

# *Toxoplasma gondii* scavenges host-derived lipoic acid despite its *de novo* synthesis in the apicoplast

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**In contrast to other eukaryotes, which manufacture lipoic acid, an essential cofactor for several vital dehydrogenase complexes, within the mitochondrion, we show that the plastid (apicoplast) of the obligate intracellular protozoan parasite *Toxoplasma gondii* is the only site of *de novo* lipoate synthesis. However, antibodies specific for protein-attached lipoate reveal the presence of lipoylated proteins in both, the apicoplast and the mitochondrion of *T. gondii*. Cultivation of *T. gondii*-infected cells in lipoate-deficient medium results in substantially reduced lipoylation of mitochondrial (but not apicoplast) proteins. Addition of exogenous lipoate to the medium can rescue this effect, showing that the parasite scavenges this cofactor from the host. Exposure of *T. gondii* to lipoate analogues in lipoate-deficient medium leads to growth inhibition, suggesting that *T. gondii* might be auxotrophic for this cofactor. Phylogenetic analyses reveal the secondary loss of the mitochondrial lipoate synthase gene after the acquisition of the plastid. Our studies thus reveal an unexpected metabolic deficiency in *T. gondii* and raise the question whether the close interaction of host mitochondria with the parasitophorous vacuole is connected to lipoate supply by the host.**

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## Introduction

Parasites of the phylum Apicomplexa comprise a large group of medically important protozoa, including *Plasmodium falciparum* and *Toxoplasma gondii*. These parasites are characterized by their obligate intracellular lifestyle, which may provide protection from immune attack. Intracellular

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replication also provides a potentially rich and homogeneous source of nutrients for survival. *T. gondii* is capable of growth in virtually any nucleated vertebrate host cell, and is known to be auxotrophic for tryptophan, arginine, and purines (Pfefferkorn, 1984; Fox *et al.*, 2004). The parasite also scavenges cholesterol from the host cell, although this is not strictly required for survival (Coppens and Joiner, 2001). However, *T. gondii* can grow for extended periods in cells whose protein synthesis has been shut down, and indefinitely in confluent fibroblasts with very low metabolic rates (Pfefferkorn and Pfefferkorn, 1981; Gurnett *et al.*, 1995), arguing for a high degree of self-sufficiency. The complete spectrum of metabolite synthesis and salvage in these parasites remains to be defined.

Most apicomplexan parasites harbor a distinctive secondary endosymbiotic organelle dubbed the ‘apicoplast’ (Köhler *et al.*, 1997; Foth and McFadden, 2003), which carries out a variety of biosynthetic functions (Ralph *et al.*, 2004) and is essential for parasite survival (Fichera and Roos, 1997). The apicoplast participates in heme biosynthesis, using a pathway that is partitioned unusually, between the mitochondrion, cytosol, and plastid (Ralph *et al.*, 2004). The apicoplast is also thought to synthesize isoprenoids, via the methylerythritol 4-phosphate pathway typically associated with plastids (Jomaa *et al.*, 1999); it is not known whether these compounds enter into the parasite cytoplasm (e.g. formation of ubiquinone, dolichol, and so on), or are required solely for housekeeping functions (e.g. for isopentenylated tRNAs). Perhaps the best-characterized apicoplast metabolic pathway is the synthesis of fatty acids, via a type-II fatty acid synthase complex (FAS-II) typically found in prokaryotes rather than eukaryotes (Waller *et al.*, 1998; McLeod *et al.*, 2001). Fatty acid synthesis requires the precursor acetyl-coenzyme A, which is presumably synthesized by the plastid pyruvate dehydrogenase (PDH) complex (Foth *et al.*, 2005).

In this context, we have investigated the dependency of *T. gondii* parasites on the cofactor lipoic acid (LA), responsible for an essential protein modification of the E2 subunits of 2-oxo acid dehydrogenase complexes, including PDH, 2-oxoglutarate dehydrogenase (OGDH), branched-chain 2-oxo acid dehydrogenase (BCDH) (Perham, 2000), and the H-protein of the glycine decarboxylase system (Douce *et al.*, 2001). In addition, LA can also function as an antioxidant in aerobic cells (Moini *et al.*, 2002). In the dehydrogenase reaction, LA serves as a swinging arm, transferring an activated acyl group to coenzyme A. LA is covalently attached to specific lysine residues in the E2 protein lipoylation domains. In eukaryotes, this process typically takes place within mitochondria, whereas plants and algae also lipoylate a plastid isoform of PDH-E2 (see Supplementary Figure S-7A; Mooney *et al.*, 2002).

Free LA salvaged from exogenous sources is coupled to lipoyl domains by lipoate protein ligase (LplA in *Escherichia coli*; Morris *et al.*, 1995). The bacterial lipoate ligase reaction is ATP-dependent, whereas in mammals ligation requires

preactivation of lipoate by a GTP-dependent lipoate-activating enzyme (Fujiwara *et al*, 2001). Alternatively, LA is synthesized *de novo* from octanoyl-acyl carrier protein (generated by the FAS-II system), via the action of two enzymes, lipoic acid synthase (LipA; Miller *et al*, 2000) and octanoyl-acyl carrier protein:*N*-octanoyltransferase, LipB (see Supplementary Figure S-7B; Zhao *et al*, 2005).

