at 1:2000 (a kind gift from Gary Ward, University of Vermont (21)).

**Western Blotting**—Immunofluorescence microscopy was performed as described previously (17) and used rat anti-HA antibodies (Roche Applied Science) at 1:100 and anti-GRAB antibodies at 1:200 (a kind gift from Gary Ward, University of Vermont (21)).

**Stable Isotope Labeling and Metabolomic Analyses**—Yeast strains were grown on YP (1% yeast extract, 2% peptone) or yeast synthetic complete (SC) medium supplemented with 0.67% Difco yeast nitrogen base (BD Biosciences), containing either 2% (w/v) D-galactose and 0.05% (w/v) D-glucose (GalGlu), 4% (w/v) D-glucose or 2% (w/v) raffinose as the carbon source(s). For solid media, 2% agar was added. Selections were carried out on plates lacking leucine (SGal-Glu−Leu). Counter-selections against plasmids containing a URA3 marker were done on 5-fluoroorotic acid plates (23) supplemented with GalGlu (5’FOAGalGlu). Liquid culture with 4% (w/v) D-glucose was used to repress expression from the GALL promoter. Strains subjected to spotting assay were grown overnight in YPGalGlu, kept for 24 h below an A600 < 1, and then grown for 8 h in YPRaf. The cell density was normalized to an A600 = 0.5 before being spotted on YPGalGlu, SGalGura GalGlu, and SC 5’FOAGalGlu.

**Yeast Strain Construction**—The yeast strain Saccharomyces cerevisiae W1536 (Δelo2Δelo3/YCp33 GALI ScELO3 (URA3)) (Δade2, Δade3, his3-11, his3-12, trp1-1, ura3-1, elo2::kanMX4, elo3::kanMX4) has been described before (24–26). The yeast strain was transformed with either one of the following plasmids (all LEU2): YCp111 GALI-TgELO-B, YCp111 GALI-TgELO-C, or YCp111 GALI-TgELO-A on SCGallGlu-Leu. (Primers used in the construction of these plasmids are listed in supplemental Table S3.) Candidate transformants on SCGalGlu-Leu plates were picked and streaked on 5’-FOAGalGlu plates to select against the presence of the YCp33 GALI ScELO3 plasmid. Complementation of the Δelo2Δelo3 mutations was indicated by the production of a large amount of FOA-resistant colonies. Presence of the plasmids carrying T. gondii constructs was verified by the ability of the FOA-resistant colonies to grow on SCGalGlu-Leu. These candidates were used in further experiments. Strain W1536-5B Δtsc13/ YCplac33 GALI ScTSC13 (MATa; Δade2, Δade3, his3-11, his3-12, trp1-1, ura3-1, tsc13::kanMX4) was transformed with 0.5 N methanolic HCl (50 μL, Sigma), and the capillaries were sealed under vacuum and incubated at 95 °C overnight. Samples were neutralized with 10 μL of pyridine (20 min) before being transferred to GC-MS vials, dried, and derivatized by addition of 25 μL of N-methyl-N-(trimethylsilyl)-trifluoroacetamide/trimethylchlorosilane. Samples were analyzed on an Agilent 7890A-5975C GC-MS system. Splitless injection (injection temperature 280 °C) onto a 30 m + 10 m × 0.25 mm DB-5MS + DG column (J&W, Agilent Technologies) was used, using helium as the carrier gas. The initial oven temperature was 80 °C (2 min), followed by temperature gradients to 140 °C at 30 °C/min, from 140 to 250 °C at 5 °C/min, and from 250 to 265 °C at 15 °C/min. The final temperature was held for 10 min. Data analysis was performed using Chemstation software (MSD Chemstation D.01.02.16, Agilent Technologies). Abundance and label incorporation was calculated as described previously (22). Data shown are the average of three technical replicates and their standard deviation. They are representative of two biological experiments. Statistical significance was evaluated by Wilcoxon Rank Sum Testing with continuity corrections, using the R data analysis package (version 2.14.0), where p values of <0.05 were considered significant.
YCp111 GAL1-TgTSC13 on SCGAlGlu-Leu, and colonies complemented by *T. gondii TSC1* were identified and isolated in an analogous fashion as described above. Finally, yeast mutants complemented by *T. gondii PHS1* were generated in a similar manner, using strain W1536-5B Δphs1/pYES2 PHS1 (MATα; Δade2, Δade3, his3-11, his3-12, trpl-1, ura3-1, psh1::kanMX4) transformed with YCp111 GAL1-TgPHS1.

**Preparation of ER Extracts from Yeast Cells**—Yeast cells were grown to exponential phase (2·10⁶ cells·ml⁻¹) and then harvested by centrifuging for 15 min at 1300 × g, 4 °C. The pellet was washed with sterile water and suspended in ice-cold lysis buffer. The cells were disrupted with glass beads and centrifuged for 15 min at 15,000 × g at 4 °C to remove the cell debris. The supernatants were centrifuged for 90 min at 85,000 × g in a Sorvall Ti70 rotor at 4 °C to generate pellet (P85) and supernatant (S85) fractions. The P85 was suspended in lysis buffer and used for the elongase assay. The protein concentrations were determined by the method of Lowry using bovine serum albumin as the standard. Equivalent amounts of total lysate, P85, and S85 were precipitated by adding trichloroacetic acid (TCA) to a 10% final concentration and processed for immunoblotting.

**Yeast Fatty Acid Analysis**—Yeast cultures were inoculated from an overnight starter culture to an A₆₀₀ of 0.05 in 100 ml of YPGalGlu, allowed to grow to an A₆₀₀ of 0.5, and harvested by 5 min centrifugation at 4300 × g. Cells were washed in 50 ml of water and harvested by centrifugation. The cell pellet was flash-frozen in liquid nitrogen and stored at −70 °C. Fatty acid analysis was performed following methods published earlier (27) with minor modifications. Briefly, the cell pellets were extracted with 1 ml of chloroform/methanol/formic acid (10:10:1) followed by a second extraction with 1 ml of chloroform/methanol/water (5:5:1 v/v). The combined organic phases were washed with an equal volume of 1 M KCl in 0.2 M phosphoric acid and dried. Lipids were dissolved in 1 ml of toluene and treated with 1 ml of methanolic HCl (prepared by adding 0.5 ml of acetyl chloride to 5 ml of cold dry methanol) overnight at 50 °C. The solution was washed with 1 ml of 4 M KCl; the organic phase was separated, and the aqueous phase was extracted with 1 ml of hexane. The organic phases were pooled, washed with 0.5 ml of 0.5 M ammonium bicarbonate, dried, redissolved in 100 µl of hexane, and subjected to gas chromatography on an HP6890 gas chromatograph with an HP5973 mass analyzer and equipped with a J&W Scientific DB624 (30 m × 0.32 mm, stationary phase 1.8 µm) column. 1 µl was injected splitless (detector block 250 °C) and subjected to a temperature program from 60 to 250 °C (20 °C/min) and a hold period of 35 min, flow 2 ml/min. Masses were scanned from m/z 35 to 800, and a Wiley 275 spectral library was used for identification.

