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Characterization of a second sterol-esterifying enzyme in *Toxoplasma* highlights the importance of cholesterol storage pathways for the parasite

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Summary

Lipid bodies are eukaryotic structures for temporary storage of neutral lipids such as acylglycerols and sterol esters. Fatty acyl-CoA and cholesterol are two substrates for cholesteryl ester (CE) synthesis via the ACAT reaction. The intracellular parasite *Toxoplasma gondii* is incapable of sterol synthesis and unrelentingly scavenges cholesterol from mammalian host cells. We previously demonstrated that the parasite expresses a cholesteryl ester-synthesizing enzyme, TgACAT1. In this paper, we identified and characterized a second ACAT-like enzyme, TgACAT2, which shares 56% identity with TgACAT1. Both enzymes are endoplasmic reticulum-associated and contribute to CE formation for storage in lipid bodies. While TgACAT1 preferentially utilizes palmitoyl-CoA, TgACAT2 has broader fatty acid specificity and produces more CE. Genetic ablation of each individual *ACAT* results in parasite growth impairment whereas dual ablation of *ACAT1* and *ACAT2* is not tolerated by *Toxoplasma*. Δ ACAT1 and Δ ACAT2 parasites have reduced CE levels, fewer lipid bodies, and accumulate free cholesterol, which causes injurious membrane effects. Mutant parasites are particularly vulnerable to ACAT inhibitors. This study underlines the important physiological role of ACAT enzymes to store cholesterol in a sterol-auxotrophic organism such as *Toxoplasma*, and furthermore opens up possibilities of exploiting TgACAT as targets for the development of antitoxoplasmosis drugs.

Keywords

Protozoa; toxoplasmosis; fat storage organelle; cholesterol homeostasis; acyl-CoA:cholesterol acyltransferase; ACAT inhibitors

Introduction

Cholesterol is a contradictory molecule: it is a vital part of membranes; however, its overabundance in cells leads to inflammation and oxidative damages. Mammalian cells have developed several protective mechanisms against cholesterol overloading (reviewed in Chang *et al.*, 2006). Among the cholesterol homeostatic mechanisms are: i) the reverse cholesterol transport, which delivers cholesterol to extracellular lipoproteins, ii) the conversion of cholesterol into oxysterols, which triggers cholesterol catabolism, and iii) the esterification and storage of cholesterol within cytoplasmic inclusions. Cholesteryl esters (CE), i.e. cholesterol with long-chain fatty acids linked to the hydroxyl group, are less polar

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Supplemental information

This section includes three Figures and one Table.

than free cholesterol, and represent the biologically inert storage forms, mainly confined to cytoplasmic lipid bodies. When needed, CE can then be de-esterified by hydrolysis and released from lipid bodies for the formation of new membranes. In general, the cycle of cholesterol esterification and hydrolysis provides the major short-term buffering of cholesterol levels in cells.

Among the members of the superfamily of membrane-bound acyltransferases, acyl-CoA:cholesterol acyltransferase enzymes (ACAT) are dedicated to the synthesis of CE. The sterol ester biosynthetic machinery is evolutionary conserved, and genes encoding sterol-esterifying enzymes have been identified in Vertebrates, plants, Invertebrates such as nematodes and insects, the fungi (yeast) branch and protozoa, but not in prokaryotes (reviewed in Athenstaedt and Daum, 2006). Higher eukaryotes express two enzymes, ACAT1 and ACAT2, which share 44-47% of amino acid homology. Concerning the substrates, both enzymes utilize cholesterol or oxysterols, but different acyl-CoA: 16:0, 18:1, 18:2 for ACAT1 and ACAT2, and 20:4 for ACAT1 (summarized in Chang *et al.*, 2009). Mammalian ACAT 1 and ACAT2 are endoplasmic reticulum (ER)-resident proteins; however they differ in their tissue distribution and membrane topology. While ACAT1 is found in various cells and tissues, ACAT2 expression is restricted to hepatocytes and intestinal mucosal cells. The distinctive patterns of tissue distribution of ACAT1 and ACAT2 correlate with the separate functions of these enzymes: ACAT1 generates intracellular CE for storage in lipid bodies to maintain appropriate cholesterol availability in cell membranes whereas ACAT2 produces CE that are assembled in the ER and incorporated into apoB-containing lipoproteins to promote cholesterol absorption. Confirming these observations, *ACAT1*-deficient mice lack CE droplets but have no apparent defects in lipoprotein production or cholesterol absorption. In contrast, *ACAT2*-knockout mice produce apoB-containing lipoproteins with low CE content in the liver, and are protected from hypercholesterolemia. Similar to higher eukaryotic cells, *Saccharomyces cerevisiae* possesses two sterol *O*-acyltransferase-encoding genes, *ARE1* and *ARE2* (reviewed in Daum *et al.*, 2007). The two gene products are located in the ER; they vary in their sterol substrate specificity (ergosterol for *ARE2* and sterol precursors for *ARE1*) but have similar specificity towards fatty acids (Zweytick *et al.*, 2000). The two yeast ARE have different physiological functions, depending on growth conditions. Interestingly, deletion of both *ARE1* and *ARE2* results in complete abolishment of sterol esterification, yet the double mutant remains viable.

Unlike mammals, protozoa are incapable of cholesterol *de novo* synthesis, and therefore their survival strictly depends on their ability to retrieve cholesterol from their environment. The intracellular protozoan parasite *Toxoplasma gondii* multiplies in a parasitophorous vacuole (PV) formed in the cytoplasm of mammalian cells. The parasite scavenges cholesterol from plasma low-density lipoproteins (LDL) by re-routing host lysosomes to its PV, and further internalizes cholesterol using membrane-associated transport proteins (Coppens *et al.*, 2000; 2006; Ehrenman *et al.*, 2010). Previous studies have reported the deleterious effects of well-known ACAT inhibitors on *T. gondii* (Sonda *et al.*, 2001; Ohshiro and Tomoda, 2011), which has suggested endogenous ACAT activities in the parasite. First genetic evidence was provided by our earlier work, in which we identified two isoforms of an ACAT-related enzyme, designated TgACAT1 α and TgACAT1 β sharing 18% sequence identity with human ACAT1 (Nishikawa *et al.*, 2005). Like their counterparts, the TgACAT1 isoforms reside in the ER and have a broad sterol substrate specificity to form CE. Host LDL and fatty acids scavenged by *Toxoplasma* can serve as ACAT activators, resulting in stimulation of CE synthesis and lipid droplet biogenesis in the parasite. Contrariwise, selected ACAT inhibitors dramatically alter the plasma membrane architecture of *Toxoplasma*, and consequently impair parasite growth. Together, this demonstrates the importance for *T. gondii* to maintain an optimal amount of intracellular

cholesterol, and consequently its vulnerability towards interference with the cholesterol storage pathways.

Here, we have extended our studies on the molecular equipment used by *Toxoplasma* to synthesize CE, and reinvestigated the physiological importance of cholesterol storage for this parasite. We have identified and characterized a second ACAT-related gene product, named TgACAT2, and compared the activity and substrate specificity of TgACAT2 with the previously reported TgACAT1. We have genetically disrupted either *ACAT1* or *ACAT2* and analyzed the consequence of such deletions on parasite viability and CE content. Our results show that the activity of at least one ACAT enzyme is essential for CE formation and parasite viability. We conclude that TgACAT1 and TgACAT2 have non-redundant roles to store host-derived cholesterol in parasite lipid bodies, and that the storage cholesterol function is essential in the cholesterol auxotroph *T. gondii*.