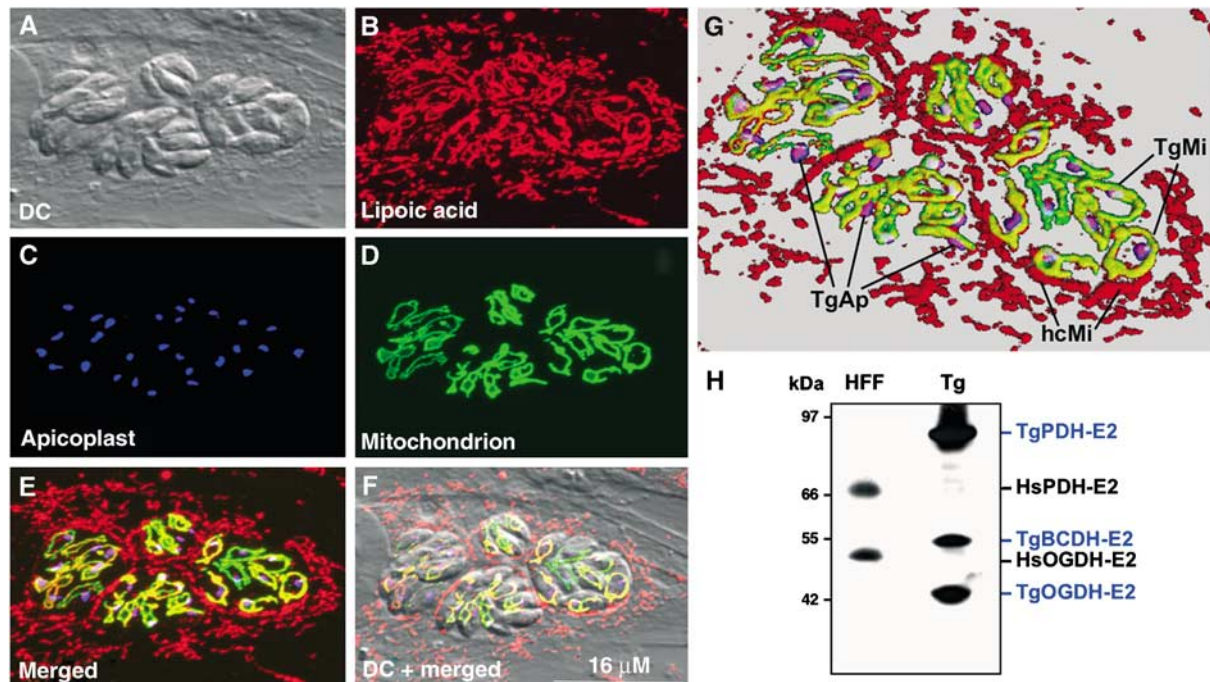
Plant and algal plastids harbor LipA and LipB but no LplA (see Yasuno and Wada, 2002, and references therein), and this is also true for the apicoplast of *T. gondii* and *P. falciparum* (see Supplementary Figure S-7A, Mooney *et al*, 2002; Thomsen-Zieger *et al*, 2003; Wrenger and Müller, 2004). In contrast, eukaryotic mitochondria (including those in plants) typically contain all three enzymes—an arrangement that may allow the organelle to respond to a shortage of exogenous LA by upregulating synthesis. Remarkably, the mitochondria of both *Toxoplasma* and *Plasmodium* are predicted or have been shown to contain an LplA enzyme, but no evidence for LipA nor LipB targeting to this organelle could be obtained by green fluorescent protein (GFP)-fusion studies, making it very likely that these parasites possess only one compartment capable of LA synthesis (Thomsen-Zieger *et al*, 2003; Wrenger and Müller, 2004). This raises several important questions regarding the subcellular allotment of lipoylated proteins in apicomplexans, the relationship of LA synthesis in the apicoplast and LA salvage in the parasite mitochondrion, and the potential role of the host in the provision of LA (Günther *et al*, 2005).

In this report, we demonstrate that LA synthesis in the *T. gondii* apicoplast is critically dependent on the FAS-II pathway within this organelle. Disruption of apicoplast LA synthesis does not affect lipoylation in the parasite mitochondrion, however, indicating that mitochondrial LA is taken up from the host, rather than being acquired from the apicoplast. Thus, the secondary loss of enzyme paralogs during the evolution of these parasitic organisms has yielded a case where intracellular compartmentalization requires exploitation of host resources, despite the presence of all enzymes necessary for synthesis of LA.

## Results

### Presence of lipoylated proteins in the apicoplast and mitochondrion

As a first approach to study lipoylation in *T. gondii*, we used a polyclonal antibody specific for protein-bound LA to determine the intracellular distribution of LA-containing proteins. This commercial antibody has been used in a number of studies for the detection of lipoylated proteins in species like bacteria, yeast, plants, and mammals (Humphries and Szveda, 1998; Sasaki *et al*, 2000). The two compartments expected to be potential locations for LA-containing proteins (mitochondrion and apicoplast) were genetically tagged with GFP (mitochondrion) or with an hemagglutinin (HA)-tagged apicoplast-resident protein (ferredoxin NADP<sup>+</sup>-reductase) to facilitate subcellular identification (Thomsen-Zieger *et al*,



**Figure 1** Detection of lipoylated proteins in the apicoplast and mitochondrion of *T. gondii* and host cell mitochondria by confocal immunofluorescence microscopy and immunoblot analysis. Infected HFF cells (A) were stained with polyclonal anti-LA antibody and Cy3-labeled secondary antibody (B). Apicoplast-resident HA-FNR was visualized using anti-HA and secondary Cy5-labeled antibodies (C). The mitochondrion could be detected by GFP fluorescence (see Materials and methods) (D). In (E) and (F), all three fluorescent channels are merged. Pink color indicates colocalization of lipoylated proteins with the apicoplast, yellow with the mitochondrion of the parasites. (G) Represents a partial blow-up of (E), which was also volume-rendered using VolumeJ software. The background color was changed to gray for better visualization of organelles. Some host cell mitochondria surrounding the parasitophorous vacuolar membrane are indicated (hcMi). Parasite mitochondria (TgMi) and apicoplasts (TgAp) are also outlined. Note the close association between apicoplast and mitochondrion. (H) Immunoblot analysis of uninfected host cells (HFF) and purified *T. gondii* tachyzoites (Tg) with polyclonal anti-LA antibody. The positions and names of host (Hs) and parasite (Tg) lipoylated proteins reacting with this antibody are indicated (see text for details).