**RESULTS**

**Toxoplasma Fatty Acid Metabolism**—In apicomplexans, the enzymes of the FASII pathway are localized to the apicoplast (4). In plants, the homologous chloroplast pathway is the sole source of de novo-synthesized fatty acids. We have previously constructed a *T. gondii* mutant with a conditional defect in this pathway. Although the growth of this mutant was severely reduced upon inhibition of FASII, no difference was observed in the rate of fatty acid biosynthesis as measured using [1⁴C]acetate (9). We considered that FASII may not use acetyl-CoA generated from exogenous [1⁴C]acetate. To test this, we have established a new metabolic labeling procedure, in which the incorporation of 1³C-derived from [U⁻⁷¹C]glucose into the entire cellular pool of fatty acids is detected by GC-MS analysis (Fig. 1, A–C). When infected fibroblasts were labeled with [U⁻¹⁷⁷C]glucose, label was rapidly incorporated into tachyzoite lipids that were recovered from extracellular stages after host cell lysis and parasite egress. [U⁻¹⁷⁷C]Glucose labeling of extracellular tachyzoites resulted in negligible labeling of parasite lipids indicating that de novo fatty acid synthesis occurs predominantly in intracellular stages and decreases dramatically following egress (data not shown). Host fibroblast cells are also capable of synthesizing fatty acids via a FASI system. In principal, 1³C derived from exogenous [1⁷³C]glucose could be incorporated into host fatty acids and subsequently scavenged by intracellular tachyzoites. To investigate the extent to which fibroblasts synthesize fatty acids, uninfect fibroblasts were metabolically labeled with [U⁻¹⁷⁷C]glucose, and incorporation of 1³C into fatty acids was assessed by GC-MS. In contrast to the intracellularly labeled tachyzoites, negligible label was detected in the uninfected fibroblast lipids (Table 1). These experiments, together with the data presented below, indicate that 1³C labeling of tachyzoite fatty acids with [1³C]glucose represents de novo fatty acid biosynthesis within the parasite rather than uptake of labeled host lipids. In this context, it is notable that significant differences occur in the extent to which different fatty acids are labeled. Although the long and very long chain fatty acids, C14:0, C16:0, C16:1, C18:1, C20:1, and C26:1, were strongly labeled (>60%) others, including C18:0, C22:0, and C24:1, were labeled to less than 20%. These differences could reflect differences in the turnover and achievement of isotopic equilibrium in different fatty acids pools or, more likely, differences in the rate of salvage of “unlabeled” host fatty acids. Regardless, the results indicate that a number of major fatty acids in intracellular stages are primarily synthesized de novo rather than taken up from the host.

**Apicoplast FASII Is Required for Synthesis of Long Chain Fatty Acids**—The detection of multiple mass isomers of the long chain fatty acids, differing by 2 atomic mass units in [U⁻¹⁷⁷C]glucose fed-parasites (Fig. 1B) indicated multiple rounds of incorporation of [1³C]acetoy-CoA and [1²C]acetyl-CoA into these chains, consistent with the operation of a FAS system. To investigate the contribution of the apicoplast-located FASII complex to cellular fatty acid synthesis, fibroblasts were infected with the *T. gondii* ΔACP/ACPi mutant that expresses components of the apicoplast FASII complex under the control of a tetracycline-regulated promoter (9). When *T. gondii* ΔACP/ACPi-infected fibroblasts were incubated with [U⁻¹⁷⁷C]glucose in the absence of ATc, high levels of 1³C incorporation were observed in tachyzoite lipids (Fig. 1, D and E). In contrast, when labeling was done in the presence of ATc, resulting in selective down-regulation of parasite FASII activity, the labeling of tachyzoite fatty acids was strongly inhibited. In particular, labeling of the long chain saturated and unsaturated fatty acid C14:0 and C16:0 was reduced by >80% in the pres-
ence of ATc (Fig. 1E). In a control experiment, ATc was found to have no effect on the labeling of wild type tachyzoite fatty acids, indicating that ATc by itself does not inhibit [U-13C]glucose uptake and catabolism to acetyl-CoA or fatty acid synthesis (Fig. 1C and Table 1). These experiments provide further direct support for the conclusion that labeling of parasite fatty
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### Table 1

| Cell type | RH | HFF |
|-----------|----|-----|
| +/− ATc  |    |     |
| C14:0     | 87.73 (± 3.86) | 84.68 (± 0.50) | ND |
| C16:0     | 56.56 (± 0.58)  | 59.01 (± 1.09)  | 0.00 (± 0.00) |
| C18:0     | 12.60 (± 0.55)  | 12.64 (± 0.73)  | 0.00 (± 0.00) |
| C20:0     | 23.64 (± 12.81) | 16.84 (± 6.41)  | 0.00 (± 0.00) |
| C22:0     | 1.14 (± 2.95)   | 4.23 (± 1.05)   | 0.00 (± 0.00) |
| C24:0     | 14.57 (± 4.41)  | 15.38 (± 1.53)  | 0.00 (± 0.00) |
| C16:1     | 65.15 (± 1.62)  | 63.76 (± 0.47)  | 0.00 (± 0.00) |
| C18:1     | 57.97 (± 3.88)  | 54.59 (± 0.15)  | 1.76 (± 1.77)  |
| C20:1     | 70.41 (± 0.82)  | 68.62 (± 0.71)  | 0.00 (± 0.00) |
| C22:1     | 6.81 (± 8.79)   | 9.76 (± 3.44)   | 0.00 (± 0.00) |
| C24:1     | 1.31 (± 1.87)   | 0.00 (± 2.58)   | 0.00 (± 0.00) |
| C26:1     | 82.83 (± 4.14)  | 80.37 (± 2.75)  | ND |

Adapting fatty acids with [U-13C]glucose reflects parasite *de novo* synthesis rather than salvage. They also suggest that the FASII pathway is directly responsible for the *de novo* synthesis of saturated long chain fatty acids (C14:0 and C16:0) that are utilized, at least in part, for the synthesis of unsaturated and very long chain fatty acids.

