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Article

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Synthesis versus Salvage of Ester- and Ether-linked Phosphatidylethanolamine in the Intracellular Protozoan Pathogen *Toxoplasma gondii*

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**Abbreviations**: AID: auxin-induced degron; Cas9, clustered regularly interspaced short palindromic repeats-associated protein 9; CCT: phosphocholine cytidylyltransferase; CDP: cytidine 5’-diphosphate; CRISPR: clustered regularly interspaced short palindromic repeat; CT: cytidylyltransferase; DHFR-TS: a pyrimethamine-resistant mutant of dihydrofolate reductase-thymidylate synthase; ECT: phosphoethanolamine cytidylyltransferase; EK: ethanolamine kinase; EPT: ethanolamine phosphotransferase; ER: endoplasmic reticulum; HFF: human foreskin fibroblast; HXGPRT: hypoxanthine-xanthine-guanine phosphoribosyl transferase; IAA: indole-3-acetic acid; PSD: phosphatidylserine decarboxylase; PtdCho: phosphatidylcholine; PtdEtn: phosphatidylethanolamine; PtdGro: phosphatidylglycerol; PtdIns: phosphatidylinositol; PtdSer: phosphatidylserine; PtdThr: phosphatidylthreonine; PSS: phosphatidylserine synthase; PTS: phosphatidylthreonine synthase; PV: parasitophorous vacuole; sgRNA: single guide RNA; smHA: Spaghetti monster-HA

**ABSTRACT**

*Toxoplasma gondii* is a prevalent zoonotic pathogen infecting livestock as well as humans. The exceptional ability of this parasite to reproduce in several types of nucleated host cells necessitates a coordinated usage of endogenous and host-derived nutritional resources for membrane biogenesis. Phosphatidylethanolamine is the second abundant phospholipid in *T. gondii*, but how its requirement in the acutely-infectious and fast-dividing tachyzoite stage is satisfied remains enigmatic. This work reveals that the parasite deploys *de novo* synthesis and salvage pathways to pacify its demand for ester- and ether-linked PtdEtn, respectively. Auxin-mediated depletion of the
phosphoethanolamine cytidylyltransferase (ECT) caused a lethal phenotype in tachyzoites due to impaired invasion and cell division, disclosing a vital role of the CDP-ethanolamine pathway during the lytic cycle. In accord, the inner membrane complex and mitochondrion were disrupted concurrent with a decline in major phospholipids. Not least, integrated lipidomics and stable isotope analyses of the TgECT mutant unveiled the endogenous synthesis of ester-PtdEtn, and salvage of ether-linked lipids from host cells. In brief, this study demonstrates how T. gondii operates various means to produce distinct forms of PtdEtn while featuring the therapeutic relevance of its de novo synthesis.

INTRODUCTION

The protozoan phylum Apicomplexa comprises many common intracellular pathogens, such as Toxoplasma, Plasmodium, Eimeria and Cryptosporidium. Toxoplasma gondii – the only known species in the genus – has a remarkable ability to infect many types of nucleated cells in various vertebrates; it is therefore recognized as one of the most successful pathogens. The natural infection of the hosts by T. gondii starts by ingesting the oocysts or cysts in the contaminated food. The sporozoite and bradyzoite stages released from the oocyst or cyst, respectively, infect the gastric epithelium and further develop into a highly infectious, promiscuous and fast-dividing tachyzoite stage, which reproduces in other tissues, eventually causing necrosis by perpetual lytic cycles. Upon physicochemical and immune stress, some tachyzoites differentiate into quiescent and encysted bradyzoites, establishing chronic infection. For intracellular reproduction within host cells, the parasite must produce sufficient membrane biomass via its synthesis and salvage pathways, many of which bestow excellent anti-parasitic therapeutic targets.

Glycerophospholipids are a major ingredient of the biomembranes, including in tachyzoites of T. gondii. Phosphatidylcholine (PtdCho), phosphatidylethanolamine (PtdEtn), phosphatidylethanolamine (PtdThr), phosphatidylserine (PtdSer), phosphatidylinositol (PtdIns), phosphatidyglycerol (PtdGro) and phosphatidylserine (PtdOH) are typical phospholipids present in tachyzoites and required for the optimal lytic cycle. Besides their customary roles for parasite replication, many phospholipids have recently emerged as key players in calcium homeostasis and signal transduction, contributing to the gliding motility, invasion and egress of tachyzoites. The parasite is able to synthesize phospholipids using precursors acquired from the host cell. Further, it can salvage certain phospholipid species/probes from the extracellular and/or intracellular milieu.

PtdEtn is the second most common phospholipid in tachyzoites, present in ester and ether-linked forms. The ester-PtdEtn can be obtained by several routes, including distinct PtdSer decarboxylases (PDSs) located in the parasite mitochondrion and parasitophorous vacuole, CDP-ethanolamine a.k.a. Kennedy pathway in the endoplasmic reticulum, and via P4-ATPase-mediated flipping in the plasma membrane. Its endogenous synthesis through the CDP-ethanolamine pathway requires a diacylglycerol scaffold that can be generated by the tachyzoites. Though not yet studied in T. gondii, ether-linked PtdEtn contains an alkyl-glycerol backbone, made by an alkylglycerone phosphate synthase (AGPS) in mammalian cells. In functional terms, the conical shape of ester-PtdEtn provides the membrane curvature, regulating the budding, fusion and fission events and thereby facilitating the membrane-protein interactions. On the other hand, the shape of ether-PtdEtn allows stronger intermolecular hydrogen bonding between headgroups, resulting in decreased membrane fluidity.
This work examined the biogenesis and physiological relevance of ester- and ether-PtdEtn during the lytic cycle. We studied the phosphoethanolamine cytidylyltransferase (ECT), which catalyzes ethanolamine kinase-derived phosphoethanolamine to CDP-ethanolamine in the parasite cytosol. Our data show that ECT is essential for the asexual development of tachyzoites in human cells. Its conditional depletion by mutagenesis impairs the amount and synthesis of ester-PtdEtn species, whereas ether-PtdEtn remains unaffected. Likewise, we discovered that intracellular parasites could salvage ether-linked PtdEtn from the host cells.