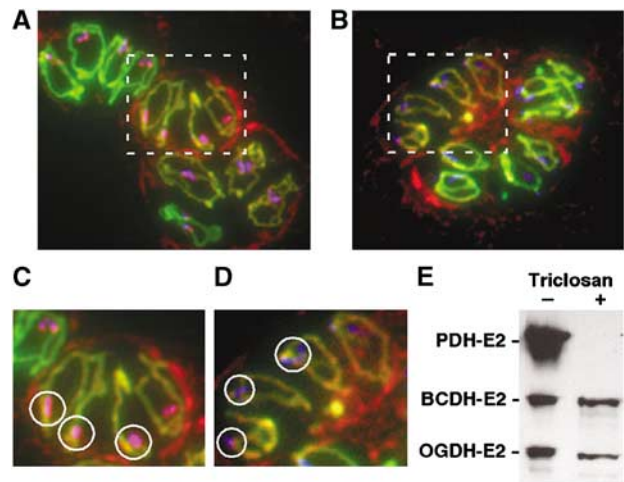
2003). As shown in Figure 1B, polyclonal anti-LA antibodies gave a complex staining pattern when reacted with infected human foreskin fibroblasts (HFF), due to the fact that host cell mitochondria are also stained by this antibody (Sasaki *et al*, 2000). However, in the merged picture it is clearly evident that both the apicoplast and mitochondrion of *T. gondii* harbor lipoylated proteins.

Using extracts from purified *T. gondii* tachyzoites, the polyclonal anti-LA antibody recognized three proteins on immunoblots (Figure 1H). Gene predictions and EST data from the *T. gondii* genome resource (www.ToxoDB.org) provide convincing evidence that the lipoylated proteins are apicoplast-targeted PDH-E2 (band at ~92 kDa) and mitochondrial BCDH-E2 (~54 kDa) and OGDH-E2 (~47 kDa) (Thomsen-Zieger *et al*, 2003). Although *T. gondii* also possesses a homolog of the H-protein of the glycine decarboxylation system, no band of the expected size (~22 kDa) could be identified, perhaps owing to low abundance or lack of antibody recognition. The polyclonal anti-LA antibody also stained host cell mitochondria, and consequently two proteins known to be PDH-E2 (74 kDa) and OGDH-E2 (50 kDa) were also visible on immunoblots of uninfected HFF (Figure 1H), whereas this antibody does not recognize human BCDH-E2, the H-protein, or the dihydro-lipoamide dehydrogenase-binding protein (E3BP) in mitochondrial preparations (Sasaki *et al*, 2000). We confirmed that the 92 kDa band is indeed the apicoplast-targeted PDH-E2 by immunoprecipitation and N-terminal sequencing (see Supplementary Figure S-1). We could not obtain enough material for this analysis to positively identify the other two lipoylated proteins of *T. gondii*.

### Lipoylation in the apicoplast is dependent on functional FAS-II

Having established a facile method to follow compartment-specific lipoylation in *T. gondii*, we asked whether pharmacological disruption of FAS-II in the apicoplast would have an effect on lipoylation in this organelle or the mitochondrion. *T. gondii* differs from *P. falciparum* in that it has not only a type-II FAS in the apicoplast but also a giant multimodular type-I FAS protein that localizes to the parasite mitochondrion (M Crawford, G Zhu and DS Roos, unpublished results). However, the proteins required for *de novo* LA synthesis (LipA and LipB) localize to the apicoplast (Thomsen-Zieger *et al*, 2003). Inhibition of the FAS-II system is predicted to deplete the apicoplast of octanoyl-acyl carrier protein (ACP), the precursor of LA synthesis (Zhao *et al*, 2003). Triclosan is an enoyl-ACP reductase (ENR) inhibitor that specifically targets the apicoplast type-II system at low concentrations (<1 µg/ml) (McLeod *et al*, 2001). Treatment of intracellular *T. gondii* with 0.3 µg/ml triclosan resulted in the loss of lipoylated TgPDH-E2 signal (Figure 2A–D), whereas fluorescence in the mitochondrion of the parasite (and also of the host cells) was not visibly affected. Adding a surplus of free LA to the culture medium during triclosan treatment did not restore antibody reactivity (see Figure 4), indicating that LA is either unable to enter the apicoplast or that apicoplast LipB (like *E. coli* LipB) cannot ligate free LA to acceptor proteins (Zhao *et al*, 2003). The IFA results were confirmed by immunoblot experiments (Figure 2E).

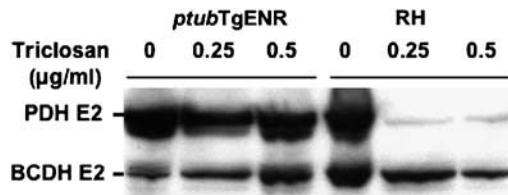
These results show that a functional FAS-II machinery is required for lipoic acid synthesis in the apicoplast, but not for



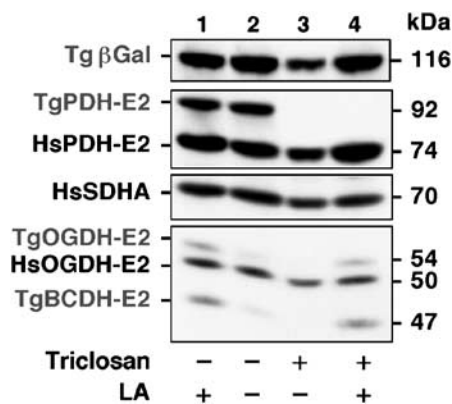
**Figure 2** Reduction of apicoplast, but not mitochondrial, lipoylation upon triclosan treatment. (A) Untreated tachyzoites 16 h post-infection, (B) triclosan-treated parasites after 36 h in the presence of 0.3 µg/ml triclosan. The merged images are composites of mitochondria (green), polyclonal anti-LA (red), and apicoplast (blue). The whole color plate with individual images can be found in Supplementary Figure S-8. (C, D) Enlarged images of the dashed areas in (A) and (B), respectively. In three exemplary regions, circles indicate the presence of colocalization of lipoylated proteins within the apicoplast (C, pink color) or its absence due to triclosan treatment (D, only blue color). (E) Immunoblot analysis of lipoylated proteins from purified *T. gondii* from untreated or triclosan-treated samples (0.3 µg/ml for 48 h) using polyclonal anti-LA antibody.

salvage within the parasite mitochondrion. We ruled out that the diminished signal of *T. gondii* PDH-E2 under triclosan treatment (Figure 2) was owing to protein degradation by following the lipoylation of a transgene consisting of the N-terminal apicoplast targeting sequence and the three lipoyl attachment sites from TgPDH-E2 fused to yellow fluorescent protein (YFP) (see Supplementary Figure S-2). This construct stained only the plastid, providing additional evidence that PDH-E2 in *T. gondii*, like in *P. falciparum*, is apicoplast-resident (Foth *et al*, 2005).