**Growth Defects Due to Loss of FASII Pathway Are Only Partially Restored by Chemical Complementation with Myristic and Palmitic Acid**—We have previously shown that loss of FASII activity leads to inhibition of parasite growth (9). As loss of FASII results in a deficiency in myristic and palmitic acid synthesis, we investigated whether nutritional supplementation of FASII-deficient parasites with these fatty acids would restore growth. Parasite lines expressing red fluorescent protein were cultured in 96-well plates in medium supplemented with fatty acids complexed to BSA at concentrations ranging from 20 nM to 2 µM. These fatty acid concentrations have no effect on the growth of the wild type parasites (data not shown). As expected, intracellular growth of the ΔACP/ACPi mutant was markedly reduced after 6 days in the presence of ATc (Fig. 2A) (9). Growth inhibition in the presence of ATc was delayed by 2 days in the presence of a combination of 1 µM myristic and 1 µM palmitic acid. However, fatty acid supplementation did not restore wild type growth or prevent death over longer periods, possibly reflecting inefficient transport to the parasite vacuole, increased fatty acid demand at later time points, and/or the involvement of FASII in other lipid biosynthetic pathways, such as the synthesis of lipoic acid.

**Parasites Express an Endoplasmic Reticulum-associated Fatty Acid Elongation System**—Several very long chain fatty acids were labeled with [U-13C]glucose in the absence of FASII suggesting that additional fatty acid biosynthetic pathways are active during intracellular tachyzoite development. Bioinformatic analysis of the *T. gondii* genome indicates that these parasites may have a functional fatty acid elongase system. Using the sequences of the components of the yeast elongation pathway as query in similarity searches, we found three candidate genes coding for fatty acid elongases and a single candidate for each of the downstream reductases and the dehydratase in *T. gondii* (see supplemental Table S1).

We evaluated the coding sequence for all six genes by cDNA and rapid amplification of cDNA ends-PCR followed by subcloning and sequencing. This analysis validated the computationally predicted gene models for four genes: ELO-A–C and the dehydratase, which is annotated as a hypothetical protein tyrosine phosphatase-like domain-containing protein in the *T. gondii* genome database. We established a diverging coding sequence for the presumptive enoyl-reductase and keto-acyl-CoA reductase (see “Experimental Procedures” and supplemental Table S1 for details). The coding sequences were amplified and introduced into a parasite expression vector that appended a C-terminal epitope tag. These constructs were transfected into parasites, and stable transgenic lines were established through drug selection and cloning (see “Experimental Procedures” for details). The six epitope-tagged proteins localized to the same perinuclear structure in the parasite, as determined by immunofluorescence assays. For comparison, we transfected these lines with an expression plasmid for Der1-GFP (17), a marker for the *T. gondii* endoplasmic reticulum. This marker co-localized with the epitope-tagged proteins indicating that these enzymes localize to the endoplasmic reticulum (Fig. 3) (note that ELO-C had a more restricted pattern at the apical face of the nuclear envelope reminiscent of the previously characterized ER exit site (28)). These observations are consistent with reports from other organisms where the components of the fatty acid elongation pathway have been found to form a multienzyme complex on the membrane of the endoplasmic reticulum (29, 30).

**Candidate T. gondii Genes Rescue Lethal Yeast Fatty Acid Elongation Mutants**—We next tested the molecular function of each of the six enzymes by heterologous expression in well-defined yeast mutants lacking equivalent enzymes. For these phenotypic complementation experiments, we constructed yeast expression vectors under the control of the galactose-activated/glucose-repressed GAL1 promoter. Constructs encoding the putative *T. gondii* fatty acid elongases were transformed into *S. cerevisiae* strain W1536Δelo2Δelo3/YCp33 GAL1-ScELO3 (26). This mutant lacks the genes for both fatty acid elongase 2 and 3. This is a lethal double mutation (27), and the strain is only viable due to episcopal expression of elongase 3 from a GAL1 promoter. Following transformation with parasite test plasmids, loss of the *URA3*-containing yeast plasmid was achieved by counter-selection on media containing the *URA3* activated toxic substrate 5′-fluoroorotic acid. *T. gondii* ELO-B and ELO-C but not ELO-A rescued the growth deficiency of the *Δelo2Δelo3* mutant strain (Fig. 4A). Functional complementation was further validated by incubation of microsomal fractions prepared from the two respective complemented strains with radiolabeled malonyl-CoA and palmitoyl-CoA (31). As expected, this resulted in chain elongation of the substrate (data not shown). Similarly, when expressed in yeast cells, the *T. gondii* candidates for dehydratase and enoyl reductase were found to complement the growth of mutants in their respective yeast homologs (*S. cerevisiae* Phs1p and Tsc13, see Fig. 4B and “Experimental Procedures” for detail). Overall, these experiments suggest that the *T. gondii* candidate genes indeed encode the enzymes of the fatty acid elongation system. We cannot make firm conclusions for TgELO-A and TgKCR that failed to complement. This may be due to lack of expres-
sion or poor interaction of the heterologous enzymes with the native yeast machinery.

Genetic Ablation of Fatty Acid Elongases in *T. gondii*—To further investigate the functional significance of the fatty acid elongase system in *Toxoplasma*, we generated conditional parasite mutants for the condensing enzymes of the pathway. A parasite strain expressing epitope-tagged ELO-B under a tetracycline-regulated promoter from an ectopic locus was con-

FIGURE 2. Growth of ΔACP/ACP parasites in media supplemented with myristic and palmitic acid. Growth of ΔACP/ACP parasites stably expressing a dTomatoRFP transgene was evaluated by measuring parasite fluorescence daily in a 96-well culture format (35). Parasites were grown in normal growth media supplemented with fatty acid-free BSA (A) or media supplemented with 1 μM of each myristic and palmitic acid coupled to BSA (3:1 molar ratio) (B). Parasites were grown in the presence (black) or absence (white) of ATc. The average of fluorescence intensity (arbitrary units) for three independent replicates is shown, and error bars represent the standard deviation for each data point. Continuous culture in BSA-conjugated fatty acids (or higher serum supplementation) did not lead to continuous growth of the ACP mutant in the presence of Atc. FA, fatty acids.