Results

Cloning and molecular characterization of a second ACAT-related enzyme in *Toxoplasma*

Through searches for sequence homology to signature motifs of *O*-acyltransferases in the *Toxoplasma* genome database (www.ToxoDB.org), we previously identified a gene coding for an acyl-CoA:cholesterol acyltransferase (ACAT)-like enzyme, named TgACAT1 (Nishikawa *et al.*, 2005). Additional BLAST searches in the *Toxoplasma* genome database have divulged the existence of second ACAT enzyme (TG GT1_114790; location on chromosome VIII) that we named TgACAT2 based on order of discovery. The TgACAT2 ORF was amplified by PCR from a cDNA library. The ORF is 1,623 nucleotides long, consists of 15 exons and encodes a polypeptide of 540 amino acids, with a predicted molecular weight of 63.4-kDa (Fig. 1). Sequence comparison between the previously reported TgACAT1 and TgACAT2 indicates 55.7% identity (80.1% similar) in 433 aa overlap between the two parasite enzymes.

Like TgACAT1, TgACAT2 has all the major hallmarks of the ACAT family (Fig. 1). First, the protein displays the common features of the superfamily of membrane-bound *O*-acyltransferases (Hofmann, 2000; Cases *et al.*, 1998) with an invariant histidine positioned within a long hydrophobic region (His456). Second, the specific motifs shared between ACAT and DGAT1 enzymes (Bouvier *et al.*, 2000) are present in TgACAT2; these motifs include a putative invariant serine (Ser244) and the remarkably conserved motif [AExxRFGDRx FYx DWWN] corresponding to the fatty acid binding site, starting at Ala391. Third, the TgACAT2 sequence also harbors the putative cholesterol binding site [H/YSF] comprised of a serine (Ser244) residue surrounded by aromatic and/or basic amino acids (Guo *et al.*, 2001) present in all the identified ACAT enzymes so far [Phe243SF]. Fig. S1 shows partial sequence alignment of TgACAT2 with the two human enzymes, ACAT1 and ACAT2, confirming the high degree of conservation of the ACAT motifs in the parasite sequence. Pairwise comparison of TgACAT2 with human ACAT1 or ACAT2 indicates 25.2% identity (58.8% similar) in 437 aa overlap and 25.8% identity (53.8% similar) in 535 aa overlap, respectively. The transmembrane region prediction program (http://ulrec3.unil.ch/software/TMPRED_form.html) favors seven membrane helix spanning domains for TgACAT2. A unique characteristic of TgACAT1 (isoform α only) is the presence of a unique hydrophilic serine-rich region at the N-terminus (~50% serine between amino acids 24-107), which was predicted to contribute to the overall stability of TgACAT1 (Nishikawa *et al.*, 2005). However, this long serine stretch is not present in the TgACAT2 sequence.

Like *TgACAT1*, *TgACAT2* localizes to the parasite ER

We next examined the intracellular localization of *TgACAT2*. For this purpose, we created a stable line of *Toxoplasma* expressing C-terminally HA-tagged *TgACAT2* under control of the strong tubulin promotor. To verify the expression of exogenous *TgACAT2*-HA, we performed SDS-PAGE and immunoblot analysis using anti-HA antibodies from lysates of transgenic parasites, and detected a single band at ~62- kDa (Fig. 2A). Apparently, overexpression of *TgACAT2* did not affect the parasite growth, as determined by uracil incorporation assay (Fig. 2B, panel a). The protein localization in *TgACAT2*-HA-expressing *T. gondii* was assayed by immunofluorescence assays (IFA) using anti-HA antibodies. Data in panel b in Fig. 2B illustrate a labeling of cortical tubular extensions and a perinuclear staining as well, as clearly visible on parasites at higher magnification (Fig. 2B, panel c). These observations indicate that *TgACAT2* localizes to the ER, as previously shown for *TgACAT1* (Nishikawa *et al.*, 2005).

Expression of *TgACAT2* in mammalian cells lacking ACAT activities stimulates lipid body biogenesis and restores cholesterol esterification

We next conducted experiments to probe the enzymatic activity of *TgACAT2* by expressing the protein in a cell line of mouse embryonic fibroblasts (MEF) lacking its unique ACAT gene (*ACAT1*), and therefore the ability to form any steryl esters (MEF *ACAT1*^{-/-}; Meiner *et al.*, 1996) in order to examine the potential ability of *TgACAT2* to restore ACAT activity in the mutant cell, namely the resuming of cholesterol esterification activity. We first analyzed the intracellular localization of *TgACAT2* when ectopically expressed in MEF wild-type (MEF *ACAT1*^{+/+}). The cells were transfected with an expression plasmid containing *TgACAT2*-HA and immunolabeled with antibodies against HA to examine the distribution of *TgACAT2*. Expectedly, a fluorescence pattern corresponding to cortical and perinuclear ER was discernible (Fig. 2C). MEF *ACAT1*^{-/-} were then transfected with the same plasmid, and IFA data using anti-HA antibodies show similar ER localization (Fig. 3A).

The ability of *TgACAT2* to esterify cholesterol was then evaluated by examining the re-emergence of lipid bodies in MEF *ACAT1*^{-/-} upon expression of *TgACAT2*. The adipose differentiation-related protein (ADRP, also named perilipin 2 or adipophilin) is a major lipid body-coating protein that is particularly important for *de novo* formation and maturation of lipid bodies after their release from the ER (Murphy *et al.*, 2012). MEF *ACAT1*^{-/-} were transiently transfected with p*TgACAT2*-HA for 24-h, and to visualize potentially newly formed lipid droplets, cells were immunolabeled with two different antibodies against ADRP (anti-ADRP-1 and anti-ADRP2). Remarkably, in cells expressing *TgACAT2*, a fluorescent labeling of small spherical structures disseminated throughout the cytosol was apparent with either antibody against ADRP (Fig. 3A). These structures exhibited an annular staining that is typical for lipid body-coating proteins. This indicates that lipid body formation was reconstituted in MEF *ACAT1*^{-/-} following expression of *TgACAT2*.

We next engineered a MEF *ACAT1*^{-/-} cell line stably expressing *TgACAT2*-HA to characterize the cell lipid content. We first wanted to confirm the presence of canonical lipid bodies containing neutral lipids in expressors by incubating the cells with Nile Red, a fluorescent lipid body dye. Cells expressing the parasite enzyme displayed a bright punctuate fluorescent staining in contrast to mock-transfected cells, which confirms the induction of lipid body formation by *TgACAT2* (Fig. 3B). To correlate the presence of lipid bodies with reconstituted cholesterol esterification activity mediated by *TgACAT2*, we monitored the synthesis of CE in MEF *ACAT1*^{-/-} stably expressing *TgACAT2*-HA, compared to MEF wild type and mock-transfected cells. Radiolabeled palmitate as a fatty acyl substrate for cholesterol esterification was added to the medium for 2-h. First we

verified the ability of the three cell lines to equally internalize same amounts of radioactive palmitate; no statistical difference for palmitate uptake was observed between the cell lines (Fig. 3C, panel a). Lipid fractions of the cells were then resolved by thin layer chromatography (TLC) and the levels of [^{14}C]palmitate-labeled CE were quantified. Clearly, expression of TgACAT2 in MEF ACAT1 $^{-/-}$ cells restored the ability of the cells to synthesize CE to almost MEF wild-type levels. To confirm these data, we transfected mammalian AC29 cells, another cell line lacking ACAT activities following chemical mutagenesis (Cadigan *et al.*, 1988) with pTgACAT2 or the empty vector, to compare the production of cholesteryl palmitate with CHO wild-type cells (Fig. 3C, panel b). In accordance with our data collected from MEF ACAT1 $^{-/-}$, AC29 cells transfected with pTgACAT2 were also able to synthesize cholesteryl palmitate. Levels of cholesteryl palmitate in TgACAT2-transfected cells corresponded to about half to those of CHO wild-type cells.

Together, this demonstrates that TgACAT2 is capable of catalyzing the cholesterol esterification reaction and triggering the storage of CE in lipid bodies.