To verify the specificity of triclosan treatment on the apicoplast lipoylation pattern, we analyzed transgenic *T. gondii* overexpressing the predicted target of triclosan, enoyl ACP reductase (Fab I or ENR; McLeod *et al*, 2001). Proper apicoplast localization was confirmed through the use of a C-terminal HA tag (data not shown). By immunoblot analysis, overexpression of *T. gondii* ENR (TgENR) resulted in increased levels of drug tolerance up to 0.5 µg/ml triclosan, revealed by lipoylation levels of TgPDH-E2 similar to untreated cultures (Figure 3). These data provide direct evidence that the effect of low levels of triclosan on lipoylation is due to the inhibition of TgENR and not to a secondary effect. We attempted to increase triclosan resistance by engineering and expressing various mutants of TgENR (e.g. A231V) based on orthologous positions of mutant ENR proteins of Fab I of *E. coli* or *P. falciparum*, insensitive to the drug (Kapoor *et al*, 2004). However, no increased resistance could be observed over the background of wild-type TgENR overexpression (data not shown). This may reflect improper folding of these mutants when overexpressed, or that alternative mutations not expected from the prokaryotic precedent must be selected to confer a higher level of triclosan resistance. For



**Figure 3** Complementation of the triclosan-mediated lipoylation defect by recombinant overexpression of *T. gondii* enoyl-ACP reductase (TgENR). A stable *T. gondii* clone recombinantly expressing the predicted triclosan target (apicoplast TgENR) with a C-terminal HA tag (see Materials and methods) was created. Immunoblot analysis with anti-LA antibody probed against lysates of purified *T. gondii* (ptubTgENR-HA) and wild-type parasites (RH) treated with the indicated triclosan concentrations for 48 h reveals the specific effect of TgENR overexpression on TgPDH-E2 lipoylation in triclosan-treated cultures.



**Figure 4** Lipoylation of mitochondrial *T. gondii* proteins in LA-deficient medium. HFF infected with RHβ1 parasites were cultivated in LAM5<sup>-</sup> in the absence or presence of 1 µM exogenous LA for 48 h and then analyzed by SDS-PAGE and immunoblotting with polyclonal anti-LA antibodies (lanes 1–2). The same experiment was also performed in the presence of 0.5 µg/ml of triclosan (lanes 3–4). Mitochondrial parasite proteins TgBCDH-E2 and TgOGDH-E2 showed greatly reduced lipoylation signals in the absence of LA (lane 2). In addition, lipoylation of TgPDH-E2 was reduced in the absence of fatty acid synthesis (lane 3). Parasite-encoded β-galactosidase (TgβGal) and the 70 kDa subunit of human mitochondrial succinate-ubiquinol oxidoreductase (complex II; HsSDHA), respectively, served as loading control for each cell type.

triclosan levels above ~0.5 µg/ml, we observed inhibition of parasite growth for all parasites, including those overexpressing TgENR isoforms, indicating that a secondary effect unrelated to TgENR inhibition is occurring at higher triclosan concentrations. This result is similar to the effect of triclosan on *Trypanosoma brucei* (Paul *et al*, 2004), where multiple targets are suggested, and likely explains our inability to develop triclosan-resistant *T. gondii* using global mutagenesis screens (M Crawford, unpublished data).

#### Mitochondrial lipoylation depends on host-derived LA

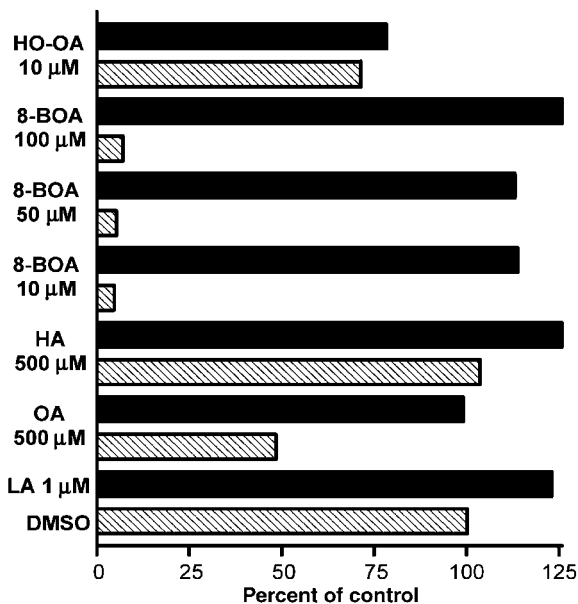
We then turned our attention to the lipoylation of mitochondrial proteins which was unaffected by pharmacological inhibition of LA production in the apicoplast. To test if LA could be provided by the host cell, we established cell culture conditions using a chemically defined LA-free culture medium (LAM5<sup>-</sup>). A drastic reduction in the anti-LA staining