**RESULTS**

*T. gondii encodes a putative ethanolamine cytidylyltransferases*

Our former work has reported the occurrence of a functional CDP-ethanolamine pathway in the tachyzoites, albeit we could not identify a potential AGPS in the parasite genome. In the following studies, we described the first and last enzymes of *de novo* PtdEtn synthesis, *i.e.*, ethanolamine kinase (EK) and ethanolamine phosphotransferase (EPT). Here, we identified the ECT protein, which uses phosphoethanolamine and CTP as the substrates to produce CDP-ethanolamine. By comparing with homologs from the model organisms, *Saccharomyces cerevisiae, Homo sapiens, Mus musculus* and *Trypanosoma brucei*, we found a potential ECT in the *Toxoplasma* database (TGGT1_310280, ToxoDB, Fig 1). Unlike mammalian and kinetoplastid ECTs, which encompass about 400 residues and share a phylogenetic node, apicomplexan homologs are much longer, forming a distinct own clade. *TgECT* encodes 573 residues, while *PfECT* and *EfECT* contain 1128 amino acids. Apicomplexan ECTs possess an unusual N-terminal extension of >100 residues compared to canonical homologs. *TgECT* is exceptional with a long extension of about 500 amino acids, resulting in a 3x larger protein than its non-apicomplexan counterparts (Fig 1A).

*TgECT harbors two tandem cytidylyltransferase domains of circa 100-150 residues parted by a linker region, analogous to its counterparts in other organisms. This contrasts with the bacterial CTP:glycerol-3-phosphate cytidylyltransferase or eukaryotic CTP: phosphocholine cytidylyltransferase (CCT), which contains only a single CT domain. Sequence alignment of the typical ECT domains also identified conserved residues (Fig S1). All signature motifs of the cytidylyltransferase family occur in *TgECT*: HSGH and HVGH; an RTEGISTS motif; KWVDEVI and RVVDEVI regions (Fig S1). Homology modeling of *TgECT* domains based on the *HsECT* structure (PDB code, 3ELB) suggests that the HSGH, HVGH and RTEGISTS are involved in forming a catalytic center, whereas the KWVDEVI and RVVDEVI enable the dimerization of the N- and C-terminal CT domains (Fig 1B). The N-terminal CT domain in *HsECT* and *PfECT* are critical for their catalytic activity, which is likely to hold true for *TgECT* (Fig 1A).

*TgECT is a cytosolic protein refractory to genetic deletion in tachyzoites*

The first step of the CDP-ethanolamine pathway producing phosphoethanolamine occurs in the cytosol, and the third/last reaction synthesizing PtdEtn happens in the endoplasmic reticulum. Here, we examined the localization of ECT to decipher the subcellular location of CDP-ethanolamine formation. We generated a transgenic parasite strain expressing ECT fused to a C-terminal spaghetti monster (10xHA) by 3'-genomic tagging. A donor amplicon with the 5' and 3' homology sequences flanking the pyrimethamine-resistant DHFR-TS expression cassette (selection marker) was co-
transfected into tachyzoites with a CRISPR construct encoding Cas9 and a gene-specific sgRNA (Fig 1C). The drug-resistant parasites were cloned by limiting dilution and screened by PCR. Immunofluorescent staining revealed a punctate distribution of ECT-3xHA within the parasite, co-localizing with a known cytosolic marker, aldolase (TgAld)\textsuperscript{28}. To assess the physiological relevance of ECT during the lytic cycle, we attempted to delete the gene in tachyzoites via CRISPR/Cas9-assisted double homologous recombination. Our multiple experiments to achieve a viable knockout mutant were futile, however, implying that this protein is indispensable for tachyzoite survival.

**Conditional knockdown of ECT reveals its essentiality in tachyzoites**

In the following work, we made a conditional mutant of TgECT based on an auxin-induced degron, which directs the tagged proteins to proteasomal degradation upon incubation with indole-3-acetic acid (IAA)\textsuperscript{29}. A CRISPR/Cas9 construct encoding Cas9 and sgRNA to cleave the ECT-3'UTR was co-transfected with an amplicon for homology-directed repair at the target locus in tachyzoites. The donor sequence contained 5' and 3' homology arms (40 bp each) flanking the AID-3xHA motif for 3'-insertional tagging and DHFR-TS selection marker (Fig 2A). The genomic integration was examined by PCR screening using crossover-specific primers. Unlike the parental strain, the TgECT-AID-3xHA mutant showed a band of 5.5 kb (Fig 2A-B), confirming the 3'-gene tagging, which was verified by DNA sequencing. Immunoblot revealed a protein of \( \sim 180\)-kDa corresponding to AID-3xHA-tagged ECT in the transgenic strain cultured without IAA (Fig 2C). The protein expression was not detectable after IAA treatment of the mutant parasites. As predicted, no HA signal was observed in the parental strain irrespective of IAA exposure. Yet again, ECT localized with TgHsp90 in the parasite cytosol\textsuperscript{30} (Fig 2D), excluding any artifact of AID-3xHA tagging. The AID-mediated regulation of the protein by IAA was also confirmed by immunofluorescence assay (Fig 2E).