TgACAT1 and TgACAT2 utilize LDL-cholesterol as substrate, but differ in their fatty acid specificity

We next planned experiments to compare the enzymatic activities of TgACAT1 and TgACAT2 and determine the nature of substrates preferentially used by these enzymes. MEF ACAT1 $^{-/-}$ transiently expressing either TgACAT1 or TgACAT2 tagged with HA were incubated with tritiated cholesterol associated with LDL in the medium for 2-h and the levels of [^3H]cholesteryl esters were quantified (Table I). No differences were observed in the amounts of radioactive cholesterol taken up from the medium by the two transgenic cells. However, TLC analyses showed significantly higher levels of radioactive CE in TgACAT2-expressing MEF ACAT1 $^{-/-}$ than TgACAT1-expressing MEF ACAT1 $^{-/-}$.

We next performed studies to assess the fatty acyls that are preferentially used by either TgACAT. We selected oleate, palmitate, stearate and arachidonate that are known to be avidly taken up by *Toxoplasma* (Quittnat *et al.*, 2004) and incorporated in CE (Lige *et al.*, 2011): among total CE, cholesteryl oleate represents 42%, cholesteryl palmitate 26%, cholesteryl stearate 3.4% and cholesteryl arachidonate 2.6%). MEF ACAT1 $^{-/-}$ cells transiently expressing either TgACAT1 or TgACAT2 were exposed to [^3H]palmitic acid (16:0), [^3H]stearic acid (18:0), [^3H]oleic acid (18:1) or [^3H]arachidonic acid (20:4) for 3-h prior to quantification of labeled CE produced by the two cell types. No difference in amounts of internalized fatty acids by TgACAT1- and TgACAT2-expressing cells was noticed (Table II). Cells expressing TgACAT1 preferentially incorporated palmitate and, to a lesser extent, oleate into cholesterol. We also detected the production of CE using radioactive stearate or arachidonate though the abundance of stearate- and arachidonate-labeled CE was ~6 and ~9-times less than CE containing oleate, respectively. By contrast, cells expressing TgACAT2 synthesized equal amounts of CE containing palmitate acid, stearate, oleate acid or arachidonate.

Together, this suggests that both parasite enzymes utilize host-derived cholesterol as a substrate. TgACAT2 forms more CE than TgACAT1 in MEF ACAT1 $^{-/-}$ cells and utilizes a broad range of fatty acyls. In contrast, TgACAT1 shows marked preference for palmitoyl-CoA over other fatty acyl-CoA.

***Toxoplasma* lacking either ACAT1 or ACAT2 develops poorly and has reduced size body**

We previously reported the high vulnerability of *Toxoplasma* towards ACAT inhibitors (Nishikawa *et al.*, 2005). To further assess the physiological importance of the ACAT

activities in the parasite and provide more insights into TgACAT1 and TgACAT2 functions, we focused on genetically disrupting the *ACAT1* or *ACAT2* gene. A fusion PCR-based method was used for *ACAT1* or *ACAT2* deletion by their replacement with the selectable marker HXGPRT; successful homologous recombination was confirmed by PCR (Fig. S2). Despite their very slow growth in cultured cells compared to the parental strain (Δ Ku80 Δ HXGPRT or Δ Ku80), clones lacking either *ACAT1* or *ACAT2* could be identified. The growth rate of the Δ ACAT1 or Δ ACAT2 strains in fibroblasts was quantified by uracil incorporation assays 24-h post-invasion (p.i.; Fig. 4A). In accordance with our functional data identifying TgACAT2 as the major contributor to CE formation in the parasite, the Δ ACAT2 also showed the more dramatic growth defect with a growth rate lowered by ~4-fold as compared to a ~3-fold reduction in the Δ ACAT1 mutant. To further characterize the observed phenotype, we next looked more specifically at the ability of mutant parasites to invade host cells by enumerating intracellular parasites 20-min p.i. (Fig. 4B). As compared to the parental strain both mutant parasite strains showed a ~50% reduced invasion efficiency. This suggests that the growth defect observed in the Δ ACAT1 strain is largely due to impaired host cell invasion, while the deletion of TgACAT2 appears to have a broader effect, affecting the host cell invasion as well as other post-invasion events.

We then looked at the morphology of the mutant strains and their organization within the PV by optical microscopy. Mutant parasites were immunolabeled with antibodies against the plasma membrane marker SAG1 (Fig. 4C). Both Δ ACAT strains were characterized by a very small and globular cell body as compared to the parental strain typified by a long and lunate cell body (dimension: $\sim 1 \times 2\text{-}\mu\text{m}$ for parasite mutants vs. $1.5 \times 7\text{-}\mu\text{m}$ for control parasites), which might be due to abnormalities in their lipid membrane composition. In addition, parasite mutants were disordered in their PV and were rarely organized as typical 'rosettes', which may reflect asynchronous divisions. Complementation of the mutant strains with the respective coding sequences completely restored the growth deficiencies (Fig. S3 A-B), confirming that the observed defects were specific to the deletion of the *ACAT1* or *ACAT2* gene, and therefore to the reduced ACAT activities in either mutant.

Host-derived LDL is the major source of cholesterol for *Toxoplasma* (Coppens *et al.*, 2000). Profusion of LDL in the culture medium leads to massive uptake of cholesterol by the parasite, suggestive of an uncontrolled uptake of this lipid by *T. gondii*. Nevertheless, as an efficient strategy to balance increasing cholesterol levels, the parasite is able to esterify cholesterol for subsequent storage in lipid bodies (Nishikawa *et al.*, 2005). The number of lipid bodies correlates with the amounts of LDL in the medium. We hypothesized that exposure of our mutant parasite strains to excess exogenous cholesterol would lead to a toxic accumulation of free cholesterol causing membrane damages and parasite death. Hence, we next challenged the Δ ACAT1 and Δ ACAT2 strains by incubating them in medium containing 20-times more LDL than normal conditions and examined their morphology (Fig. 4D). As compared to control parasites, parasite mutants showed grosser morphological alterations including distortion of the parasite body shape compared to mutants incubated under control conditions (10% FBS with 0.1 mg LDL/ml) at 24-h p.i. Additionally, the applied stress seemed to induce defects in cytokinesis as evidenced by asynchronous divisions and slow individualization of nascent parasites. After 2 days incubation with excess LDL, the mutants had formed smaller and disorganized PVs as compared to the parental strain and appeared to have lost their capability to egress.

Together, these results reveal an essential function of TgACAT activities in controlling cholesterol levels in the parasite, which is required for parasite intracellular development and differentiation.

The Δ ACAT1 strain shows plasma membrane defects whereas the Δ ACAT2 strain accumulates lipids in the PV

We next undertook EM studies to scrutinize the morphological abnormalities of parasites lacking *ACAT1* or *ACAT2* compared to control parasites. Panel A of Fig. 5 shows two PVs of the parental strain of *Toxoplasma* characterized by an electron-dense cytoplasm. Several lipid bodies surrounded by ER elements in close proximity to the mitochondrion were visible. Besides the presence of a tubulo-vesicular network of membranes, putatively ascribed to promote nutrient delivery to the parasites (Sibley *et al.*, 1995), few structures were present within the PV. In contrast, the Δ ACAT1 strain had an electron-lucent cytoplasm and accumulated dense material and membranous structures into the PV, likely originating from the parasite cytoplasm upon rupture of the plasma membrane (Fig. 5B). The presence of parasite “ghosts” lacking cytoplasmic constituents supports this assumption. Interestingly, this phenotype has been also observed for *Toxoplasma* treated with ACAT inhibitors (Nishikawa *et al.*, 2005). In addition, some parasite organelles were dramatically altered such as the mitochondrion showing swollen cristae. By comparison, the Δ ACAT2 strain exhibited large deposits of osmiophilic material, likely lipids, both in the PV lumen and the cytoplasm (Fig. 5C). This may reflect gross lipid disorders as a consequence of a perturbation of the balance between CE and free cholesterol.