FIGURE 3. *T. gondii* fatty acid elongation pathway is localized to the parasite endoplasmic reticulum. Transgenic parasite lines were allowed to infect coverslip cultures and were fixed and processed for immunofluorescence assay 24 h later. Transgenic proteins were marked with an HA (A–C) or a Myc (D–F) epitope tag detected with suitable antibodies (red channel). All strains carried a Der1GFP marker (green) previously shown to localize to the endoplasmic reticulum (17). DAPI and phase contrast images are shown for comparison. See supplemental Table S1 for reference to *T. gondii* gene models and “Experimental Procedures” for details on gene identification and cloning. Note that all six candidate fatty acid elongation enzymes are found to be associated with the endoplasmic reticulum. Tagged ELO-C produces a more restricted pattern at the apical side of the nuclear envelope.
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constructed. In this background, we replaced the chromosomal copy of this gene with a chloramphenicol resistance cassette using homologous recombination. Chloramphenicol-resistant parasites were screened by PCR to identify the clones with genetic disruption for ELO-B. Using a similar strategy, we also engineered conditional mutants for ELO-C and ELO-A (see “Experimental Procedures” for details and note that some deletions were constructed using plasmids (9) and others with cosmids (19)). Deletion of the respective locus was confirmed for each mutant by Southern blot (Fig. 5, G–I). We evaluated the ATc dependence of the expression of each of the genes by immunofluorescence assay and Western blot assays. For immunofluorescence assay, mutant parasites were grown in the absence and presence of ATc for 24 h prior to fixation and processing. For Western blots, parasites were grown in ATc for 0–4 days. Both assays show rapid loss of the tagged proteins upon ATc addition. The proteins are essentially undetectable after 24 h of treatment (Fig. 5). To determine the effect of the loss of these proteins on parasite growth, we next performed plaque assays. Confluent host cell monolayers were infected with the mutant parasites in the absence or presence of ATc. Plaques in the host monolayer were visualized by staining after 10 days. We detected no significant difference in plaque number or size for each of the three elongase mutants (Fig. 5L). The ACP mutant was used as a positive control, and reduced plaque formation was readily observed as described previously (9). We conclude that individual loss of ELO-A, ELO-B, or ELO-C does not impede parasite growth in tissue culture.

T. gondii ELO Proteins Are Required for the Synthesis of Very Long Chain Fatty Acids—We do not detect a growth phenotype in single elongase-deficient parasites. This could reflect the possibility that fatty acids synthesized by the elongase system are not essential, the ELO enzymes are functionally redundant (27), and/or the intracellular parasites can compensate for the loss of the pathways by salvaging necessary fatty acids from the host. To determine the specific biochemical role of individual elongases, the fatty acid biosynthesis capabilities of each mutant were initially analyzed using [14C]acetate labeling. Parasites were grown in the absence or presence of ATc, liberated from their host cells, and incubated in fatty acid-free medium supplemented with 10 μCi of [14C]acetate. Lipids were extracted after 4 h, and the corresponding fatty acid methyl esters were analyzed by thin layer chromatography (Fig. 6). The loss of ELO-B resulted in a moderate yet reproducible decrease in the labeling of all long chain fatty acids. Loss of ELO-C produced the complete loss of labeling in a single very long chain fatty acid. In addition, we noted a marked increase in the abundance of a shorter fatty acid, suggesting that this may be a precursor that accumulates in the absence of the downstream enzyme (Fig. 6C). Ablation of ELO-A did not produce recognizable changes in the labeling pattern. Although these analyses indicate clear changes in the biosynthetic capabilities of two of our mutants, they are limited in their sensitivity and resolution. In particular, they do not provide accurate measurement for the length and saturation of the affected fatty acids.

FIGURE 4. T. gondii genes complement yeast fatty acid elongation mutants. Yeast mutants with deletions in specific genes encoding fatty acid elongation enzymes were transformed with plasmids carrying T. gondii candidate genes as detailed under “Experimental Procedures.” The resulting strains were used to conduct spotting assays on YPGalGlu media and demonstrate complementation (panels on the left). Control experiments using the same yeast cultures were done on media that select against the yeast rescue plasmid and thus indicate that phenotypic complementation is due to presence of the T. gondii constructs only (5’ FOA-GalGlu), or media that indicate presence of the yeast rescue plasmids only (ScUra-GalGlu) (center and right panels, respectively). Yeast cultures were progressively diluted prior to plating from left to right as indicated. A, complementation analyses for the yeast elongase ScΔelo2Δelo3 double mutant and the yeast (8) enoyl-CoA reductase (ScECR) and dehydratase (ScΔDEH) mutants, respectively. Note that under restrictive conditions (5’ FOA-GalGlu) growth depends on the presence of the T. gondii genes. Only experiments that resulted in phenotypic complementation are shown here. See Table 2 for full detail on the genotype of all yeast strains.
ELO-B and ELO-C Produce Monounsaturated Long Chain Fatty Acids in a Two-step Mechanism—To further investigate the substrate specificity of the different *T. gondii* fatty acid elongases, each of the ELO mutant parasites was metabolically labeled with [13C]glucose in absence or presence of ATc, and fatty acid mass isotopomers were analyzed by GC-MS. The labeling of saturated long chain fatty acids up to C16:0 was unaffected by the individual loss of activity of ELO-A, ELO-B, and ELO-C (Fig. 7), consistent with their synthesis being regulated by FASII. Loss of ELO-A activity was associated with a marked reduction in labeling of saturated and unsaturated fatty acids with chain lengths of 18–24 carbons. Ablation of ELO-A also resulted in a reduction in the cellular levels of these fatty acids. Significantly, both the abundance and level of labeling of the unsaturated fatty acid C16:1 increased in the absence of ELO-A. These results suggest that ELO-A is required for efficient elongation of C16:0 or C16:1 to longer species. In the absence of ELO-A, de novo synthesized C16:0 is apparently channeled into increased synthesis of C16:1.

In contrast, loss of expression of ELO-B or ELO-C resulted in enhanced labeling of long chain fatty acids but reduced labeling of several very long chain fatty acids. Specifically, down-regulation of ELO-B activity severely ablated labeling of the monounsaturated very long chain fatty acids C20:1, C22:1, C24:1, and C26:1 (Fig. 7A and Table 2). However, down-regulation of ELO-C activity led to a highly selective loss of labeling of C26:1...
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Loss of elongases results in selective reductions in the rate of synthesis and cellular levels of unsaturated fatty acids. Intracellular tachyzoites of the three ∆ELO mutants were labeled with [U-13C]glucose in the presence (black) or absence (white) of ATc as detailed in Fig. 1. A level of 13C incorporation into the major fatty acids (FA) of the isolated tachyzoites. Error bars represent standard deviation where n = 3–4. B, changes in the relative abundance of individual fatty acids in ∆ELO tachyzoites in the presence of ATc as measured by GC-MS (values are given relative to the abundance of this fatty acid species in the no ATc control). Results are indicative of >3 individual replicates. Individual values for all experiments are listed in Table 4. Fatty acids for which significant changes in labeling were observed in the presence and absence of ATc using the Wilcoxon Rank Sum Test (p values less than 0.05) are indicated with an asterisk.