A conditional mutant enabled us to evaluate the significance of ECT for the parasite’s lytic cycle in HFF cells. We set up plaque assays, which indicate the overall growth fitness of *T. gondii* (Fig 2F). The parental strain exhibited normal growth regardless of IAA in culture, and so did the TgECT-AID-3xHA mutant in the absence of auxin (Fig 2F, Fig 5A). In contrast, the IAA-induced depletion of ECT completely arrested the parasite growth, as shown by an absence of plaques in the host-cell monolayer. These results resonate with the preceding observation that the ECT gene cannot be deleted, ascertaining its physiologically-critical role in *T. gondii*.

**TgECT is required for the parasite invasion and proliferation**

We next examined the cause of a lethal phenotype in ECT-depleted tachyzoite cultures by additional assays, such as the replication, endodyogeny (cell budding), egress, invasion and gliding motility (Fig 3). For the first two experiments, we chose time points corresponding to early (24 h) and late (40 h) parasitized cultures (Fig 3A-B). The replication was quantified by enumerating ‘parasites per vacuole’ and calculating the fraction of vacuoles with variable numbers of progeny. Unlike the parental strain, which remained unaffected by IAA, the ECT mutant had a much higher fraction of small vacuoles (1-8 parasites) in auxin-treated cultures at both time points compared to untreated samples (Fig 3A). The large vacuoles with >16 parasites were barely present under ECT-depleted conditions, contrasting with the control cultures. In agreement, the assessment of endodyogeny demonstrated a strongly impaired daughter cell formation only in the IAA-treated TgECT-AID-3xHA mutant (Fig 3B).
The conditional mutant also exhibited a defect in natural egress following the completion of tachyzoite replication (Fig 3C). To test whether the observed phenotype was due to slower replication, we scored induced egress in response to zaprinast—a potent phosphodiesterase inhibitor that triggers the premature exit of intracellular tachyzoites by activating the cGMP signaling. Indeed, the egress phenotype was rescued by zaprinast (Fig 3D). Notably, ECT-depleted parasites invaded host cells very poorly (Fig 3F), implying the inability of the mutant to establish a new infection. Since the invasion process is driven by gliding motility, we assayed the latter phenotype (Fig 3E). Our data disclosed a marked decline in the motile fraction and trail length of the IAA-exposed mutant but not in control samples. The observed defects in the parasite motility, invasion and replication culminated in a highly reduced yield of the conditional mutant incubated with auxin (Fig 3F). Its tachyzoite count dropped by ~90% upon IAA treatment compared to the control cultures.

**Loss of ECT impairs the inner membrane complex, mitochondrion, and phospholipids**

The tachyzoite motility and invasion are driven by complex machinery, termed glideosome, embedded in the inner membrane complex. The staining of daughter cells (Fig 3B) also pointed to a malfunctioning organelle, which we examined further by visualizing TgGap45—a well-characterized protein essential for the glideosome function—in tachyzoites of the TgECT-AID-3xHA strain (Fig 4A). Culturing mutant with IAA for 24 and 48 hours caused an increasingly irregular and weaker staining of TgGap45. The IMC labeling was patchy with intermittent poor to no staining (see arrowheads, Fig 4A). As PtdEtn is important for the optimal mitochondrial functioning in mammalian cells, we also stained TgPSD1mt in the tachyzoite mitochondrion (Fig 4B). The mutant without IAA exhibited a normal lariat-shaped morphology spread throughout the parasite body. Upon exposure to auxin for 24 h, the organelle appeared shrunken and fragmented. The phenotype was accentuated upon prolonged incubation (48 h) with IAA.

Encouraged by our phenotyping results and given a putative role of ECT in PtdEtn synthesis, we performed a lipidomic analysis of the TgECT-AID-3xHA and parental strains (Fig 5). Total lipids were isolated from parasites, resolved and analyzed by HPLC-MS. Compared to the parental and untreated mutant controls, we witnessed about a 50% decline in phospholipid content of the ECT-depleted strain (Fig 5A). A significant decrease in all major phospholipid classes of IAA-treated mutant was seen, whereas no change was apparent in the parental strain (Fig 5B). The abundance of shown lipids plummeted by 30-50% upon ECT depletion. We also measured levels of the ester and ether-linked PtdEtn (Fig 5D). None were altered in -/+ IAA cultures of the parental strain. A knockdown of ECT in the mutant caused a decline in ester-PtdEtn but not in ether-lipid (Fig 5D). Hence, the ratio of ester vs. ether-PtdEtn was changed from 2.6:1 in untreated cultures to 1.1:1 in the ECT-depleted mutant.

**Knockdown of ECT causes differential regulation on ester- and ether-linked lipids**

We next plotted the magnitude of change in all detectable phospholipid species irrespective of abundance as volcano plots to illustrate the fold-change vs. statistical significance upon IAA-induced loss of ECT (Fig 6A-B). None of the lipid species in the parental strain were affected above a threshold of ≥2-fold (p-value ≤0.01) (Fig 6A). The TgECT-AID-3xHA strain, on the other hand, displayed substantial regulation of several lipid species corresponding to PtdCho, PtdEtn, PtdIns,
PtdSer, PtdThr, lyso-PtdCho and lyso-PtdIns (Fig 6B, Fig S2-S3). Among downregulated ester-phospholipids, PtdEtn, PtdSer and PtdThr species were most evident (Fig 6B). A few others, namely C36:1, C38:4, C40:5 PtdEtn, C42:5 PtdSer, C36:4, C38:1 PtdCho and C20:4 lyso-PtdIns were upregulated. Interestingly, ether-lipids belonging singularly to PtdEtn and PtdCho were upregulated or unaltered in the mutant (Fig 6C, 7A).