Both Δ ACAT1 and Δ ACAT2 strains have reduced levels in CE and accumulate free cholesterol

Our morphological analyses strongly suggest a key role for ACAT enzymes to adjust proper levels of free cholesterol in *Toxoplasma*. Hence, we next analyzed the sterol composition of the Δ ACAT1 and Δ ACAT2 strains to determine the relative contribution of TgACAT1 and TgACAT2 to the overall parasite CE synthesis. Sterols located in membranes or stored in lipid bodies were first visualized by fluorescence microscopy using filipin and Nile Red, respectively (Fig. 6A). Compared to control parasites, the cellular distribution of free cholesterol was unaltered in Δ ACAT1 and Δ ACAT2 parasites as a positive filipin signal was observed on the plasma membrane. Though, the Δ ACAT parasites showed a dramatic decrease in the number and size of lipid bodies as illustrated by a weak Nile Red fluorescence signal.

Next, we performed lipid analyses to assess the content of free cholesterol and CE in the Δ ACAT1 and Δ ACAT2 strains as compared to the parental strain. No significant difference in the total amount of cholesterol (free + esterified cholesterol) was observed between the three strains (Fig. 6C). The total cholesterol content also remained unchanged in the parental strain exposed to the ACAT inhibitor DuP128. Expectedly, incubation in excess LDL led to a significant increase of total cholesterol amounts (by 170%) in the parental strain as a result of avid uptake of cholesterol-LDL by *T. gondii*.

To determine the ratio of free cholesterol vs. cholesterol that has been esterified in our strains, we incubated the parasites with radioactive palmitate prior to lipid extraction and TLC to monitor the synthesis of cholesteryl palmitate (Fig. 6C). In the parental strain, the fraction of cholesteryl palmitate constituted about one third of the total cholesterol ($10.9 \mu\text{g}$ cholesteryl palmitate.mg protein⁻¹ vs. $33.5 \mu\text{g}$ total cholesterol.mg protein⁻¹). The synthesis of cholesteryl palmitate was significantly decreased in the Δ ACAT strains and dropped to $2.49 \mu\text{g}$.mg protein⁻¹ and $1.25 \mu\text{g}$.mg protein⁻¹ in the Δ ACAT1 and Δ ACAT2 strains respectively ($P < 0.033$). By comparison, the production of cholesteryl palmitate was reduced to $4.4 \mu\text{g}$.mg protein⁻¹ in parasites treated with DuP128. This indicates that the amount of free cholesterol was elevated by 130%, 140% and 125% in the Δ ACAT1 strain, Δ ACAT2 strain and DuP128- treated parasites, respectively, as compared to the untreated parental strain. Expectedly, a substantial production of cholesteryl palmitate by the parasites

exposed to excess LDL was observed; however, the amount of free cholesterol in these parasites remained in the same range as calculated for parasites cultivated in normal medium.

Loss of either *ACAT* gene renders the mutant parasites particularly vulnerable to *ACAT* inhibitor treatment

In order to completely abolish the synthesis of CE by the parasite, we attempted to generate a double knockout strain. However, our multiple attempts to delete the *ACAT2* gene in the Δ *ACAT1* parasites and vice-versa, were unsuccessful, suggesting that expression of at least one an *ACAT* gene is required to ensure the parasite survival. We therefore performed quantitative real-time PCR to test for transcriptional co-regulation of the two *ACAT* genes. Expectedly, the transcript of *TgACAT2*, being the major *ACAT* enzyme in *Toxoplasma*, was more abundant and appeared at cycle 16, as compared to *TgACAT1* which was visible at cycle 22 (Fig. 7A). Besides, the deletion of *ACAT1* showed no changes in gene expression of the *ACAT2* gene, and vice versa (Fig. 7B). In agreement with our functional data showing that the two *ACAT* enzymes are involved in the production of different pools of CE in the parasite, this further confirms that the two genes are non-redundant, and are not able to complement each other, which corroborates our observation that the double deletion is lethal.

Hence we used a pharmacological approach to further decrease the CE production in each single Δ *ACAT* strain. Mutant parasites were incubated for 24-h with two different *ACAT* inhibitors, DuP128 or CI976, and parasite growth was monitored by uracil incorporation assays (Table III). As compared to the parental parasites treated with DuP128 or CI976, the Δ *ACAT1* and Δ *ACAT2* mutants displayed significantly higher sensitivity to treatment with *ACAT* inhibitors. For example, upon DuP128 exposure, Δ *ACAT1*, Δ *ACAT2* and the parental strain showed growth reduction of 63%, 75% and 53% respectively, as compared to untreated parental parasites. A similar effect was observed with CI976 as the growth rate of the Δ *ACAT1*, Δ *ACAT2* and the parental strain was reduced by 53%, 66 %, and 20% respectively, as compared to untreated parental parasites. After exposure to these inhibitors for 24-h, both mutant parasites showed signs of atrophy and were barely identifiable inside host cells.

Altogether, these data highlight an essential function of cholesterol esterification in *Toxoplasma*, which cannot tolerate the simultaneous loss of both *ACAT* activities, and establish a protective role for *TgACAT1* and *TgACAT2* to overcome the accumulation of cytotoxic free cholesterol.

Discussion

Toxoplasma contains several species of CE with predominance of cholesteryl oleate and palmitate (Lige *et al.*, 2011). The parasite is able to esterify its own cholesterol by *ACAT*-like enzyme previously characterized (Nishikawa *et al.*, 2005). In this paper, we have extended our investigations on cholesterol storage in *Toxoplasma* by identifying a second *O*-acyltransferase member that catalyzes the synthesis of CE. Sterols are essential lipid components of eukaryotic membranes, which harbor multiple essential functions ranging from signal transduction to protein lipidation. Likewise, *Toxoplasma* contains cholesterol in its membrane; however, the parasite does not have the enzymatic machinery to synthesize the steroid core and relentlessly takes up sterols from the host cell (Coppens *et al.*, 2000). The preferential source of cholesterol for *Toxoplasma* is plasma LDL internalized into late endosomes and lysosomes. When LDL is present in the medium, the parasite seizes cholesterol and transports the lipid into the ER, where cholesterol becomes esterified prior to storage in lipid bodies. In *Toxoplasma*, lipid bodies represent a reservoir for exogenously

acquired lipids that are surplus to the immediate requirements of membrane formation. In medium poor in LDL, *T. gondii* consumes its stores of cholesterol, and slows down its cycle of replication (Coppens *et al.*, 2000; Nishikawa *et al.*, 2005). The presence of two ACAT enzymes both involved in the esterification and storage of cholesterol may relieve *Toxoplasma* from an unremitting salvage of cholesterol and enable it to survive episodic variations of LDL-cholesterol concentrations in the environment.