**TABLE 2** Percent labeling of fatty acids derived from [U-13C]glucose-fed tachyzoites

| Cell type | ACP | ELOA | ELOA | ELOA | ELOB | ELOB | ELOC | ELOC |
|-----------|-----|------|------|------|------|------|------|------|
| +/−ATc    |     |      |      |      |      |      |      |      |
| C14:0     | 54.08* (± 0.99) | 7.66* (± 1.78) | ND   | ND   | ND   | ND   | ND   | ND   |
| C16:0     | 33.58* (± 0.68) | 6.06* (± 0.22) | 45.17 (± 0.64) | 44.24 (± 0.19) | 50.01 (± 0.45) | 46.34 (± 1.47) | 45.65 (± 1.28) | 45.14 (± 0.35) |
| C18:0     | 2.38* (± 0.51)  | 0.26* (± 0.26) | 10.02* (± 0.50) | 3.08* (± 0.03) | 8.40 (± 0.40) | 10.18 (± 0.54) | 5.95 (± 0.53) | 7.02 (± 0.18) |
| C20:0     | 0.00 (± 0.00)   | 0.00 (± 0.00) | 17.79* (± 3.14) | 2.28* (± 1.60) | 0.00 (± 0.00) | 20.99 (± 8.83) | 0.00 (± 0.00) | 1.97 (± 7.81) |
| C22:0     | 0.00 (± 0.00)   | 0.00 (± 0.00) | 9.27 (± 1.61)  | ND   | 1.26 (± 2.64) | 4.62 (± 3.55) | 0.00 (± 2.47) | 0.00 (± 0.00) |
| C24:0     | 0.00 (± 4.82)   | 1.23 (± 0.08) | 10.21* (± 1.47) | 3.75* (± 0.12) | 4.18 (± 0.42) | 8.87 (± 3.04) | 3.12 (± 0.81) | 3.17 (± 1.14) |
| C16:1     | 17.76* (± 1.76) | 10.01* (± 3.17) | 35.63 (± 3.90) | 47.04 (± 1.82) | 41.37 (± 2.78) | 25.38 (± 7.47) | 23.94 (± 4.33) | 36.37 (± 2.07) |
| C18:1     | 16.37* (± 0.86) | 4.96* (± 0.15) | 31.95* (± 0.25) | 5.42* (± 0.25) | 20.15 (± 0.08) | 27.88 (± 0.64) | 28.86 (± 0.53) | 28.83 (± 0.19) |
| C20:1     | 24.05* (± 2.99) | 11.21* (± 0.61) | 45.56* (± 1.48) | 21.54* (± 3.51) | 40.60* (± 1.72) | 8.46* (± 1.93) | 43.64 (± 0.96) | 45.09 (± 0.83) |
| C22:1     | 0.00 (± 1.82)   | 1.05 (± 1.50) | 0.51 (± 1.26) | 0.55 (± 0.82) | 20.12* (± 3.21) | 0.00* (± 0.19) | 4.91 (± 1.38) | 24.13 (2.22) |
| C24:1     | 0.31 (± 0.52)   | 0.00 (± 1.58) | 8.36 (± 1.75)  | 5.29 (± 4.18) | 9.43* (± 2.07) | 2.63* (± 1.14) | 8.10 (± 2.10) | 8.39 (± 1.70) |
| C26:1     | 36.45 (± 11.48) | 27.56 (± 1.86) | 59.32* (± 4.71) | 47.30* (± 3.37) | 70.80* (± 2.30) | 0.00* (± 0.00) | 59.41* (± 3.28) | 0.00* (± 0.00) |

and the accumulation of C22:1. These results suggest that ELO-B is largely responsible for the elongation of C18:1/C20:1 to C22:1, whereas the primary role of ELO-C is to elongate fatty acids from C22:1 to C26:1. This model is also supported by our measurements of overall fatty acid abundance (Fig. 7B). Loss of ELO-C results in a severe drop in the abundance of only C26:1, in contrast, loss of ELO-B affected several shorter monounsaturated fatty acids in addition to C26:1. This is reminiscent of the situation in S. cerevisiae, where ScElo2p (equivalent to TgELO-B) is capable of elongating fatty acids up to 24 carbons, preferring a substrate with <22 carbon length. Likewise, ScElo3p and TgELO-C are alike in preferential production of
very long chain fatty acids. Note that both *T. gondii* enzymes complement the yeast double mutant indicating that they can have overlapping and slightly broader specificity.

To distinguish between the potential activities of ELO-B, we returned to the complemented yeast strains and analyzed their fatty acid contents. The wild type yeast, mutant Δelo2Δelo3 complemented *S. cerevisiae* ELO3, as well as the mutant complemented by expression of TgELO-B were grown on YPGal-Glu medium. Total lipids were extracted and analyzed by GC-MS (see under “Experimental Procedures”). As shown previously, when compared with wild type, the mutant shows a much decreased level of fatty acids C20–24, indicating that FASII is inactive (see GC-MS tracks in Fig. 8, A and B). An overlay of the chromatogram of fatty acid methyl esters from this strain and the wild type is shown. A, enlargement of the region of the trace showing C22 and C24 methyl esters. B, region showing C26 methyl esters.

**DISCUSSION**

Intracellular pathogens need to either scavenge or synthesize lipids to maintain the integrity of their own membranes and/or the membrane of specialized vacuoles within which they survive and proliferate. Lipid and fatty acid salvage mechanisms have been shown to be important for the intracellular growth of several intracellular bacteria. Similarly, apicomplexan parasites have been shown to salvage lipids from the host endocytic pathway or cytoplasmic lipid bodies (12, 32–34, 36). Nonetheless, recent bioinformatic, genetic, and biochemical studies have suggested that intracellular *T. gondii* tachyzoite stages express multiple pathways of fatty acid biosynthesis and may be dependent on *de novo* synthesis of fatty acids for the synthesis of bulk and specialized lipid classes (1). To dissect this complex network of uptake and synthesis, we have combined reverse genetics with metabolomic analyses. We define the function of individual fatty acid synthesis pathways and have developed a model that incorporates these findings (Fig. 9). The FASII pathway is localized to the apicoplast (Fig. 9, shown in green) and is thought to utilize acetyl-CoA derived from glycolytic triose phosphates that are imported into the apicoplast via a phosphate translocator (19, 37). Our labeling studies provide strong support for this model. Specifically, although [14C]acetate was not effectively incorporated into FASII products, 13C derived from [U-13C]glucose was strongly incorporated into this pathway. Ablation of FASII activity resulted in a global reduction in fatty acid labeling. This was most apparent for the saturated long chain fatty acids C14:0 and C16:0, indicating that FASII is largely responsible for maintaining the cellular levels of these fatty acids. The pattern of 13C labeling of these fatty acids in FASII-competent parasites was also consistent with *de novo* synthesis from short chain (∼C4) fatty acid precursors. Synthesis of this precursor is most likely dependent on apicoplast FabD (38), but apicoplast ACP has also been demonstrated to be capable of self-malonylation (39). Note that *T. gondii* also expresses two differentially localized acetyl-CoA carboxylases, one in the apicoplast the other in the cytoplasm (40), that could provide malonyl-CoA for FASII and the elongation pathway, respectively. These findings demonstrate that the apicoplast, like the plant chloroplast, is the main cellular site of fatty acid *de novo* synthesis.
TABLE 4
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Values indicated are % of relative response units of total fatty acid methyl esters. *p values were calculated using a two-tailed Student’s t test, setting not detected fatty acid species to 0. The results of fatty acid extractions from two independent sets of cultures are shown. Fatty acyl species were identified by GC/MALDI mass spectroscopy as described under “Experimental Procedures.” ND means not detected.