The interlinked synthesis of phospholipids motivated us to find patterns in their co-regulation. A loss of ECT altered several species, especially those shared by PtdSer, PtdThr and PtdEtn. C34:2, C38:6, C38:7, C40:7 PtdSer and C38:6, C38:7 PtdThr matched the trend to PtdEtn species (Fig S3A), suggesting their synthesis by the base-exchange PSS and PTS enzymes in *T. gondii* [7]. There was barely a common PtdEtn and PtdCho species (Fig 6B), ratifying the absence of a lipid methyltransferase [5]. Equally, no correlation of PtdEtn species with PtdIns was seen, as expected by sovereign routes of their syntheses [12,25]. Strikingly, some species of PtdCho (36:4, 38:1), PtdEtn (36:1, 38:4, 40:4, 40:5) and PtdSer (42:5) were induced in the ECT-deprived mutant (Fig S3B), pointing to a counterbalancing mechanism. None of the specified PtdEtn species, however, was 13C-labeled in extracellular tachyzoites or in those grown in pre-labeled host cells (Fig 7B-C, see below). Such ester-PtdEtn species are therefore likely formed by PtdSer decarboxylases [10,21].

**Tachyzoites can salvage ether-linked PtdEtn from the host environment**

Unlike its ester form, ether-PtdEtn species were increased or unaffected (Fig 7A), prompting us to search the enzymes of ether-PtdEtn synthesis in *T. gondii*. As mentioned above, our bioinformatic analysis did not find a suitable candidate in the parasite genome. We, therefore, hypothesized that the Kennedy pathway provides the ester-linked PtdEtn and the production of ether-lipids depends on the parasite milieu. To test this notion, we performed the stable isotope labeling (13C2-ethanolamine) followed by lipidomics, which enabled us to distinguish the PtdEtn species synthesized by the parasite from those salvaged from the host cells (Fig 7B). Akin to previously-reported 13C6-myoinositol labeling of PtdIns [12], host-free parasites labeled with 13C2-ethanolamine were analyzed to discern the endogenously-made PtdEtn, while tachyzoites cultured in pre-labeled host cells were deployed to deduce the occurrence of a salvage pathway. We also performed 13C2-choline labeling to exclude the contribution of ECT to the CDP-choline pathway (not shown).

Lipidomic analysis of the extracellular parasites revealed enrichment of 13C2-ethanolamine in several ester-PtdEtn species, such as C34:1 (>5 fold), C34:2 (~11 fold), C36:2 (~10 fold), C38:4 (~6 fold) and C40:5 (~6 fold) (Fig 7C), which confirmed the presence of a functional CDP-ethanolamine pathway [5,10]. The IAA-mediated ECT depletion impaired the isotope labeling of C34:1, C34:2, and C36:2 species, consistent with the observed decline in their content (Fig 7A). By contrast, the 13C2-labeling of C38:4 and C40:5 PtdEtn were not affected by IAA (Fig 7C) in accord with their unperturbed or increased levels (Fig 7A). Tachyzoites grown in 13C2-ethanolamine-labeled host cells did not show isotopic enrichment in any of the ester-PtdEtn species ruling out salvage of these lipids (Fig 7C). Inversely, we witnessed an opposite phenomenon for ether-PtdEtn species (A34:2, A36:5) that were 13C2-enriched (~4-5x) in intracellularly-grown but not in host-free parasites (Fig 7D). Other lipid species (A38:5, A38:6, A40:6) were labeled equally under both settings (~2-3x), and as expected, no change in 13C2-labeling of ether-PtdEtn was seen upon depletion of ECT. The data
entail that ether-PtdEtn species are synthesized primarily \textit{de novo} using the CDP-ethanolamine pathway, while the ether-linked PtdEtn species are salvaged by the parasite from its host cell.

**DISCUSSION**

Understanding the metabolic basis of intracellular parasitism in apicomplexan parasites has been central to pathogen-host research. Over the last three decades, \textit{Toxoplasma gondii} has evolved into a model parasite to discover apicomplexan biology due to the relative ease of its culture and advanced tools for genome engineering and mutant phenotyping. Albeit actively studied, lipid synthesis, trafficking, salvage, sensing and signaling still remain poorly appreciated in Apicomplexa.

Exploring a phosphoethanolamine cytidylyltransferase (ECT), this work determined that tachyzoites of \textit{T. gondii} deploy distinct routes to fulfill their need for the ester- and ether-linked PtdEtn species (Fig 8). While the first set of lipids is generated through the Kennedy pathway, the second type is salvaged from the host cells. ECT is an essential protein facilitating parasite invasion and replication during the lytic cycle. These features and its phylogenetic divergence from the mammalian homologs make \textit{Tg}ECT an excellent drug target for therapeutic inhibition of acute toxoplasmosis.

Our work suggests a role of CDP-ethanolamine pathway in making ester-PtdEtn. The levels of many ester-PtdEtn species were reduced in the ECT mutant; however, unperturbed and upregulated amount of certain others indicates the functional presence and contribution of additional routes. Ester-PtdEtn can indeed be generated by PSD1\textsubscript{mt} and PSD1\textsubscript{pv} (Fig 8)\textsuperscript{10,21}. Moreover, at least the extracellular tachyzoites are able to salvage PtdEtn\textsuperscript{15,20}, and it is plausible that when intracellular, they can acquire PtdSer-derived PtdEtn made in the parasitophorous vacuole by catalysis of PSD1\textsubscript{pv}. Finally, the host endoplasmic reticulum and mitochondria recruited onto the vacuolar membrane are major sites of PtdSer and PtdEtn synthesis in mammalian cells and possibly serve as extra source of these lipids. Interestingly, the PSD1\textsubscript{mt}-knockout tachyzoites can survive in prolonged cultures and show upregulation of the CDP-ethanolamine route \textsuperscript{10}, but the opposite seems not true as ECT is essential for the lytic cycle. We propose that distinct reactions drive the synthesis of discrete ester-PtdEtn species, and those encoded by the Kennedy pathway cannot be equipoised by other means, leading to a lethal phenotype in the ECT mutant.