of the predicted mitochondrial *T. gondii* proteins, BCDH-E2 (54 kDa) and OGDH-E2 (47 kDa), was observed under these conditions, whereas the PDH-E2 staining remained unaffected (Figure 4, lane 2 and Supplementary Figure S-6A). Adding 1 µM LA to LAM5<sup>-</sup> at the beginning of the experiment resulted in the lipoylation of both bands at normal levels (Figure 4, lane 1 and Supplementary Figure S-6A). Similar results were seen by immunofluorescent analysis, clearly indicating the disappearance of the mitochondrial signal in LA-depleted medium (see Supplementary Figure S-3). Combining both triclosan treatment and LA depletion from medium, we could obtain parasites which had a drastic reduction in anti-LA reactivity in all three proteins usually lipoylated (Figure 4, lane 3). The addition of exogenous LA together with triclosan rescued mitochondrial (but not plastid) lipoylation in the parasites (Figure 4, lane 4). In all experiments, lipoylation signals from the two host cell mitochondrial proteins were not significantly affected. This can be explained by the relative insensitivity of human cells to triclosan treatment (Liu *et al*, 2002) and the potential ability of host cell mitochondria to counteract LA depletion with LA synthesis (see Discussion). In contrast, actively dividing *T. gondii* tachyzoites, particularly early in the infection cycle, probably have a high demand for exogenous LA. In addition, extracellular *T. gondii* seem unable to incorporate substantial amounts of exogenous LA into nonlipoylated mitochondrial TgBCDH-E2 and TgOGDH-E2 (see Supplementary Figure S-4). Taken together, these data show that no mitochondrial LA synthesis is present in *T. gondii* and that LA produced within the apicoplast is not appreciably delivered outside of this organelle. This strict compartmentalization therefore seems to preclude an exchange of LA compounds.

#### Lipoate analogs kill *T. gondii* in the absence but not in the presence of LA

Surprisingly, *T. gondii* could be cultivated for prolonged times in LA-depleted medium (three intracellular replication cycles were followed), although LA supplementation of LAM5<sup>-</sup> consistently resulted in improved growth (see Figure 5). It is likely that residual LA supply from the host cell is responsible for *T. gondii* growth in LA-depleted medium (see Discussion). At least OGDH is predicted to be an essential enzyme for the parasite as it provides succinyl-CoA, a precursor of the heme biosynthetic pathway (Ralph *et al*, 2004). Omitting succinate (present at 1 mM in LAM5<sup>-</sup>) from the medium resulted in a moderate but significantly reduced growth rate in the absence of LA (Supplementary Figure S-5). This would be consistent with a requirement for LA by *T. gondii* for succinyl CoA synthesis via OGDH. LA depletion might be partly compensated for by high amounts of succinate in the medium through conversion to succinyl-CoA via parasite succinyl-CoA ligase, but this aspect needs further investigation.

Assuming that *T. gondii* depends on exogenous LA, we tested whether the LA precursor octanoic acid (OA) and the LA analog 8-bromooctanoic acid (8-BOA) could compete with endogenous uptake of LA and interfere with *T. gondii* growth in LAM5<sup>-</sup>. The rationale was that in the absence of LA, both compounds might instead be incorporated substantially by LplA, as is the case for bacteria (Ali *et al*, 1990; Zhao *et al*, 2003). Given that the incorporation of the initial sulfur is presumed to occur at C8 and would be blocked in 8-BOA, this



**Figure 5** Inhibitory effects of LA analogs on *T. gondii* growth and protein lipoylation. RHβ1 were cultured in LAM5<sup>-</sup> in the presence of the indicated concentrations of fatty acid analogs in the absence (striped bars) or presence of 1 μM LA (black bars) in 48-well plates for 72 h and then assayed for β-galactosidase activity as a means for parasite growth as described previously (Seeber and Boothroyd, 1996). Fatty acid abbreviations are given in the text. OD values of the untreated control were then set as 100% growth and the other values calculated accordingly. Note the consistent beneficial effect on growth by LA supplementation in untreated cells.

compound cannot be converted to functional LA (White, 1980). The same should be true for 8-hydroxyoctanoic acid (HO-OA). In contrast, OA could potentially serve as sulfur acceptor but only in the presence of LipA (see Supplementary Figure S-7B; Zhao *et al*, 2003). As can be seen in Figure 5 and Supplementary Figure S-6, low amounts of 8-BOA blocked intracellular growth of *T. gondii* in LA-deficient medium, whereas OA started to become toxic only at much higher levels (500 μM). Interestingly, when 1 μM LA was added together with 1–100 μM 8-BOA or 500 μM OA, their toxic effect was totally reversed. Adding 500 μM heptanoic acid (HA) had no effect on *T. gondii* growth, indicating specificity for chain length and ruling out a general effect of fatty acids on the parasite. Surprisingly, HO-OA differed from 8-BOA in that it had some growth-inhibitory effect that could not be counteracted by LA supplementation. This difference might be explained by the fact that ω-hydroxylated fatty acids can be rapidly converted to dicarboxylic acids (Sanders *et al*, 2005, and references therein), which are potentially cytotoxic by inhibiting mitochondrial functions (Passi *et al*, 1984). Neither 8-BOA nor OA in LAM5<sup>-</sup> showed adverse effects on host cell growth when tested at the concentrations used above (data not shown). Reduced glutathione (GSH) could not substitute for LA in 8-BOA treated cells, even at 100-fold higher concentrations (data not shown), suggesting that the antioxidant properties of LA are not responsible for the detoxifying effect on 8-BOA treated cultures. Immunoblot and IFA analysis with polyclonal anti-LA antibodies revealed the appearance of the signals corresponding to the putative TgOGDH-E2 and TgBCDH-E2 proteins upon LA supplementation in 8-BOA-, HA-, or OA-treated cells (see Supplementary