The presence of more than one sterol esterification enzyme in *Toxoplasma* is not *a priori* out-of-the-ordinary since yeast and mammalian cells also have two ACAT-related enzymes. However, while yeast or mammalian cells use their enzymes to perform different physiological activities (reviewed in Daum *et al.*, 2007 and Athenstaedt and Daum, 2006), *Toxoplasma*, at first glance, appears to employ TgACAT1 and TgACAT2 to execute the same activity: the catalysis of cholesterol esterification and compartmentalization of CE within cytoplasm inclusions. In support of this idea, we showed that disruption of either *ACAT1* or *ACAT2* gene leads to reduced intracellular levels of CE and sparse lipid bodies. Nevertheless, our studies identified two slight differences in TgACAT1 and TgACAT2 activities. First, our results derived from heterologous expression of each parasite enzyme in ACAT-deficient mammalian cells showed that TgACAT2 forms more CE than TgACAT1, suggesting that TgACAT2 could be the major contributor of CE for the parasite. Supporting this notion are the higher level of expression of the *TgACTA2* transcript as compared to *TgACAT1* (cycle 16 vs. 22), and the more severe growth defect observed in the Δ ACAT2 strain as compared to Δ ACAT1 parasites (75% for Δ ACAT2 vs. 65% for Δ ACAT1 compared to the parental strain). Interestingly, the observed growth defect could be mostly (TgACAT1) or partially (TgACAT2) attributed to a reduced invasion efficiency, which was lowered to ~47% and ~54%, respectively. Apparently, deletion of parasite ACAT results in altered cycles of cholesterol esterification and accumulation of free cholesterol, which causes an abnormal membrane lipid composition and indirectly affects host cell invasion. Moreover, the deletion of TgACAT2 appears to have a broader effect, also impairing post-invasion events, demonstrating the greater overall role of TgACAT2 for the parasite viability. Second, TgACAT1 and TgACAT2 differ in their specificity for fatty acyl substrates. TgACAT1 has inherent higher acyl donor specificity than TgACAT2 and prefers palmitic acid over other fatty acids, which is the most predominant fatty acid found in *T. gondii* (Tomavo *et al.*, 1989; Quittnat *et al.*, 2004; Nishikawa *et al.*, 2005; Naget et Boothroyd, 1989; Chaudhary *et al.*, 2005; Mazumdar and Striepen, 2007; Ramakrishnan et al., 2012). One possible explanation for differences in fatty acyl selection between the parasite enzymes could be that, as proposed for mammalian ACAT (Joyce *et al.*, 2000), subtle differences in the enzyme topology in the ER membranes that may influence the access to fatty acids of TgACATs.

In microorganisms, the biogenesis and accumulation of lipid bodies is an adaptive response to nutrient depletion that marks the cessation of growth and the entry into a quiescent phase. The number and the size of lipid bodies in *Toxoplasma* vary between the different parasite developmental stages and environmental conditions (Dubey, 1998). The oocyst form of *Toxoplasma*, e.g. sporozoite, which can survive in soil for years without dividing, contains larger stores of lipids than the replicative intracellular tachyzoite form that is adapted to live in nutrient-rich habitats as offered by mammalian cell interiors. Our data reveal, nevertheless, that storage cholesterol synthesis is essential for the survival of the tachyzoite form. For example, pharmacological inhibition of ACAT activities in the parasite results in poor survival. The loss of individual ACAT gene induces a similar phenotype in the parasite. Interestingly, though causing a severe growth defect, the loss of individual ACAT genes and the consequent changes in CE pools are tolerated by *Toxoplasma*, suggesting a plasticity in cholesterol metabolism and a partial redundancy of the two ACAT genes. However, our qRT-PCR analyses did not show a co-regulation at the transcriptional level of

the two parasite ACAT genes. Furthermore, simultaneous loss of both ACAT genes seems lethal to the parasite, as demonstrated by our incapability to generate a double deletion mutant and the complete growth arrest upon treatment of the single mutant strains with ACAT inhibitors. Hence, TgACAT1 and TgACAT2 appear to be incapable of fully complementing each other, and the expression of at least one ACAT gene is absolutely required for the parasite survival.

Since a lack of production of plasma LDL-cholesterol by the mammalian host seems improbable, why is *Toxoplasma* so vulnerable to impairment of ACAT activities? A possible explanation might lie in the fact that the parasite scavenges LDL-cholesterol by uncontrolled uptake mechanisms, and engorges itself with cholesterol proportionally to the amount of this lipid in the environment. In case of large amounts of intracellular LDL-cholesterol, the parasite has no other option than detoxifying cholesterol by conversion into CE which have lower biological toxicity than free sterols, and thereby provide a safe sink for cholesterol. This is supported by the observed phenotypes of the Δ ACAT parasites showing a ruptured plasma membrane, which suggests that these mutants might have become osmotically fragile upon excess free cholesterol in the plasma membrane. In comparison, yeast lacking *ARE1* and *ARE2* have no growth defect due to their ability to prevent an accumulation of free cholesterol in the ER via a stringent regulation of ergosterol *de novo* synthesis as well as ergosterol export from the ER using two ABC transporters (Sandager *et al.*, 2002; Jacquier and Schneider, 2012). Mutant yeast lacking these ABC transporters cannot grow normally, demonstrating the importance of sterol egress for sterol homeostasis (Wilcox *et al.*, 2002). So far, an analogous mechanism of cholesterol efflux could not be identified in *Toxoplasma*, offering an alternative explanation for the higher sensitivity of the parasite towards interference with cholesterol homeostatic pathways.

Another possibility might be that lipid bodies in *Toxoplasma* may have more sophisticated functions than just temporary lipid and energy storage. In multicellular organisms, lipid bodies fulfill numerous additional roles including lipid transport, steroid sequestration, protein deposit, membrane trafficking and signaling (reviewed in Murphy, 2012). Hence, malfunctioning lipid bodies can increase the risk of developing diseases in humans, e.g., lipodystrophy in case of too little lipid body production; obesity, hepatic steatosis and atherosclerosis in case of overproduction of lipid bodies; several cancers and Parkinson's and Alzheimer's disease associated with abnormal lipid body function. Interestingly, a *Toxoplasma* strain lacking a Niemann-Pick, type C1-related protein (NCR1) forms abundant and large lipid bodies in comparison to wild-type parasites (Lige *et al.*, 2011). An overall increase in the CE content has been detected in the Δ NCR1 strain, and yet these mutants are more virulent *in vitro* and *in vivo* than wild-type parasites. This information highlights that the regulation of lipid metabolism including the synthesis of CE in *Toxoplasma* may be more complex than expected for a unicellular organism.

Relatively little information on lipid body content in protozoa is available in the literature. The ciliated *Tetrahymena pyriformis* contains triacylglycerols (TAG; Borowitz and Blum, 1976) and can synthesize CE from cholesterol retrieved from the medium (Billheimer *et al.*, 1989). The flagellate *Trypanosoma brucei* accumulates lipid bodies and a unique kinase has been localized at the surface of these structures (Flaspohlet *et al.*, 2010). The apicomplexan *Plasmodium falciparum* expresses a single member of the MBOAT family that is involved in TAG synthesis and storage in lipid bodies (Vielemeyer *et al.*, 2004; Palacpac *et al.*, 2004a; Coppens and Vielemeyer, 2005), and that is essential for the parasite viability (Palacpac *et al.*, 2004b). *Plasmodium* does not store cholesterol retrieved from the host cell. *Toxoplasma* contains three biosynthetic enzymes TgDGAT1, TgACAT1 and TgACAT2 that contribute to the storage of neutral lipids (Quittnat *et al.*, 2004; Nishikawa *et al.*, 2005; this study). We showed previously that TgDGAT1 is an essential enzyme and highlighted here

the importance of ACAT activities for the parasite. Future research will be needed to provide insights into the biochemical properties of lipid bodies (e.g., protein content) and the interactions of these essential structures with cellular process (e.g., interorganelle communication) to uncover unique pathways in the life cycle of neutral lipids in *Toxoplasma*.

Experimental Procedures

Chemicals and antibodies

All chemicals were obtained from either Sigma Chem. Co. (St. Louis, MO) or Fisher (Waltham, MA) unless indicated otherwise. Solvents and standards for chromatography were of the highest analytical grade. Silica gel 60 TLC plates were from EM Science (Gibbstown, NJ). CI976 and DuP128 were kindly provided by A. Rodriguez (Johns Hopkins University). Low-density lipoprotein particles (LDL: density 1.019 to 1.063 g.ml⁻¹) were isolated from human serum and prepared as described (Coppens *et al.*, 2000). Radiolabeled reagents included: [1,2-³H]cholesterol (sp act: 58 Ci.mmol⁻¹), [5,6-³H]uracil (sp act: 45 Ci.mmol⁻¹), [9,10-³H]oleic acid (sp act: 12 Ci.mmol⁻¹), [1-¹⁴C]palmitic acid (sp act: 57 mCi.mmol⁻¹), [9,10-³H]palmitic acid (sp act: 57 mCi.mmol⁻¹), [9,10-³H]stearic acid (sp act: 55 mCi.mmol⁻¹) purchased from Moravsek Biochemicals, Inc. (Brea, CA), and [5,6,8,9,11,12,14,15-³H]arachidonic acid (sp act: 210 mCi.mmol⁻¹) from Dupont NEN Corporation (Boston, MA). Primary antibodies used in this study were mouse anti-SAG1 generously given by J.F. Dubremetz (University of Montpellier), rabbit anti-adipose differentiation-related proteins (or ADFP) from Novus Biologicals, LLC (Littleton, CO), and mouse anti-HA from Babco (Richmond, CA).

