Ceramide biosynthesis is critical for establishment of the intracellular niche of *Toxoplasma gondii*

**Highlights**
- *De novo* sphingolipid synthesis is active at the ER of *Toxoplasma gondii*
- *T. gondii* scavenges sphingomyelins but synthesizes ceramide phosphoethanolamines
- *TgSPT1* is required for establishment of the intracellular niche during invasion
- Defects in *T. gondii* sphingolipid synthesis affect chronic infection *in vivo*

**Authors**
Mary Akinyi Nyonda, Joachim Kloehn, Piotr Sosnowski, ..., J. Thomas Hannich, Gerard Hopfgartner, Dominique Soldati-Favre

**Correspondence**
dominique.soldati-favre@unige.ch

**In brief**
*Toxoplasma gondii* is known to salvage sphingolipids from its host but has retained a ceramide biosynthesis pathway. Nyonda et al. demonstrate that the parasite’s sphingolipidome differs from that of its host because of active synthesis, which is important for establishment of the intracellular niche *in vitro* and for chronic infection.
Ceramide biosynthesis is critical for establishment of the intracellular niche of *Toxoplasma gondii*

Mary Akinyi Nyonda, 1 Joachim Kloehn, 1 Piotr Sosnowski, 2 Aarti Krishnan, 1 Gaëlle Lentini, 1 Bohumil Maco, 1 Jean-Baptiste Marq, 1 J. Thomas Hannich, 3,4 Gerard Hopfgartner, 2 and Dominique Soldati-Favre 1,5,*

1Department of Microbiology and Molecular Medicine, University of Geneva, Geneva, Switzerland
2Life Sciences Mass Spectrometry, Department of Inorganic and Analytical Chemistry, University of Geneva, 24 Quai Ernest Ansermet, 1211 Geneva 4, Switzerland
3Department of Biochemistry, NCCR Chemical Biology, University of Geneva, Quai Ernest-Ansermet 30, Geneva, Switzerland
4Present address: Molecular Discovery Platform, CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, 1090 Vienna, Austria
5Lead contact
*Correspondence: dominique.soldati-favre@unige.ch

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SUMMARY

*Toxoplasma gondii* possesses sphingolipid synthesis capabilities and is equipped to salvage lipids from its host. The contribution of these two routes of lipid acquisition during parasite development is unclear. As part of a complete ceramide synthesis pathway, *T. gondii* expresses two serine palmitoyltransferases (*Tg*SPT1 and *Tg*SPT2) and a dihydroceramide desaturase. After deletion of these genes, we determine their role in parasite development in vitro and in vivo during acute and chronic infection. Detailed phenotyping through lipidomic approaches reveal a perturbed sphingolipidome in these mutants, characterized by a drastic reduction in ceramides and ceramide phosphoethanolamines but not sphingomyelins. Critically, parasites lacking *Tg*SPT1 display decreased fitness, marked by reduced growth rates and a selective defect in rhoptry discharge in the form of secretory vesicles, causing an invasion defect. Disruption of de novo ceramide synthesis modestly affects acute infection in vivo but severely reduces cyst burden in the brain of chronically infected mice.