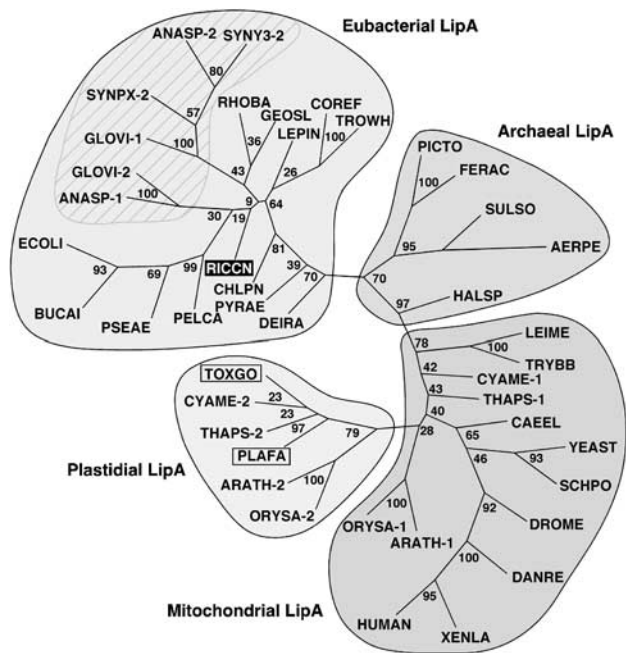
Figure S-6). Collectively, these experiments show that LA analogs can inhibit the growth of *T. gondii* in LA-depleted medium, although possibly by affecting different targets (see Discussion).

### Phylogenetic analysis reveals secondary loss of mitochondrial LA synthesis genes

To determine the evolutionary events that might have led to the unique cellular distribution of apicomplexan LipA, we analyzed the phylogenetic relationship of LipA proteins from different organisms (Figure 6). Remarkably, eukaryotic LipAs are more similar to the archaeal proteins than to those of α-proteobacteria, the closest extant relatives to the presumed endosymbiont that gave rise to mitochondria. This suggests that LipA did not arrive in eukaryotes with this endosymbiont. Likewise, plastidial LipAs (including those of Apicomplexa) do not cluster with those of cyanobacteria, as would be expected if they derived from the primary endosymbiont leading to the first plastid. Rather, they form a sister group to the nuclear-encoded mitochondrial proteins, which likely arose through gene duplication and subsequent acquisition of plastid targeting sequences in the different species. Most importantly, the completely sequenced genomes of the red alga *Cyanidioschyzon merolae* and of the diatom *Thalassiosira pseudonana* both contain two LipA and two LipB genes, respectively (see Supplementary Table S-I). Both organisms clearly require LA in both, the mitochondrion and plastid as each possesses PDH-E2 subunits with targeting sequences predicting either mitochondrial or plastid localization (Supplementary Table S-I). Accordingly, one of their LipAs clusters early with the mitochondrial sequences, whereas the predicted plastid-localized protein groups with the apicomplexan and plastidial LipAs (Figure 6). As there is strong evidence that Apicomplexa and diatoms share a common ancestry that predates the acquisition of the red algal endosymbiont (Harper *et al*, 2005), it is plausible that *T. gondii* and other Apicomplexa lost their mitochondrial LipA (and LipB) genes after the arrival of the apicoplast.

## Discussion

Lipoic acid is an essential cofactor for all organisms dependant on a functional citric acid cycle and on pyruvate to acetyl-CoA conversion via PDH. Most of these organisms possess the genes essential for the synthesis of LA from fatty acid synthesis precursors, LipA and LipB (Cronan *et al*, 2005b). In addition, LA can also be scavenged from the environment or host using LplA, as is the case for *Listeria monocytogenes* and other bacteria that are LA auxotrophs (O’Riordan *et al*, 2003). In *Caenorhabditis elegans*, individual LipA and LplA knockdowns have no severe effect at the two-cell stage but become lethal at the postembryonic stage (Sonnichsen *et al*, 2005). Comparable results have recently been obtained in mammals. Embryos of mice that have both genomic copies of LIAS (the mammalian LipA homolog) deleted die at an early implantation stage (between 7.5 dpc and 9.5 dpc; Yi and Maeda, 2005). These data indicate that maternal LA is sufficient to support cell growth only in the very early stages of embryogenesis. A loss of lipoylation capability should be similar to the functional loss of the E2 subunits of 2-oxo acid dehydrogenases since the non-lipoylated proteins are enzymatically inactive (Perham, 2000).



**Figure 6** Maximum likelihood phylogenetic analysis of LipA protein sequences. Selected sequences representing major taxa whose genome sequences have been determined entirely were analyzed as described in Materials and methods. All eukaryotic sequences were analyzed for the presence of N-terminal organellar targeting sequences using various bioinformatic tools (see Supplementary Table S-1). Their known or predicted organellar location correlates very well with their phylogenetic clustering (Supplementary Table S-1). Numbers at the basis of the nodes are bootstrap values of 100 replicates. The different clades are outlined (cyanobacteria within the eubacterial clade are striped), the  $\alpha$ -proteobacterium *Rickettsia prowazekii* is highlighted, and the two apicomplexan LipA's are boxed. Organism identification codes are according to the UniProt group.

Apicomplexa are unique because they possess only one PDH complex located exclusively in the apicoplast, as shown recently for *P. falciparum* (Foth *et al*, 2005) and in this study for *T. gondii*. Our data provide direct evidence that pharmacological disruption of FAS-II in the apicoplast using triclosan only affects lipoylation of TgPDH-E2. Its lipoylation under drug treatment could be restored by expression of enoyl-ACP reductase (TgENR) in the apicoplast. Surprisingly, although lipoylation was restored, transgenic overexpression of wild-type or mutant TgENR isoforms with mutations predicted to confer triclosan resistance (Ala231Val, Ala231Ser) (Kapoor *et al*, 2004) did not alter the growth sensitivity of *T. gondii* to triclosan (data not shown). This suggests that TgENR is not the sole molecular target of this compound. Indeed, there is accumulating evidence indicating that this drug also interferes with the physicochemical properties of bacterial or eukaryotic membranes, thereby disrupting the function of membrane-resident proteins (Villalain *et al*, 2001; Lygre *et al*, 2003; Paul *et al*, 2004).