Mammalian cell lines, culture conditions and parasite propagation

The mammalian cell lines used in this study are the primary HFF (ATCC CRL-1635), CHO cells (ATCC CCL-163), the AC29 mutant lacking ACAT activity from mutagenized CHO cells (Cadigan *et al.*, 1988) generously given by Dr Ta-Yuan Chang (Dartmouth Medical School) and the MEF derived either from ACAT1^{+/+} or ACAT1^{-/-} mice generated by targeted gene disruption (Meiner *et al.*, 1996) kindly provided by Dr Robert Farese (University of California). HFF and CHO cells were cultivated in Dulbecco's modified Eagle medium and the MEF cultures in RPMI 1640 medium. Both media were supplemented with 10% FCS, 2 mM glutamine and penicillin/streptomycin (100 units.ml⁻¹ per 100 µg.ml⁻¹). The RH strain tachyzoite of *Toxoplasma gondii* was used throughout this study and was propagated *in vitro* by serial passages in monolayers of HFF (Roos *et al.*, 1994). Intracellular parasites were purified from host cells by density gradient using Nycodenz and isopycnic centrifugation as detailed previously (Coppens *et al.*, 2000). To evaluate parasite viability, the incorporation of [³H]Uracil incorporated into the parasites was measured as described (Roos *et al.*, 1994). Invasion assays were performed as previously described (Huynh *et al.*, 2003). Twenty-four-well plates were infected with 10⁷ parasites per well and incubated for 20 min at 37°C before fixation. Slides were differentially stained with anti-SAG1 antibodies for as per the red-green invasion assay.

Cloning of full-length cDNA encoding TgACAT2 and transient transfection of TgACAT2-HA in *Toxoplasma*

BLAST searches in the *Toxoplasma* database (www.ToxoDB.org) identified a second ACAT homolog in *T. gondii* (TgACAT2: TGGT1_114790). The 1.6-kb ORF was amplified from a *T. gondii* cDNA library generously provided by V.B. Carruthers (University of Michigan) using the primers F-ACAT2 and R-HAACAT2, and cloned into plasmid pYFP (BglIII/AvrII) allowing the expression of C-terminally HA-tagged TgACAT2-HA under control of the tubulin promoter (see Table SI for the sequences of primers used in this

study). The obtained cDNA fragment was subsequently sequenced and deposited in GenBank under the accession number JX441364. To study the subcellular localization of ACAT2 in *Toxoplasma*, extracellular parasites ($\sim 10^7$, RH strain) were transfected with TgACAT2-HA by electroporation as described (Soldati and Boothroyd, 1997) and the protein localization was determined by immunofluorescence using mouse-anti-HA antibodies.

Plasmid constructs for expression in mammalian cells for functional studies

TgACAT2-HA was cloned into the mammalian expression plasmid pcDNA3.1⁺ (EcoRI/HindIII) using the primers ACAT-ecor/ACAT-Hin resulting in the expression of a C-terminally HA-tagged protein under control of the *pCMV* promoter. The construct was transfected into ACAT1-deficient cells using the lipofectamine method as described (Quittnat *et al.*, 2004). Transfected cell lines were selected under neomycin (800 μ g/ml) selection, after 2 days or 2 weeks for transient or stable expression of TgACAT2-HA respectively. In one assay, a plasmid containing TgACAT1-HA (Nishikawa *et al.*, 2005) was introduced into ACAT1-deficient cells for transient expression to monitor CE production in comparison MEF ACAT1^{-/-} expressing TgACAT2-HA. The transfection % was measured by counting fluorescent cells expressing TgACAT1-HA or TgACAT2-HA detected by IFA using anti-HA antibodies. Comparable % transfection corresponding to $24 \pm 3\%$ and $27 \pm 5\%$ were observed between TgACAT1- and TgACAT2-expressing MEF ACAT1^{-/-}, respectively.

Genetic disruption of TgACAT1a or TgACAT2 gene

To create the Δ ACAT1 or Δ ACAT2 strain, we PCR amplified 600bp 5'- and 3'-UTR fragments of TgACAT1 (primer pairs ACAT1-P1/ACAT1-P4 and ACAT1-P5/ACAT1-P2) and TgACAT2 (primer pairs Acat2-P1/Acat2-P4 and Acat2-P5/Acat2-P2) as well as the hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) selectable marker cassette (primer pairs ACAT1-P3/ACAT1-P6 or Acat2-P3/Acat2-P6) and subsequently fused the 3 fragments via PCR as described (Lige *et al.*, 2011). About 10^7 extracellular tachyzoites of the Δ ku80 strain (Huynh *et al.*, 2009) were transfected with $\sim 50\mu$ g of the knockout construct and transgenic parasites were selected using mycophenolic acid and xanthine as described (Donald *et al.*, 1996). Stable parasites were cloned and screened for homologous crossover and the consequent absence of the respective ACAT gene locus by the different PCR reactions depicted in Fig. S2.

Complementation of the Δ ACAT1 and Δ ACAT2 strains by negative selection of HXGPRT

To generate the complementation construct the above mentioned 5'- and 3'-UTR fragments of ACAT1 and ACAT2 were fused adjacent to the respective gene coding sequences. The 3 individual PCR fragments were obtained by PCR using the primer pairs ACAT-P1/A1comp-4, A1comp-3/A1comp-6 and A1comp-5/ACAT-P2 for ACAT1, as well as the primer combinations Acat2P1/A2comp-4, A2comp-3/A2comp-6 and A2comp-5/Acat2P2 for ACAT2. $\sim 50\mu$ g of the complementation constructs were transfected into the corresponding knockout strain by electroporation and stable integration achieved by selection with 2-hydroxy-6-mercaptopurine (0.85 mg.ml^{-1}). Resistant parasites were analyzed for homologous integration of the coding sequence by screening PCR using the primer combinations shown in Fig. S3.

Quantitative real-time PCR

Total RNA was isolated from the Δ KU80, Δ ACAT1, and Δ ACAT2 parasites using the RNeasy Mini Kit prior to reverse transcription of 100 ng RNA each using random hexamer primers. qPCR was performed using primers listed in Table S1. Transcript abundance was

calculated by comparison of the ct values with the housekeeping transcripts (TGGT1_124740, GT1; TGGT1_037840, Elf1a).

Lipid measurements

Cholesterol concentration: Total cholesterol content was measured by an enzymatic and colorimetric assay using a reagent mixture from Roche Diagnostics Corp. (Indianapolis, IN). **CE synthesis:** Mammalian cells or parasites were incubated for 2-h in medium containing 0.2 mM radioactive FA bound to albumin (1% BSA, FA:BSA molar ratios of 2:1) or with 1 mg.ml⁻¹ of [³H]cholesterol incorporated into LDL to monitor the formation of radioalabeled CE. Cell isolation, lipid extraction and separation by TLC using petroleum ether/diethyl ether/acetic acid (80:20:1) were performed as described (Coppens *et al.*, 1995).