The physiological relevance of the Kennedy pathway has also been studied in other parasitic protists, including \textit{T. brucei} and \textit{P. bergei} \textsuperscript{36,37}. The underlying enzymes are reported to be essential for these parasites even though they harbor functional PSD enzymes. Depletion of \textit{Tb}ECT impaired the parasite proliferation, correlated with a reduced PtdEtn content, unusual cell morphology and altered mitochondrial structure in \textit{T. brucei} \textsuperscript{36}. These findings resound phenotypic observation in ECT-depleted tachyzoites of \textit{T. gondii}. Conditional loss of \textit{Tg}ECT abrogated the parasite invasion and replication, concurrent with abnormal inner membrane complex and fragmented mitochondrion that can be attributed to a dysregulated phospholipid composition or possibly to PtdEtn species enriched in the indicated organelles. Indeed, both exist in proximity and are proposed to facilitate the lipid trafficking and calcium homeostasis in tachyzoites\textsuperscript{14}, which may be affected upon ECT depletion. Furthermore, a skewed ratio of ester- and ether-linked PtdEtn in the mutant may perturb the membrane fluidity and cytokinesis, as described in mammalian cells \textsuperscript{23}. 


Our data also suggest that PtdEtn serves as a donor for synthesizing PtdSer and PtdThr via ethanolamine-to-serine or threonine exchange reaction catalyzed by PSS and PTS proteins, respectively (Fig 8). Notably, a few PtdEtn species (36:1, 38:4, 40:4, 40:5) were unaffected or even elevated upon depletion of ECT. They are likely made by PtdSer decarboxylation, which merits lipidomic analysis of the PSD1mt and PSD1pv mutants. No parallels were evident in the modulation of PtdCho and PtdIns species with that of PtdEtn, reverberating with the independent routes of syntheses for the former two phospholipids. Importantly, this study reveals the salvage of host-derived ether-PtdEtn species by intracellular parasites (Fig 8). While tachyzoites are known to produce diacylglycerol for making ester-PtdEtn, a bona fide AGPS for producing the alkyl-glycerol scaffold of ether-PtdEtn could not be found in the parasite genome. The presence of ether-linked lipids in tachyzoites is enigmatic. They are reported to have "redox" effects in mammalian cells. Certain cultured cells lacking them become more sensitized to oxidative damage. Equally, the pro-oxidant feature of these lipids has also been described. Future work should focus on dissecting the salvage machinery and functional importance of ether-lipids in tachyzoites of T. gondii.

In conclusion, this work demonstrates TgECT as a vital enzyme contributing to the synthesis of ester-PtdEtn. Its conditional depletion impairs the membrane biogenesis, replication and invasion in tachyzoites. We reveal a collaboration of endogenous synthesis, interconversion and salvage pathways to produce PtdEtn. In particular, the data show previously-unknown scavenging of host-derived ether-linked PtdEtn species by the parasite. Last, our study offers a sound basis for therapeutic targeting of PtdEtn synthesis in T. gondii.

MATERIAL AND METHODS

Biological reagents and resources
The RH△ku80△hxgprt-TIR1 strain of T. gondii, the pLinker-AID-3xHA-HXGPRT plasmid for AID-3xHA tagging and anti-TgAld antibody were provided by David Sibley (Washington University, St. Louis, USA). Other primary antibodies binding to TgGap45, TgHsp90, and TgIMC3 were offered by Dominique Soldati-Favre (University of Geneva, Switzerland), Sergio Angel (IIB-INTECH, Buenos Aires, Argentina) and Marc-Jan Gubbels (Boston College, MA, USA), respectively. The anti-TgPSD1mt sera were obtained from FriendBio Bioscience and Technology (China). Other primary antibodies against the hemagglutinin (HA) epitope and Tsgag1 were obtained from Sigma-Aldrich (Germany) and ThermoFisher Scientific (Germany), respectively. The secondary antibodies for immunostaining (Alexa488, Alexa594, IRDye 680RD, 800CW) and oligonucleotides (Table S1) were purchased from Life Technologies (Germany). The cell culture reagents were procured from PAN Biotech (Germany). Other standard chemicals were supplied by Sigma-Aldrich and Carl Roth (Germany). The reagent kits for gene cloning were acquired from Analytik Jena and Life Technologies (Germany). Lipid standards were delivered by Avanti Polar Lipids (USA).

Parasite and host cell cultivation
Human foreskin fibroblasts (HFFs) were used as the host cells to propagate tachyzoites of T. gondii. Cells were harvested by trypsinization (0.25% trypsin-EDTA) and seeded in flasks, dishes or coverslips, as per the requirement of individual assays. The confluent monolayers were infected on alternate days to maintain tachyzoites. Cultures were performed in DMEM (Dulbecco’s Modified Eagle’s Medium) supplemented with glucose (4.5 g/L), fetal bovine serum (10%, PAN Biotech,
Germany), glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/mL), streptomycin (100 μg/mL) and minimum Eagle’s non-essential amino acids (100 μM each) at 37°C and 5% CO₂ in a humidified incubator. For most assays, tachyzoites were released from late-stage parasitized cultures by scraping and squirting through 23- and 27-gauge syringes. The extracellular parasites were utilized directly for downstream assays.