Our experiments show that the mitochondrion of *T. gondii* also harbors lipoylated proteins not visibly affected by triclosan treatment. On the other hand, cultivating *T. gondii* in LA-depleted medium clearly indicates that LA has to be scavenged from the medium (host cell) for optimal lipoylation of the mitochondrial proteins. Despite *de novo* synthesis of LA within the apicoplast, this cofactor is not substantially

diverted to the mitochondrion. However, we cannot rule out entirely that very small amounts, undetectable by our experiments, escape from the apicoplast and allow the survival of *T. gondii* in LA-depleted medium. Current knowledge of the biochemistry of LA synthesis in bacteria indicates that introduction of sulfur into octanoate by LipA occurs in the lipoyl domain-bound form of OA (Zhao *et al*, 2005). Therefore, LA supply to the mitochondrion would only be possible by LA release through TgPDH-E2 breakdown. Its extent is unknown but is considered only minor (see also below). Importantly, if lipoate synthesis from the apicoplast is required as an LA supply for the mitochondrion, LipA/LipB genes should be found in all apicoplast-bearing Apicomplexa. This is not the case at least for *Theileria parva* and *T. annulata*. Both parasites have lost the entire FAS-II pathway in the apicoplast and do not contain genes for PDH, LipA, or LipB (Pain *et al*, 2005). However, LplA is still present in both organisms (see Supplementary Table S-1). Consequently, their mitochondrial proteins (BCDH-E2 and OGDH-E2) can only be lipoylated by LA salvaged from the cellular environment.

A likely source for residual LA in LA-depleted medium in our experiments is the recycling or synthesis of LA in the host mitochondrion. It is well established that host mitochondria as well as endoplasmic reticulum closely associate with the PVM surrounding intracellular *T. gondii* (Jones and Hirsch, 1972; see also Figure 1G and Supplementary Figure S-6B), and this interaction is actively supported by the parasite (Sinai *et al*, 1997). Therefore, LA supply by host mitochondria or ER to the parasite is an attractive hypothesis. However, it has been shown that there is very little if any free intracellular LA present in various organisms tested (Biewenga *et al*, 1997), so liberation of protein-bound LA would be required. Only recently has a 'lipoamidase' (Lpa) from *Enterococcus faecalis* been characterized that releases LA from intact lipoylated proteins (Jiang and Cronan, 2005). No homologues have been detected in the genome databases of other organisms, and the authors concluded that other reported lipoamidase activities in eukaryotes are probably due to amidases acting on short LA-containing peptides or lipoyl-lysine derived from protein breakdown. In this respect, it is interesting to note that a recent report in yeast provided evidence for the constant release of short peptides derived from proteolysis of mitochondrial proteins, including OGDH-E2 (Augustin *et al*, 2005). Currently, no enzyme(s) that might liberate LA in mammalian mitochondria are known. One candidate could be human serum biotinidase, a protein capable of releasing biotin from proteins but which has also been reported to be active on lipoylated peptides (Nilsson and Ronge, 1992). Recently, a mitochondrial and/or ER localized isoform of biotinidase has been described (Stanley *et al*, 2004), but its activity towards lipoylated proteins was not investigated. Alternatively, LA-containing peptides or lipoyl-lysine from the host mitochondria might pass through the described pores in the parasitophorous vacuolar membrane (Schwab *et al*, 1994), with subsequent recycling of LA within the parasite by a yet unknown amidase.

Scavenging LA from the mammalian host is possible, exemplified by the bacterium *L. monocytogenes* that lacks one of the enzymes for LA synthesis but has two LplA genes that are essential for its intracellular survival (O'Riordan *et al*, 2003). Interestingly, the free-living amoeba *Dictyostelium discoideum* seems to possess only one LplA homologue in

its genome but no LipA or LipB sequences (data not shown). It should thus be dependent on scavenged LA from prey organisms (consistent with its described LA requirement in axenic culture; Franke and Kessin, 1977) and/or possess an efficient system for LA recycling. The same is principally true for *T. parva* and *T. annulata* (see above) which infect bovine T and B lymphocytes, a presumably rich source for LA due to the reported high levels of LipA transcripts in mammalian blood cells (Su *et al*, 2004). On the other hand, *Rickettsia* seem to rely solely on lipoate synthesis by LipA/LipB because no LplA homolog can be found in their genomes (Andersson *et al*, 1998). These examples highlight a surprising variability of lipoic acid metabolism in nature, to which *T. gondii* adds another interesting facet.

Genetic evidence indicates that mammalian cells also synthesize LA (Morikawa *et al*, 2001; Yi and Maeda, 2005), although no detailed metabolic studies of its mitochondrial biosynthesis have been reported yet. There is strong experimental evidence that a type-II FAS machinery is present in mammalian cells (see Cronan *et al*, 2005a, and references therein), which most likely provides the octanoyl-ACP precursor to mitochondrial LipA/LipB. It is therefore almost impossible to separate *T. gondii* from residual LA supply from its environment as no axenic culture system exists for this parasite and LA-deficient host cells would be nonviable (Yi and Maeda, 2005). Nevertheless, the specific toxic effects of either low amounts of 8-BOA or high amounts of OA (both of which are completely rescued by low amounts of LA) are strong evidence that interference with LA supply from the host cell by analogs or precursors is responsible for parasite death. How exactly they interfere needs to be determined. In the case of OA, the confinement of LipA/LipB to the apicoplast should prevent, in contrast to bacteria or mammalian mitochondria, the conversion of octanoylated to lipoylated enzymes when it is incorporated into OGDH-E2 or BCDH-E2 (Ali *et al*, 1990; Zhao *et al*, 2003). Regarding 8-BOA, its activity in any cellular system is ill defined and therefore other targets as those discussed below cannot be ruled out. At first sight its action is reminiscent of the reported irreversible inhibition of mammalian 3-ketoacyl-CoA thiolase (EC 2.3.1.16) by other brominated octanoic acid derivatives, 2-BOA and 4-bromo-2-octenoic acid, respectively, with a concomitant shutdown of mitochondrial fatty acid oxidation (Raaka and Lowenstein, 1979; Li and Schulz, 1988). However, we consider this unlikely in the case of 8-BOA. Monohalogenated fatty acids are in general much less reactive when the halogen is distant from the carboxy group (like in the  $\omega$  position in 8-BOA) compared to the  $\alpha$  position (like in 2-BOA) due to decreased lability of the halogen bond (Poidevin, 1965). Sterically, the bromo group would also be at a very different position. Structural specificity of 8-BOA's effect is further supported by our observations that neither 8-hydroxyoctanoic acid, 12-bromododecanoic acid, nor 6-bromohexanoic acid show LA-reversible toxicity at the same low concentrations (Figure 5 and data not shown). In this respect, the recently reported 3-D structure of *E. coli* LplA cocrystallized with LA (Fujiwara *et al*, 2005) might give a clue to the different toxicities of 8-BOA and OA we observe. The authors concluded that the reaction rates might be influenced by the slight differences in the van der Waals interactions between LplA and the various substrates it can accommodate. The terminal bromo group in 8-BOA