Light and electron microscopy studies

Light and epifluorescence microscopy were performed on infected cells seeded on sterile coverslips in 24-well culture dishes. IFA on parasites or mammalian cells were performed as previously described (Coppens and Joiner, 2003) using primary antibodies against HA (1:1000) or ADFP (1:100), and fluorescently labeled secondary antibodies (Invitrogen): anti-mouse and anti-rabbit IgG antibodies conjugated to either Alexa 488 or Alexa 594 diluted at 1:2000. For detection of cholesterol using filipin or lipid bodies using Nile Red by fluorescence microscopy, intravacuolar parasites were fixed in paraformaldehyde, and treated as described (Quittnat *et al.*, 2004; Coppens and Joiner, 2003). Slides were analyzed using a Nikon Eclipse E800 microscope equipped with a Spot RT CCD Camera and images were processed using the Image-Pro-Plus software (Media Cybernetics, Silver Spring, MD) before assembly using Adobe Photoshop (Adobe Systems, Mountain View, CA). For ultrastructural observation of the Δ ACAT strains by thin-section transmission electron microscopy (EM), infected cells were fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) and processed as described (Coppens and Joiner, 2003). Ultrathin sections of infected cells were stained by lead citrate and uranyl acetate before examination with a Philips CM120 EM (Eindhoven, the Netherlands) under 80 kV.

Protein determination

Protein content was determined by the bicinchoninic acid assay (Smith *et al.*, 1995), using serum albumin as a standard.

Statistical analysis

For comparison of means, *P* was determined by analysis of variance against control (ANOVA 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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TgACAT1	MLDDPLSKTRNSALATNSPRPLPSSLPSPNDLLFLSMTTITDQSSLPAALSPSSSSPSSS	60
TgACAT2	-----MSVVESKRKENG	13
	:: *	
TgACAT1	SPSSSSPSSSSPSSSSPSSVCFSSVSPSSSLPSSSVSSASLSSLASGGKLPGETSEES	120
TgACAT2	SGPVTP-----DIGGTSLLKRESKVAKSLWLRQQDTITRLEELQRQH	55
	* * : . . . : * * . * : ** . . . * . . .	
TgACAT1	LPHQQAEPVRSLEATMCSPSPEKTGPPCSPCPESCQKQEGNNTDPTVSSPCESRGSRR	180
TgACAT2	EEIIRAKERRQNSYDDLRTPLRSVDG-----SRRPSVNDINDYRG--R	97
	. : . * * . : : * . * : * * *	
TgACAT1	TMETARAGASSGKAEDHARAEGGEPAQGTHEARDKEKSGDRRPASGPGGSELDKMEKED	240
TgACAT2	PLHDQLHGAYRATLLSHHTKNLN-----LRGFLNLFILLFVIN	136
	 * : : . * . : : :	
TgACAT1	GKTFPSKLDFFDANSDLARSDFRGVAVLLFIAAIFYLVANPILRWYDSKEFVDPSSLARAM	300
TgACAT2	FRMVTDNLMYGLMIRLP--SSFRDILSNWPMMLLCFLMHFCILGAFVIERFVAAWVP---	192
	: . . . : * : . . . * . * . : : : * * : * * : : * * . . .	
TgACAT1	FDDFFFLMFMWAKLFAWSFTAYHLHLLYLRGRISRRALLFLQHLTQSAAIGYAVCSCLYN	360
TgACAT2	-----HLDWSECVLIGLNFFLVFIVPY-----LTVCNSSPSPAASALLAFSIVW	238
	:: * : . . . : : : : * . : . . *	
TgACAT1	AWPIIPAFAFVQMIAVVQFMKMSYSSNTMNFCDMRQKQTLGYPENVTLRNFCDYLFCP	420
TgACAT2	LFKLFSEHHVCLDARRALRDGNFR---ELCSNSEEAEHVRYPYSITLRHLYTFIWMF	294
	: : . . . * : * : . . . : : : : * : : : : * * . : * : : : *	
TgACAT1	VLVYEPMYRRGGFRPTYFVFKLFSMVGAMVVMYLACTSYLIPTMMRSPSMSITEAIFS-	479
TgACAT2	TMCFQFYPRVPRIRWLSVIRHVFESAACLALMKIVVDQHIVPVARNSTITEMQSIPLS	354
	. : : * * : * . : : * : * . . : : * . * : : *	
TgACAT1	-----LVFPFLFLDILIFYILFECICNLAAEITNFANRNFYDDWNSTNWDEY	527
TgACAT2	TLLVHFLDKITRLSIPNLYVWLLMFVGLFHHWCNIIAEVTRFGDRQFYLDWNASSFGEY	414
	* : * : : : * * . * : * * : * * : * * : * * : * * : * *	
TgACAT1	SRKWNKPVHRFLLRHVYMETQORYKWSHQTAAFATFLFSALLHEMILAVCFRFRVRL-YLF	586
TgACAT2	WRKWNLPPIHHFLNRHINKP-LRRARFPRIVATSVVFLISALLHEYMITVPLQLGWTGWVF	473
	**** * : * : * * : * : : : . : . * : * * * * : : * : : *	
TgACAT1	GIMLLQLPLIALG--RFYRHKKMVANAIWFACIMLG-PPLLGLAYGREWAQIHFYNAHAD	643
TgACAT2	LGFMQAAPLTYITNLSFFQRNPTVGNCFWFIFCFSGQPLGILIYWYLVGVKHGTVQQLD	533
	: : * * : * : : * . : * : : * * * * . * : *	
TgACAT1	HQPLRLF	650
TgACAT2	PSKIQIM	540
	. : : :	

Figure 1. Characteristic features of the predicted sequence of TgACAT2 showing the conserved critical motifs of the superfamily of membrane-bound *O*-acyltransferases

Alignment of the predicted ORF of TgACAT2 and TgACAT1 (isoform alpha; GenBank accession #: AY562994) using the BioEdit program, revealing the presence of many identical (*), conserved (:) or semi-conserved (.) substitutions in these proteins. Similarly to TgACAT1, TgACAT2 displays an invariant histidine (yellow) positioned within a long hydrophobic region of the superfamily of the membrane-bound *O*-acyltransferases, a conserved fatty acid binding site (turquoise) and a putative cholesterol binding site including an invariant conserved serine (magenta). Potential transmembrane segments (according to the transmembrane folding program : <http://liao.cis.udel.edu/website/servers/TMMOD/>

[scripts/frame.php?p=description](#)) are highlighted in grey and putative N-glycosylation sites (according to the NetNGLyc 1.0 server) are depicted in red.

Figure 2. Expression and localization of TgACAT2-HA in transgenic *Toxoplasma* and mammalian cells

A. Immunoblots of lysates of *T. gondii* stably expressing TgACAT2-HA and mock-transfected RH parasites (parental) revealed with anti-HA antibodies. **B.** Panel a: Uracil incorporation assay of TgACAT2-HA vs. RH parasites. HFF were infected with 5×10^6 parasites for 4-h prior to labeling with [^3H]uracil for 2-h and subsequent quantification by scintillation counting. Panels b and c: Immunofluorescence assays (IFA) of TgACAT2-HA-expressing parasites using anti-HA antibody. DAPI staining for the apicoplast (a) and the nucleus (n). Bars are 5 μm . **C.** IFA of mouse embryonic fibroblasts (MEF) transiently transfected with a plasmid containing *TgACAT2-HA* prior to IFA using anti-HA antibody. Bar is 10 μm .

Figure 3. Stimulation of lipid body production and synthesis of CE in mammalian ACAT-deficient cells expressing TgACAT2

A. IFA of MEF ACAT1^{-/-} expressing TgACAT2-HA. MEF ACAT1^{-/-} were transiently transfected with pTgACAT2-HA before double immunostaining using antibodies against HA (green) to identify transfected cells and ADRP (red) to label lipid bodies (inset). Bars are 10 μm. **B.** Fluorescence microscopy using Nile Red to visualize lipid bodies in MEF ACAT1^{-/-} stably transfected with a plasmid containing *TgACAT2-HA* or vector alone. Bar is 20 μm. **C.** Quantitative measurement of CE synthesis in MEF ACAT1^{-/-} (panel a) or AC29 cells (panel b) expressing TgACAT2-HA by thin layer chromatography (TLC). Palmitate uptake and incorporation into CE were monitored in parental cells (MEF and CHO) and transfected cells (MEF ACAT1^{-/-} and AC29) with pTgACAT2-HA or vector only. Results of palmitate uptake or cholesteryl palmitate (CP) production expressed in nmol palmitate per mg cell protein or nmol palmitate incorporated into CP per mg cell protein, respectively, are means ± SD of three separate experiments. Differences between values of palmitate incorporated into CP in cells between TgACAT2- expressed cells and vector controls are statistically significant (*, $P < 0.0005$). Representative chromatograms are shown at the top of histograms for CE synthesis. C, cholesterol; FA, fatty acids; TAG, triglycerides.