| C14 | C15 | C16:1/16 | C18:1/18 | C20:1/20 | C22:1 | C22 | C24:1 | C24 | C26 | C26-OH |
|-----|-----|----------|----------|----------|-------|-----|-------|-----|-----|-------|
| TgELO-B | 2.5 ± 1.2 | 4.4 ± 1.9 | 26.1 ± 3.8 | 36.3 ± 5.0 | 12.4 ± 3.2 | 14.8 ± 1.3 | 2.2 ± 1 | 1.6 ± 0.2 | ND/0.1 | ND |
| TgELO-C | 2.1 ± 0.6 | 3.6 ± 2.3 | 47.2 ± 3.9 | 45.0 ± 0.4 | 2.2 ± 1.6 | 0.2 / ND | ND | ND | ND | ND |
| ScELO3 | 2.5 ± 0.1 | 2.4 ± 1.5 | 40.6 ± 0.8 | 51.9 ± 1.8 | ND | ND | ND | ND | 1.1 ± 0.6 | 1 ± 0.1 |
| WT | 3.1 ± 1.4 | 1.6 ± 1.5 | 47.2 ± 6.5 | 46.1 ± 2.2 | 0.5 ± 0.1 | 0.3 ± 0.2 | 0.5 ± 0.5 | 0.1/ND | 0.6 ± 0.4 | 0.7/ND |

*p < 0.05 is in comparison with the S. cerevisiae elo2/elo3 double mutant complemented with S. cerevisiae ELO3.

*p < 0.05 is in comparison with both S. cerevisiae WT and elo2/elo3 double mutant complemented with S. cerevisiae ELO3.

De novo synthesis. There are a number of important functions for fatty acid synthesis directly in the plastid, including lipoylation, acylation of plastid proteins, and the synthesis of plastid lipids. Indeed, the loss of FASI in the apicoplast and the plant chloroplast perturbs organelle biogenesis and division leading to organelle loss (9, 41).

Does this suggest the significance for FASII to be entirely local? Following this idea, the main function of FASII could be to maintain the apicoplast to support the organelle as the site of isoprenoid precursor synthesis (42, 43). In plant chloroplasts, FASII-derived fatty acids are rapidly exported from the plastid into the cytoplasm (44). Our experiments do not formally test this and do not establish the localization of FASII-derived lipids. We note, however, that our measurements indicate that most (60–80%) of the parasite C14:0 and C16:0 are 13C-labeled, a finding that is inconsistent with FASII only supplying a small organellar pool of lipids. Moreover, our data suggest that C16:0 and C16:1 fatty acids synthesized by the apicoplast FASII complex are further elongated by the ER elongates, indicating that a significant fraction of the apicoplast-synthesized fatty acids are exported to the ER. We propose that the apicoplast is the main source of C14:0 and C16:0 for intracellular stages of T. gondii and the liver stage of Plasmodium, but in the Plasmodium erythrocyte and mosquito stages, this need can be satisfied by salvage of fatty acids from the host. A greater dependence of T. gondii tachyzoites on de novo fatty acid synthesis is further supported by our finding that loss of FASII was only partially rescued by providing excess C14:0 and C16:0 in the culture medium. Overall, these observations are consistent with important functions of FASII-derived products within and beyond the apicoplast in T. gondii.

In addition to the FASII complex, we provide direct evidence for the activity of an elaborate fatty acid elongation pathway localized to the parasite endoplasmic reticulum. This fatty acid elongation pathway consists of four enzymatic steps. The extension of the carbon chain is initiated by a condensation reaction between acyl-CoA and malonyl-CoA catalyzed by a fatty acid elongase. This is followed by reduction through ketoyl-CoA reductase, dehydration to enoyl-CoA, and final reduction to the elongated fatty acyl-CoA. We identified a full...
complement of six enzymes and support the sequence similarity-based assignments experimentally by yeast complementation analysis for several candidates. Our \[U-^{13}C\]glucose-labeling experiments in parasite mutants and heterologous expression in yeast mutants suggest that each of the elongases may act sequentially as outlined schematically in Fig. 9E. The \textit{T. gondii} elongation system generates very long chain monounsaturated fatty acids. ELO-B elongates C18:1 to C22:1 in two carbon increments. Based on the yeast data, its activity from C20:1 to C22:1 appears to be particularly robust. C22:1 is elongated in two final rounds by ELO-C to C26:1 (note that we do not fully resolve whether the synthesis of C24 is an overlapping function between the two elongases). The \textit{T. gondii} ELO-B and ELO-C pathway is the sole source of C26:1 for the parasite and does not appear to elongate saturated fatty acids. The significance of this striking preference for unsaturated fatty acids is unclear at this point. Unsaturated fatty acids have distinct physical properties resulting in differences in e.g. membrane fluidity, and those may be more suited to the needs of the parasite. It is notable that the levels of C26:1 in fibroblasts are very low (Table 1 and Fig. 10), possibly accounting for the inability to generate multiple ELO genes.

What is the source of the ELO-B substrates? Monounsaturated fatty acids can be synthesized by the desaturation of a corresponding saturated fatty acid. A route used by many organisms is the conversion of C18:0 to C18:1 oleic acid by stearoyl-CoA desaturase (45). This enzyme is present in \textit{T. gondii} (TGGT1_053030) and has been characterized in detail in \textit{P. falciparum} (46). In \textit{Plasmodium}, it was found to be localized to the membrane of the endoplasmic reticulum and thus could work hand in hand with the ELO system. This system could potentially tap into the FASII-generated fatty acid pool through the activity of ELO-A, which appears to be required for elongation of C16:0 and C16:1 to C18:0 and C18:1, respectively. Specifically, ablation of ELO-A resulted in a marked decrease in synthesis of C18:0, which was associated with the concomitant increase in both the labeling and steady state concentration of C16:0/C16:1 (Fig. 7). It is notable that only a minor pool of C18:0 is labeled with \[U-^{13}C\]glucose in wild type parasites (<10%) indicating that this fatty acid is primarily salvaged from the host. In contrast, C18:1 is labeled to a similar extent as C16:0 and C16:1 (>40%). These findings suggest that ELO-A primarily utilizes C16:0 synthesized in the apicoplast and C16:1 synthesized by the putative tachyzoite ER desaturase. The reduced cellular levels and rates of labeling of longer chain saturated/unsaturated fatty acids in ELO-A ablated parasites indicate that the C18:0 and C18:1 synthesized by ELO-A is selectively utilized by ELO-B and ELO-C, whereas the larger cellular pool of salvaged C18:0 appears to be poorly used. Collectively, these observations indicate that synthesis of very long chain fatty acids in intracellular tachyzoites involves extensive intraorganellar transport as well as some degree of substrate channeling. It should be noted that the \textit{T. gondii} genome encodes a FASI pathway (Fig. 9D), but its role in the \textit{T. gondii} fatty acid metabolism is not yet established.