**Construction of transgenic lines**

Tachyzoites (~10^7) were transfected with 10 μg plasmid constructs in filter-sterile cytomix (120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄/KH₂PO₄, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl₂ supplemented with fresh 5 mM glutathione and 5 mM ATP; pH 7.6). Parasites were then selected with the drug corresponding to the selection marker encoded by the transfected plasmid. The drug-resistant transgenic parasites were cloned by limiting dilution in 96-well plates containing HFF cells. Individual clones with desired gene manipulation were identified by screening PCR and immunostaining assays. To generate the TgECT-AID-3xHA strain, a donor amplicon comprising the AID-3xHA-TgGRA1-3'UTR and DHFR-TS selection marker flanked by short (40bp) homologous arms for a crossover at the ECT locus was co-transfected with a CRISPR-Cas9 construct in the RHΔku80Δhxgprt-TIR1 strain. To generate the TgECT-smHA strain, a plasmid construct expressing Cas9 and TgECT-specific sgRNA was transfected with a PCR amplicon comprising 10xHA and DHFR-TS expression cassette in the RHΔku80-hxgprt strain. The mutant parasites were selected by 1 µM pyrimethamine and PCR-screened for the AID-3xHA or smHA tagging of ECT.

**Indirect immunofluorescence assays**

The assay was executed, as reported earlier. Briefly, HFF cells were seeded and grown to confluence on the glass coverslips placed in 24-well plates. Freshly-released parasites were used to infect HFF monolayers. Parasitized host cells were washed with PBS and fixed in 4% paraformaldehyde for 15 mins, followed by neutralization in 0.1 M glycine/PBS. Samples were permeabilized by 0.2% Triton-X100/PBS (20 min) and blocked with 2% BSA prepared in 0.2% Triton-X100/PBS (20 min). Cells were finally stained with the matching primary (α-HA, 1:3000; α-TgGap45, 1:10000; α-TgIMC3, 1:2000; α-TgPSD1mt, 1:100; α-TgAld, 1:1000) and secondary (Alexa488, Alexa594, 1:3000) antibodies for 1 h each. Samples were washed by PBS between different treatment steps and mounted in DAPI-Fluoromount G for imaging (Carl-Zeiss, Germany).

**Lytic cycle assays**

Phenotypic assays were set up with fresh syringe-released parasites, as reported previously. Briefly, plaque assays were performed in 6-well plates by infecting confluent HFF monolayers with 200 parasites/well, followed by unperturbed incubation (-/+ 100 µM IAA in 0.1% ethanol) for 7 days. The carrier solvent was included in untreated samples. Cultures were fixed with cold methanol (15 min) and stained with crystal violet (15 min). Plaques were quantified using the ImageJ suite (NIH, Bethesda). For replication and endodyogeny assays, the confluent HFF monolayers on coverslips were infected (MoI:1, 24 h, 40 h), followed by fixation and staining with α-TgIMC3 and α-TgGap45 antibodies. The cell division was assessed by enumerating parasites replicating within their parasitophorous vacuoles. To determine the gliding motility, tachyzoites were pre-cultured with IAA for 48 h (if applicable). Afterward, 4x10^5 parasites suspended in Ca²⁺-free Hank’s balanced salt solution (-/+ IAA) were settled (400g, 5 min, room temperature), followed by incubation (15 min,
37°C) on coverslips coated with 0.01% BSA. Samples were stained by α-TgSag1 and Alexa488 antibodies to visualize the parasites and trails. Motile fractions were estimated directly on the microscope, while trail lengths were quantified using the ImageJ software.

For the invasion test, tachyzoites were pre-cultured in 100 µM IAA or the carrier solvent for 40 h and then used to infect the host cells on coverslips (Mol:10, 1 h). Samples were fixed, neutralized and stained, as reported elsewhere. The non-invaded (extracellular) parasites were immunostained for TgSag1 before detergent permeabilization of cultures. Samples were washed by PBS, permeabilized and stained for TgGap45 protein to visualize the invaded (intracellular) tachyzoites. Cultures were washed with PBS, labeled with secondary antibodies (Alexa488, Alexa594), and mounted in DAPI-Fluoromount G for fluorescent imaging (Carl-Zeiss, Germany). The percentage of invaded parasites (invasion efficiency) was counted by dual-colored parasites.

For natural egress assays, HFFs cultured on coverslips were infected with parasites (Mol:1, 40 h/64 h, -/+ 100 µM IAA). To induce egress, parasitized cultures (Mol:1, 24 h) were treated with 500 µM zaprinast (30 min), followed by two-step staining (see invasion assay) to distinguish the disrupted and intact vacuoles.

To quantify the parasite yield, host cells in dishes (3 cm) were infected by tachyzoites with an Mol of 1 and progeny was counted after 48 h.

Lipid extraction and lipidomic analysis
We selected 48 h infection for the sample collection based on the parasite yield assay. Lipids were extracted from fresh pellets of purified tachyzoites (1x10^7/sample), as reported earlier. Isolated lipids were dried under a nitrogen stream and dissolved in 100 µL of chloroform and methanol (1:1), 10 µL of which was injected onto a Acquity BEH C18 UPLC column (2.1 x 100 mm, 1.7 µm) maintained at 60°C. Separation of lipid species was achieved with a gradient of methanol and acetonitrile in water, both containing 2.5 mM ammonium acetate. The gradient elution, at a flow rate of 600 µL/min, was programmed as follows (time in min, % B): (0, 12.5), (7.5, 100), (14, 100), (14.1, 87.5), (17, 87.5). The effluent was subjected to heated electrospray ionization in a Sciex X500R QToF instrument. Data dependent MS2 spectra were collected (MS1 range 400-1050 amu; precursor >600 amu; MS2 range 50-1050 amu) with an accumulation time of 150 ms, a collision energy of 35V and a CE spread of 15V. Data processing was done using the Analytics module of SciexOS and MSDial.