Figure 4. Growth and morphology of *Toxoplasma* lacking either *ACAT1* or *ACAT2*

A. Quantification of replication rate *in vitro* of the ΔACAT1 and ΔACAT2 strains compared to the ΔKu80 parental strain. [³H]uracil incorporation was assayed using fibroblasts infected with these three strains for 24-h. Values of uracil incorporation in the parasites expressed in cpm, are the means ± SD from three separate experiments. Differences between values obtained for the two ΔACAT strains relative to the parental strain are statistically significant (*, $P < 0.05$). **B.** Quantification of invasion using the red/green assay for parasites stained with antibodies against the plasma membrane marker SAG1. Histograms represent internal parasites and data are means ± SD of triplicates, counting 20 randomly selected fields for each sample at 20× objective (*, $P < 0.05$). **C.** IFA on ΔACAT1, ΔACAT2 and ΔKu80 strains using anti-SAG1 antibodies to visualize the global shape of the parasites 36-h p.i. **D.** Same IFA on ΔACAT1, ΔACAT2 and ΔKu80 as described in C. Parasites were incubated in excess LDL (2 mg.ml⁻¹) for 24-h or 48-h prior to fixation. Bars are 5 μm.

Figure 5. Ultrastructure *Toxoplasma* lacking either *ACAT1* or *ACAT2*

A-C. Transmission electron microscopy comparing the ultrastructure of the parental strain with Δ ACAT1 and Δ ACAT2 parasites. **A.** Panel a illustrates two PV with parental parasites with well-identifiable organelles and the tubulo-vesicular network (TVN). Panel b shows a classical lipid body surrounded by ER elements and close to mitochondria (m). **B.** Panels a to c highlight the presence of abnormal material debris in the PV lumen of the Δ ACAT1 strain. Morphology of mitochondria was altered (panel b) and ghost parasites were visible (asterisk in panel c). **C.** Panels a to c illustrate abnormal accumulation of osmiophilic materials (arrows) in the vacuolar space or within the cytoplasm for the Δ ACAT2 strain. Small residual lipid bodies (LB) were visible. Scale bars are 0.5 μ m.

Figure 6. Cholesterol and CE content in *Toxoplasma* lacking either *ACAT1* or *ACAT2*

A. Fluorescence microscopic observations of Δ ACAT1, Δ ACAT2 and parental strains to visualize membrane cholesterol using filipin and lipid bodies using Nile Red. Bars are 5 μ m.

B. Quantitative measurement of total cholesterol (free and esters forms) and CE alone in Δ ACAT1, Δ ACAT2 and parental strains. Total cholesterol content was determined on parasites exposed to various culture conditions for 24-h: the parental strain cultivated in normal medium (10% FCS; Ctl), in the presence of the ACAT inhibitor DuP128 or in excess cholesterol in the medium (LDL); and the Δ ACAT1 and Δ ACAT2 parasites cultivated in normal conditions. Results of total cholesterol content expressed in μ g sterol molecules per mg cell protein, are the means $\pm$ SD from three separate experiments. Differences between values obtained for the parental strain grown in excess LDL and normal medium are statistically significant (*, $P < 0.05$). To monitor cholesterol esterification activity, parasites were exposed to radioactive palmitate for 2-h prior to lipid extraction and separation on TLC plates. Results of lipid incorporation into CE expressed in μ g palmitate incorporated into CE per mg cell protein, are means $\pm$ SD of three separate experiments. These data were compared to parental parasites under control conditions and were statistically significant (*, $P < 0.05$; **, $P < 0.01$).

Figure 7. Transcriptional profiles of TgACAT1 and TgACAT2

RT-qPCR 100 ng total RNA of Δ ACAT1, Δ ACAT2 and parental strains were subjected to first-strand cDNA synthesis using random hexamer primers. Histograms show transcript abundance (A) or fold change (B) that were calculated by normalization with the housekeeping gene transcripts (TgElf1 α and TgGT1).

Table I

Uptake of cholesterol by MEF ACAT1^{-/-} cells transfected with *TgACAT1* or *TgACAT2*, and incorporation into cholesteryl esters

	cholesterol uptake (cpm.µg cell protein ⁻¹)	CE levels (cpm.µg cell protein ⁻¹)
MEF ACAT1 ^{-/-} transfected with:		
vector	3,991 ± 601	n.d.
<i>TgACAT1</i>	4,003 ± 510	898 ± 65
		1,514 ± 110
<i>TgACAT2</i>	3,758 ± 436	

*

MEF ACAT1^{-/-} were transfected with vector alone, or plasmids containing either *TgACAT1* or *TgACAT2*. Cells were then incubated with [³H]cholesterol-LDL. Cholesterol uptake was monitored by scintillation counting. CE detection was performed by TLC analysis. The results are mean values ± SD from three incubations (*; *P* < 0.01). n.d., not detected.

Table II

Uptake of fatty acids by MEF ACAT1^{-/-} cells transfected with *TgACAT1* or *TgACAT2*, and incorporation of free fatty acids into cholesteryl esters

	FFA uptake (nmol.FFA mg cell protein ⁻¹)	CE activity (nmol FFA incorporated into CE)
MEF ACAT1 ^{-/-} transfected with:		
vector	16:0	130.1
	18:0	110.1
	18:1	118.1
	20:4	99.9
<i>TgACAT1</i>	16:0	127.0
	18:0	102.9
	18:1	100.4
	20:4	115.6
<i>TgACAT2</i>	16:0	98.4
	18:0	119.1
	18:1	127.5
	20:4	111.3

MEF ACAT1^{-/-} were transfected with the vector alone, or plasmids containing either *TgACAT1* or *TgACAT2* for 24-h. Cells were then incubated with [³H]palmitic acid (16:0), [³H]stearic acid (18:0), [³H]oleic acid (18:1) or [³H]arachidonic acid (20:4) for 3-h. Free fatty acid (FFA) uptake was monitored by scintillation counting. CE formation was performed by TLC analysis. The results are mean values from incubations performed in duplicate with less than 10% variation. n.d., not detected.

Table III

Effect of cholesterol esterification inhibitors on viability of parasites lacking TgACAT1 or TgACAT2

Strains	Parental	Δ ACAT1 [³ H]uracil incorporation in cpm (% of growth relative to untreated parental strain)	Δ ACAT2
Treatment:			
- no drug	51,902 ± 3,053 (100%)	29,008 ± 2,027 ** (56%)	19,482 ± 1,113 ** (38%)
- 5 μ M DuP128	24,506 ± 5,291 ** (47%)	19,123 ± 426 ** (37%)	12,820 ± 1,134 ** (25%)
- 10 μ M CI976	41,607 ± 3,648 * (80%)	24,480 ± 325 ** (47%)	17,731 ± 972 ** (34%)

Radioactive incorporation assays on HFF infected with the parental, Δ ACAT1 or Δ ACAT2 strains. Cells were infected for 24-h, then treated with the cholesterol esterification inhibitors, DuP128 (5 μ M) or CI976 (10 μ M), or no drug for additional 24-h prior to uracil incorporation assays to monitor parasite growth. Results are the means $\pm$ SD of three different assays. Parasite growth was significantly impaired compared to untreated parental parasites (referred as 100% growth)

*
P<0.05

**
P<0.005.