Although we demonstrate clear biochemical effects of suppressing individual ELO genes, we do not measure parasite growth impairment under tissue culture conditions. It is possible that the parasite can satisfy its needs through uptake. A recently described lipid uptake system in \textit{T. gondii} may facilitate this process (47). Alternatively, the lack of growth defects may be due to redundancy within the ELO system itself. In yeast, the loss of a fatty acid elongase 2 or fatty acid elongase 3 can be tolerated. However, loss of both elongases results in synthetic lethality. It has been suggested that the enzymes partially complement each other and that the cells can tolerate the
resulting slight differences in their fatty acid pools (27). Our yeast complementation results indicate that the \emph{T. gondii} enzymes may also have overlapping substrate specificity. To establish whether a similar redundancy exists in \emph{T. gondii}, we sought to engineer a more complete ablation of the parasite ELO system. Our first approach was to attempt to generate a conditional double mutant in ELO-B and -C. However, our attempts to generate such a mutant failed recurrently despite the fact that gene targeting was efficient for individual loci in previous experiments (data not shown). An alternative approach would be to delete a nonredundant downstream enzyme of the pathway, thereby affecting the activity of all three elongases. We attempted to generate conditional mutants for dehydratase and enoyl-CoA reductase. Again, we were unsuccessful in generating a mutant (data not shown). These negative results do not formally establish the essential nature of the \emph{T. gondii} ELO pathway, but they are consistent with such a role.

Which aspect of parasite biology generates the heavy need for elongated fatty acids served by these elaborate and potentially redundant synthesis and uptake pathways? Very long chain fatty acids are required for the synthesis of highly specialized lipids such as waxes in plants, but they are also important components of phospholipids (27, 48). Loss of the elongation ELO pathway, but they are consistent with such a role.

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\endgroup

\begin{thebibliography}{99}

\bibitem{1} Mazumdar, J., and Striepen, B. (2007) Make it or take it. Fatty acid metabolism of apicomplexan parasites. \textit{Eukaryot. Cell} \textbf{6}, 1727–1735
\bibitem{2} Valdivia, R. H. (2008) \textit{Chlamydia} effector proteins and new insights into chlamydial cellular microbiology. \textit{Curr. Opin. Microbiol.} \textbf{11}, 53–59
\bibitem{3} Bhatt, A., Molle, V., Besra, G. S., Jacobs, W. R. Jr., and Kremer, L. (2007) The \textit{Mycobacterium tuberculosis} FAS-II condensing enzymes. Their role in mycolic acid biosynthesis, acid-fastness, pathogenesis, and in future drug development. \textit{Mol. Microbiol.} \textbf{64}, 1442–1454
\bibitem{4} Waller, R. F., Keeling, P. J., Donald, R. G., Striepen, B., Handman, E., Lang-Unnasch, N., Cowman, A. F., Besra, G. S., Roos, D. S., and McFadden, G. I. (1998) Nuclear encoded proteins target to the plastid in \textit{Toxoplasma gondii} and \textit{Plasmodium falciparum}. \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{95}, 12352–12357
\bibitem{5} Hiltunen, J. K., Chen, Z., Haapalainen, A. M., Wierenga, R. K., and Kashtaniotis, A. J. (2010) Mitochondrial fatty acid synthesis. An adopted set of enzymes making a pathway of major importance for the cellular metabolism. \textit{Prog. Lipid Res.} \textbf{49}, 27–45
\bibitem{6} Goodman, C. D., and McFadden, G. I. (2007) Fatty acid biosynthesis as a drug target in apicomplexan parasites. \textit{Curr. Drug Targets} \textbf{8}, 15–30
\bibitem{7} Surolia, N., and Surolia, A. (2001) Triclosan offers protection against blood stages of malaria by inhibiting enoyl-ACP reductase of \textit{Plasmodium falciparum}. \textit{Nat. Med.} \textbf{7}, 167–173
\bibitem{8} McLeod, R., Muench, S. P., Rafferty, J. B., Kyle, D. E., Mui, E. J., Kirisits, M. J., Mack, D. G., Roberts, C. W., Samuel, B. U., Lyons, R. E., Dorris, M., Milhous, W. K., and Rice, D. W. (2001) Triclosan inhibits the growth of \textit{Plasmodium falciparum} and \textit{Toxoplasma gondii} by inhibition of apicomplexan Fab I. \textit{Int. J. Parasitol.} \textbf{31}, 109–113
\bibitem{9} Mazumdar, J. H., Wilson, E., Masek, K. A., Hunter, C., and Striepen, B. (2006) Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in \textit{Toxoplasma gondii}. \textit{Proc. Natl. Acad. Sci} \textbf{103}, 13192–13197
\bibitem{10} Vaughan, A. M., O’Neill, M. T., Tarun, A. S., Camargo, N., Pheung, T. M., Aly, A. S., Cowman, A. F., and Kappe, S. H. (2009) Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. \textit{Cell Microbiol.} \textbf{11}, 506–520
\bibitem{11} Yu, M., Kumar, T. R., Nkumah, L. J., Coppi, A., Retzlaff, S., Li, C. D., Kelly, B. J., Moura, P. A., Lakshmanan, V., Freundlich, I. S., Valderramos, I. C., Vilcheze, C., Siedner, M., Tsai, J. H., Falkard, B., Sidhu, A. B., Purcell, L. A., Gratraud, P., Kremer, L., Waters, A. P., Schiebers, G., Jacobus, D. P., Janse, C. J., Ager, A., Jacobs, W. R. Jr., Sacchettini, J. C., Heussler, V., Sinnis, P., and Fidock, D. A. (2008) The fatty acid biosynthesis enzyme FabI plays a key role in the development of liver-stage malarial parasites. \textit{Cell Host Microbe} \textbf{4}, 567–578
\bibitem{12} Charron, A. J., and Sibley, L. D. (2002) Host cells. Mobilizable lipid resources for the intracellular parasite \textit{Toxoplasma gondii}. \textit{J. Cell Sci.} \textbf{115}, 3049–3059
\bibitem{13} Gerold, P., Dieckmann-Schuppert, A., and Schwarz, R. T. (1994) Glycosylphosphatidylinositol synthesized by asexual erythrocytic stages of the malarial parasite, \textit{Plasmodium falciparum}. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. \textit{J. Biol. Chem.} \textbf{269}, 2597–2606
\bibitem{14} Zhu, G. (2004) Current progress in the fatty acid metabolism in \textit{Cryptosporidium parvum}. \textit{J. Eukaryot. Microbiol.} \textbf{51}, 381–388
\bibitem{15} Zhu, G., Li, Y., Cai, X., Millership, J. I., Marchewka, M. J., and Keithly, J. S. (2004) Expression and functional characterization of a giant Type I fatty acid synthase (CpFAS1) gene from \textit{Cryptosporidium parvum}. \textit{Mol. Biochem. Parasitol.} \textbf{134}, 127–135
\bibitem{16} Zhu, G., Shi, X., and Cai, X. (2010) The reductase domain in a Type I fatty acid synthase from the apicomplexan \textit{Cryptosporidium parvum}. Restricted substrate preference towards very long chain fatty acyl thioesters. \textit{BMC Biochem.} \textbf{11}, 46–46
\bibitem{17} Agrawal, S., van Dooren, G. G., Beatty, W. L., and Striepen, B. (2009) Genetic evidence that an endosymbiont-derived endoplasmic reticulum-associated protein degradation (ERAD) system functions in import of apicoplast proteins. \textit{J. Biol. Chem.} \textbf{284}, 33683–33691
\bibitem{18} Striepen, B., and Soldati, D. (2007) in \textit{Toxoplasma gondii, the Model Apicomplexan- Perspective and Methods} (Weiss, L. D., and Kim, K., eds) pp. 391–415, Elsevier, London
\bibitem{19} Brooks, C. F., Johnsen, H., van Dooren, G. G., Muthalagi, M., Lin, S. S., Bohne, W., Fischer, K., and Striepen, B. (2010) The \textit{Toxoplasma} apicoplast phosphate translocator links cytosolic and apicoplast metabolism and is essential for parasite survival. \textit{Cell Host Microbe} \textbf{7}, 62–73
\bibitem{20} Meissner, M., Schlüter, D., and Soldati, D. (2002) Role of \textit{Toxoplasma}}
Toxoplasma Fatty Acid Metabolism