Stable isotope labeling
The assay was performed, essentially as described previously for [13C6]-myo-inositol. To label extracellular parasites with [13C2]-ethanolamine (Sigma-Aldrich, Germany), the TgECT-AID-3xHA mutant was precultured without or with 100 µM IAA for 48 h, followed by syringe-release, PBS washing and filtering through 5 µm filter to remove the debris. Parasites (1x10^7) were then suspended in 100 µL DMEM (-/+ IAA) containing 0.5 mM [13C2]-ethanolamine and incubated at 37°C for 6 h before lipidomic analysis. For intracellular labeling of parasites in HFFs harboring [13C2]-PtdEtn, host cells in T-175 flasks were grown to confluence in 25 µM [13C2]-ethanolamine. Cultures were washed by PBS to remove the excess isotope from media and infected with parasites in the presence of 50x excess (1.25 mM) of ethanolamine to preclude the inclusion of intracellularly accumulated isotope into endogenously-produced PtdEtn of the proliferating tachyzoites. The progeny parasites were analyzed for the ester- and ether-linked PtdEtn species.
Statistical analysis and data availability

Unless specified otherwise, data shown in graphs are presented as the mean with S.E. from three experiments using representative parasite clones. Statistical analyses were performed using the GraphPad Prism program (La Jolla, CA). Significance was tested by unpaired two-tailed t-test with equal variance (*p≤ 0.05, **p≤0.01, ***p≤0.001). For the multiple comparison testing, we introduced the false-discovery rate (FDR). All underlying data are available from the authors upon request.

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Author statements

NG conceptualized and supervised the project; BR and XL performed experiments; BR, XL and NG analyzed data and drafted the manuscript; JB performed the lipidomic analysis; NG and BS acquired and managed the funding. All authors reviewed, edited and approved the manuscript. None of the authors have a conflict of interest with the contents of this article.

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FIGURE LEGENDS

**Figure 1:** *Toxoplasma encodes an ethanolamine cytidylyltransferase located in the cytosol.*

(A) Primary structure and phylogenetic proximity of ethanolamine cytidylyltransferases from representative parasitic protists and mammalian hosts. The protein sequences retrieved from the Uniprot or NCBI databases were aligned by Clustal W, and the phylogram was constructed by the MEGA 11 software (maximum likelihood method, JTT matrix model). The alignment of ECTs can be seen in Figure S1. (B) A three-dimensional homology model of the cytidylyltransferase domains in *Tg*ECT (right) and its superimposition with the crystal structure of *Hs*ECT (gray, PDB: 3ELB, left). (C) Scheme indicating the 3'-genomic tagging of *Tg*ECT with a smHA epitope. (D) Immunofluorescent co-localization of *Tg*ECT-smHA with *Tg*Ald in tachyzoites (24 h post-infection). Parasitized cultures were strained using α-HA and α-*Tg*Ald antibodies.

**Figure 2:** Conditional depletion of *Tg*ECT ablates the lytic cycle of *T. gondii* tachyzoites. (A) The CRISPR/Cas9-mediated 3'-tagging of the *Tg*ECT gene with an auxin-inducible degron and 3xHA. The *pU6-Cas9-TgECT*sgRNA construct (encoding for Cas9 and ECT-specific sgRNA) was transfected with a donor amplicon (AID-3xHA-3'UTR*Gra1*-DHFR-TS flanked by 40-bp 5'/3'-homology arms) into the RHΔku80-Δhxgprt-TIR1 parental strain. Pyrimethamine-resistant tachyzoites expressing DHFR-TS were cloned and screened by PCR. The eventual *Tg*ECT-AID-3xHA strain allowed conditional downregulation of ECT by Indole-3-acetic acid (IAA). (B) Recombination-specific screening PCR to decipher the integration of AID-3xHA at the ECT locus (see primers in panel A). (C) Immunofluorescent images confirming the co-localization of *Tg*ECT and *Tg*Hsp90 in the mutant. (D-E) IAA-dependent expression of AID-3xHA-tagged ECT shown by immunoblot and immunofluorescence methods. Parasites were cultured in the absence or presence of 100 μM IAA for 24 h. Immunostaining was done using α-HA and α-*Tg*Hsp90 (panel D) or α-HA and α-*Tg*Gap45 (panel E) antibodies. For blotting, the equivalent amount of protein for each sample was resolved on SDS-PAGE, followed by immunostaining. (F-G) Plaques produced by *Tg*ECT-AID-3xHA and parental strains (-/+IAA). Crystal violet-stained images reveal plaques (white irregular area) formed by successive lytic cycles of the stated strains in confluent HFF monolayers (blue). The plaque size as quantified by ImageJ is presented in arbitrary units (a. u.). 150-200 plaques of each strain were scored (n= 3 assays; means +/- S.E.; ***p ≤ 0.001).

**Figure 3:** *Tg*ECT is needed for the motility-dependent invasion as well as for the cell division of tachyzoites. (A-B) The replication and the budding efficiency of the *Tg*ECT-AID-3xHA and parental strains (-/+IAA). The parasitophorous vacuoles harboring tachyzoite progeny were evaluated after *Tg*Gap45 staining. The parasite budding was assessed after labeling with α-*Tg*IMC3 antibody. The bar graphs are based on 400-500 vacuoles for each sample. (C-D) Egress rates of the ECT mutant and parental strains in -/+ IAA conditions. For natural egress (C), parasitized cultures were stained 40/64 h post-infection, while zaprinast-induced egress (D) was recorded after 24 h infection. Lysed vs. intact vacuoles (panel C-D) and extracellular vs. intracellular parasites (panel E) were quantified following two-step dual-color staining. A total of >1000 events were scored for the shown bar graphs. (E) Gliding motility of tachyzoites. *Tg*Sag1-stained parasites were analyzed for the motile fraction (200-400 cells, n = 4 assays) and trail length (>60 trails per group, except for IAA-treated *Tg*ECT-AID-3xHA strain (13 trails)). (F) Invasion efficiency of the indicated mutant -/+IAA conditions. A total
of >1000 events were scored for the shown bar graphs. The data in panel A-F show data from at least three experiments (means +/- S.E.; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).