might resemble the dithiolane ring of LA in terms of space filling (8-BOA has a calculated van der Waals volume of 181.15 which is closer to that of LA (196.25) than is the volume of OA (161.87)). Therefore, compared to 8-BOA, a much higher level of OA might be required to serve as a substrate for LplA. This is consistent with studies in *E. coli* where a LipB mutant (dependent on LplA function for growth) can utilize octanoate (which is converted to LA by LipA once ligated to acceptors) for efficient growth only when present at >1000-fold higher concentrations compared to LA (Zhao *et al*, 2003). In addition, a concomitant inhibition of LplA by 8-BOA might also be possible.

Another potential target of 8-BOA could be the sodium-dependent multivitamin transporter responsible for LA as well as biotin transport into human cells (Prasad *et al*, 1998). A general inhibition of multivitamin import could explain the potent effect of 8-BOA on the parasites. However, high concentrations of biotin could not neutralize the toxicity of 8-BOA (data not shown), and the host cells are unaffected by these low concentrations of 8-BOA. This would not rule out an effect of 8-BOA on a parasite-specific LA transport system, however.

Collectively, our results suggest that apicomplexans once harbored a mitochondrion with all three enzymes required for LA metabolism but subsequently lost its mitochondrial synthesis machinery after arrival of the secondary endosymbiont. Thus, secondary loss of mitochondrial LipA and LipB ultimately contributed to the manifestation of the obligate intracellular parasitism of *T. gondii*. Therefore, LA metabolism could be potentially exploited for chemotherapeutic use. Our findings also raise the question whether the close interaction of host mitochondria and the ER with the parasitophorous vacuole is connected to LA supply by the host.

## Materials and methods

### Parasite culture and growth assays

Detailed protocols for culturing and media formulation can be found as Supplementary data at *The EMBO Journal* Online. Parasites used for most experiments were either strain RH $\beta$ 1 (Seeber and Boothroyd, 1996) expressing *E. coli*  $\beta$ -galactosidase as a convenient protein marker, or wild-type RH. LA-deficient synthetic medium (LAM5<sup>-</sup>) basically consists of Iscove's modified Dulbecco's medium supplemented with 1 mM Na-succinate, 55 nM hydrocortisone, 0.5 g/l fatty acid-free BSA, 1 ml/l iron chelating solution, 5 ml/l Lipid Mixture 1 and nine trace elements. LA, OA, 8-BOA, HO-OA, and HA were dissolved either in ethanol or DMSO and added as required from 1 M stock solutions.

LA depletion experiments were carried out as follows: host cells were grown to confluency under normal growth conditions. Before infection, the monolayer was washed once with buffer and the medium was changed to LAM5<sup>-</sup>. After 2 h, host cells were infected with a multiplicity of infection >10 with *T. gondii* tachyzoites from a freshly lysed culture for 3 h. The monolayer was washed again and fresh LAM5<sup>-</sup> was added for 24 h.

Determination of  $\beta$ -galactosidase activity of RH $\beta$ 1 as an accurate reflection of parasite numbers was performed as described (Seeber and Boothroyd, 1996) with some minor modifications detailed in the Supplementary data.

### Generation of transgenic parasites and immunofluorescence and YFP fluorescence analyses

Tachyzoites of the RH-Rep1/2 strain of *T. gondii* with a genetically GFP-tagged mitochondrion have been described (Thomsen-Zieger *et al*, 2003). The apicoplast in this strain was visualized by an HA-epitope-tagged ferredoxin NADP<sup>+</sup>-reductase. Its construction as well as that of parasites containing the N-terminus of *T. gondii* PDH-E2 fused to YFP are found as Supplementary data.

Visualization of tagged proteins in transgenic parasites was carried out either with a rat anti-HA monoclonal antibody or a mouse monoclonal anti- $\beta$ -galactosidase antibody. For localization of lipoylated proteins, two different antibodies were used. Polyclonal anti-LA antiserum (Calbiochem) is reported to recognize various lipoylated proteins in different organisms. For specific localization of lipoylated *T. gondii* PDH-E2, the anti-lipoylation domain mouse monoclonal antibody (mab) 3H-2H4 (Migliaccio *et al*, 1998) was used. In addition, rabbit polyclonal antibody raised against *T. gondii* ACP (Waller *et al*, 1998) served as an apicoplast marker. Where appropriate, mitochondria were stained with Mitotracker Red CMXRos. Details of staining conditions and the microscopes/software used can be found in the Supplementary data.

#### Immunoprecipitation, SDS-PAGE, and immunoblot analysis

These analyses followed standard protocols and can be found as Supplementary data.

#### Phylogenetic analysis

LipA protein sequences were extracted after extensive BLAST or text searches from various databases and aligned with CLUSTAL X. A subset of sequences from organisms with completely known

genome sequences, representing many major lineages, was then used for maximum likelihood phylogenetic analysis using TREE-PUZZLE 5.2, followed by bootstrap analysis with PUZZLEBOOT and final evaluation of the resulting matrices with FITCH from the PHYLIP 3.63 package (for details, see Supplementary data).

For full details of Materials and methods see Supplementary data.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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