The carbon and energy sources of the nonphotosynthetic plastid in the malaria parasite. *FEBS Lett.* **584**, 549–554

38. Prigge, S. T., He, X., Gerena, L., Waters, N. C., and Reynolds, K. A. (2003) The initiating steps of a type II fatty-acid synthase in *Plasmodium falciparum* are catalyzed by pfACP, pfMCAT, and pfKASIII. *Biochemistry* **42**, 1160–1169

39. Misra, A., Surolila, N., and Surolila, A. (2009) Catalysis and mechanism of malonyl transferase activity in type II fatty acid biosynthesis acyl carrier proteins. *Mol. Biochem. Prot.* **5**, 651–659

40. Jelenska, J., Crawford, M. J., Harb, O. S., Zuther, E., Haselkorn, R., Roos, D. S., and Gornicki, P. (2001) Subcellular localization of acyl-CoA carboxylase in the apicoplastian parasite *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 2723–2728

41. Wu, G. Z., and Xue, H. W. (2010) *Arabidopsis* β-ketoacyl-[acyl carrier protein] synthase I is crucial for fatty acid synthesis and plays a role in chloroplast division and embryo development. *Plant Cell** **22**, 3726–3744

42. Nair, S. C., Brooks, C. F., Goodman, C. D., Strum, 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 fomridomycin resistance in *Toxoplasma gondii*. *J. Exp. Med.* **208**, 1547–1559

43. 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

44. Koo, A. J., Ohlrogge, J. B., and Pollard, M. (2004) On the export of fatty acids from the chloroplast. *J. Biol. Chem.* **279**, 16101–16110

45. Stuke, J. E., McDonough, V. M., and Martin, C. E. (1990) The OLEI gene of *Saccharomyces cerevisiae* encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. *J. Biol. Chem.* **265**, 20144–20149

46. Gratraud, P., Huws, E., Falkard, B., Adjalley, S., Fidock, D. A., Berry, L., Jacobs, W. R., Jr., Baird, M. S., Vial, H., and Kremer, L. (2009) Oleic acid biosynthesis in *Plasmodium falciparum*: characterization of the stearyl-CoA desaturase and investigation as a potential therapeutic target. *PLoS ONE* **4**, e6689

47. Lige, B., Jayabalasingham, B., Zhang, H., Pypaert, M., and Coppens, I. (2009) Role of an ancestral d-bifunctional protein containing two sterol-carrier protein-2 domains in lipid uptake and trafficking in *Toxoplasma*. *Mol. Biol. Cell** **20**, 658–672

48. Bach, L., Michaelson, L. V., Haslam, R., Bellec, Y., Gissot, L., Marion, J., Da Costa, M., Boutin, J. P., Miquel, M., Tellier, F., Domergue, F., Markham, J. E., Beaudoin, F., Napier, J. A., and Faure, J. D. (2008) The very long-chain hydroxy fatty acyl-CoA dehydratase PASTICCINO2 is essential and limiting for plant development. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 14727–14731

49. Welti, R., Mui, E., Sparks, A., Wernimont, S., Isaac, G., Kirisits, M., Roth, M., Roberts, C. W., Botté, C., Maréchal, E., and McLeod, R. (2007) Lipidomic analysis of *Toxoplasma gondii* reveals unusual polar lipids. *Biochemistry* **46**, 13882–13890

50. Coppens, I., and Vlieghe-Meyer, O. (2005) Insights into unique physiological features of neutral lipids in *Apicomplexa*. From storage to potential mediation in parasite metabolic activities. *Int. J. Parasitol* **35**, 597–615

51. Gupta, N., Zahn, M. M., Coppens, I., Joiner, K. A., and Voelker, D. R. (2005) Selective disruption of phosphatidylcholine metabolism of the intracellular parasite *Toxoplasma gondii* arrests its growth. *J. Biol. Chem.* **280**, 16345–16353

21. Carey, K. L., Donahue, C. G., and Ward, G. E. (2000) Identification and molecular characterization of GRA8, a novel, proline-rich, dense granule protein of *Toxoplasma gondii*. *Mol. Biochem. Parasitol.* **105**, 25–37