**Figure 4:** Loss of ECT is associated with disrupted morphology of inner membrane complex and mitochondrion. Tachyzoites of the TgECT-AID-3xHA strain were cultured in -/+ IAA and immunostained for the expression of TgGap45 (IMC, A) and TgPSD1_{mt} (mitochondrion, B). Nuclei were visualized by DAPI. Arrowheads point to organellar anomalies.

**Figure 5:** Auxin-induced depletion of TgECT dysregulates major phospholipids in T. gondii. (A) The yield of tachyzoites cultured in the absence or presence of IAA (MoI: 1, 48 infection). (B) Comparative phospholipid content of the TgECT-AID-3xHA mutant and parental strains. Parasites were cultured (-/+IAA), followed by lipidomic analysis (n= 4 assays). (C) Relative abundance of common phospholipid classes in tachyzoites of the specified strains (-/+IAA). (D) Changes in ester- and ether-linked PtdEtn pools of the mutant and parental tachyzoites. The data in panels A-C show the means with S.E. (n =4 assays). Statistical significance was calculated by comparing the IAA-treated to untreated samples (**p≤0.01; ***p≤0.001).

**Figure 6:** Knockdown of TgECT modulates selected phospholipid species. (A) Volcano plots of the parental and TgECT-AID-3xHA (bottom) strains to depict changes in individual phospholipid species upon IAA exposure. Defined thresholds of fold-change (≥2x) and false-discovery rate-corrected p-value (≤0.01) were used to identify significantly-altered lipids (n= 4 assays). Circles of different sizes, signifying individual species, are scaled to abundance; those above the stated threshold are colored according to phospholipid class, while others are shown in gray. (B) Heatmaps showing changes in lipid species chosen from panel A. The prefix letter “A” indicates the ether-form of PtdCho and PtdEtn.

**Figure 7:** Tachyzoites can synthesize and salvage PtdEtn species. (A) Relative abundance of ester- and ether-linked PtdEtn species significantly altered upon treatment of the TgECT-AID-3xHA with IAA (see Figure 6). (B) Scheme for the stable isotope labeling of extracellular and intracellular TgECT-AID-3xHA mutant tachyzoites (-/+IAA) with [^{13}C_{2}]-ethanolamine and host-derived [^{13}C_{2}]-PtdEtn, respectively. For the latter assay, HFFs were cultured in [^{13}C_{2}]-ethanolamine to label PtdEtn, followed by propagation of tachyzoites to test the salvage of [^{13}C_{2}]-PtdEtn. The incorporation of [^{13}C_{2}] in ester- and ether-PtdEtn species of purified parasites was judged by lipidomic analysis. (C-D) Enrichment of [^{13}C_{2}]-ethanolamine in PtdEtn species of extracellular (C) and intracellular (D) tachyzoites, as described in panel B.

**Figure 8:** PtdEtn biogenesis in tachyzoites of T. gondii. The model is based on this work, as well as the previous work cited herein. Tachyzoite deploy multiple routes to produce PtdEtn. The demand for ester-PtdEtn is fulfilled primarily via the CDP-ethanolamine and PSD1_{mt} pathways. The former drives de novo synthesis of ester-PtdEtn in the ER, whereas the latter generates PtdEtn by decarboxylating PtdSer in the mitochondrion. ECT, located in the parasite cytosol, is the second and rate-limiting enzyme of the CDP-ethanolamine route. It is essential for the parasite survival and its conditional knockdown in the TgECT-AID-3xHA mutant by IAA leads to a decline in many PtdEtn, PtdSer and PtdThr species. The latter two lipids are likely made from PtdEtn via the base-exchange
reactions driven by PSS and PTS enzymes in the ER. Loss of ECT impairs the inner membrane
complex and mitochondrion, which may underlie defect in motility, invasion and cell division.
Changes in some other lipids upon depletion of ETS are not shown here for the clarity and simplicity.
The parasite’s need for ether-linked PtdEtn is met by salvage from the host cell. Whether intracellular
tachyzoites import PSD1pv-derived ester-PtdEtn is unclear. P4-ATPases residing on the parasite
surface may play a role in flipping of ether- and ester-linked PtdEtn.

SUPPORTING INFORMATION

Figure S1: Multiple sequence alignment of TgECT with homologs from selected organisms. The
primary structure of TgECT shows the position of predicted cytidylyltransferase domains and
signature motifs. The upper and lower alignment correspond to the N- and C-terminal CT domains,
which fold together into a functional protein. IDs: Toxoplasma gondii, TgECT (TGGT1_310280);
Eimeria falciformis, EfECT (EfaB_PLUS_7742.g777); Plasmodium falciparum, PfECT
(Pf3D7_1347700); Trypanosoma brucei, TbECT (Tb927.11.14140); Trypanosoma cruzi, TcECT
(TcCLB.511727.120); Leishmania mexicana, LmECT (LmjF.32.0890); Homo sapiens, HsECT
(Q99447); Mus musculus, MmECT (Q922E4).

Figure S2: Phospholipids dysregulated in tachyzoites of the TgECT-AID-3xHA mutant. Lipid species
(-/+IAA) depicted here are based on the data presented in Figure 6.

Figure S3: Selected phospholipid species upon depletion of ECT in tachyzoites. The bar graphs
show lipid species significantly decreased (A) or increased (B) upon depletion of ECT in the presence
of IAA. For additional details, refer to Figure 6.

Table S1: Oligonucleotides used in this study
Supplementary Files

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- ECTManuscriptFigS1S4.pdf