INTRODUCTION

Lipid metabolism and acquisition are central to the parasitic lifestyle of *Toxoplasma gondii*, an obligate intravacuolar pathogen that has evolved strategies to exploit host cell lipid metabolism (Shunmugam et al., 2022). *T. gondii* is the most ubiquitous member of the Apicomplexa phylum, infecting all warm-blooded animals and prevalent in one third of the global human population (Montoya and Liesenfeld, 2004). Although infection is commonly asymptomatic, it can cause severe disease in immunocompromised individuals, including individuals with HIV and chemotherapy and organ transplant recipients, or result in miscarriage or congenital anomalies when primary infection occurs during pregnancy (McAuley, 2014; Wang et al., 2017). The genus is defined by three main clonal lineages (type I, II, and III) (Howe and Sibley, 1995; Sibley and Ajoka, 2008). Type I strain parasites, including RH tachyzoites, are acutely virulent and lethal in laboratory mice (Sibley and Ajoka, 2008). Type I strain parasites, including RH tachyzoites, are acutely virulent and lethal in laboratory mice (Sibley and Boot- hroyd, 1992), whereas type II (ME49) and type III (CTG) tachyzoites display reduced virulence and readily convert into slowly dividing encysted bradyzoites, culminating in chronic infection (Sibley and Ajoka, 2008). Infection is initiated by entry into the host cell via an active invasion process associated with secretion of proteins and membranous materials in the form of secretory vesicles, detectable as so-called empty vacuoles (evacuoles) from the apically positioned rhoptry organelles (Boothroyd and Dubremetz, 2008; Hakansson et al., 2001; Nichols et al., 1983). This is followed by prompt seclusion of the parasite in a non-phagosomal parasitophorous vacuole (PV), delimited by a PV membrane (PVM) (Jones et al., 1972; Nichols et al., 1983). The PVM is derived from the host plasma membrane and substantially remodeled by parasite and host materials (Mordue et al., 1999; Suss-Toby et al., 1996). Rhoptry organelles are unique to invasive stages of apicomplexans and have been reported to contain different classes of lipids (Berteiro et al., 2008; Foussard et al., 1996). Although the discharged membranous content of the rhoptries presumably participates in formation of the PVM (Hakansson et al., 2001), the role of lipids during invasion and intracellular survival remains to be elucidated (Joiner, 1991). Crucially, the PVM acts as a nutrient acquisition gateway to host reserves (Gold et al., 2015; Lingelbach and Joiner, 1998) and as a scaffold for the cyst wall (Ferguson and Hutchison, 1987; Scholtyseck et al., 1974).
Figure 1. Cer biosynthesis is conserved and confined to the ER in *T. gondii*
(A) Schematic of the Cer biosynthesis pathway in eukaryotic organisms, including *T. gondii*. Black arrows, steps catalyzed by enzymes characterized in this study; gray arrows, steps catalyzed by enzymes not characterized in this study. Also shown is a cartoon scheme of the putative *T. gondii* Cer biosynthesis enzymes, indicating gene IDs, length in amino acids, TM domains (TMHMM 2.0), catalytic domains in gray, and motifs and signal peptides (SP) as indicated. *TgSPT1*, green; *TgSPT2*, orange; *Tg3KDHR* and *TgCERS*, silver; *TgDES*, blue.

(B) Summary of Cer biosynthesis enzymes in Apicomplexa, indicating localization in *T. gondii* based on hyperLOPIT (Barylyuk et al., 2020) and fitness score based on a genome-wide CRISPR fitness screen (Sidik et al., 2016) (color gradient: blue, dispensable; red, essential). Also shown is conservation across the apicomplexans by BLAST search on VEuPathDB.

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T. gondii synthesizes and salvages lipids that contribute to parasite metabolism, membrane synthesis, and PVM formation (Coppen, 2006; Sonda and Hehl, 2006). Sphingolipids (SLs), which comprise relatively simple ceramides (Cers) and complex lipids such as gangliosides, are major components of eukaryotic cell membranes (Barenholz, 2004; Futerman and Hannun, 2004; van Meer et al., 2008), implicated in vital cellular processes (Obeid et al., 1993; Pushkareva et al., 1995). Cer biosynthesis occurs at the cytosolic face of the endoplasmic reticulum (ER) membrane. The four-step process is initiated by condensation of serine and acyl-coenzyme A (CoA), catalyzed by a well-conserved member of the \(\alpha\)-oxoamine synthase family, serine palmitoyltransferase (SPT) (Hanada, 2003; Hanada, 2000; Weiss and Stoffel, 1997), and terminated by a dihydroceramide (dhCer) desaturase (DES), which introduces a double bond at the C4-C5 position of dhCer to yield Cer (Futerman and Riezman, 2005; Gaut et al., 2010). Further modifications of Cer into complex SLs, including sphingomyelin (SM), take place upon vesicular or Cer transfer protein (CERT)-mediated trafficking of Cers to the Golgi apparatus and plasma membrane (Gault et al., 2010). T. gondii tachyzoites contain 11 and 10 species of Cer and SMs, respectively (Lige et al., 2011) and are enriched in ceramide phosphoethanolamines (PE-Cer) (Pratt et al., 2013; Welti et al., 2007) but restricted in SM content compared to host fibroblasts (Foussard et al., 1991a; Welti et al., 2007). There is controversy concerning detection of inositol phosphoryl Cer (IPC) in lipid extracts of T. gondii (Alqaisi et al., 2018; Pratt et al., 2013; Welti et al., 2007), which is presumably synthesized by the SL synthase (TgSLS), a functional ortholog of the yeast enzyme (Pratt et al., 2013). Radiolabeling coupled with thin-layer chromatography and application of inhibitors has pointed to active synthesis of Cer, SM, and glycosylated SLs in T. gondii (Azzouz et al., 2002). More recently, catalytic activity of a TgSPT1 and a Cer synthase (TgCERS1) has been demonstrated in vitro (Koutsogiannis et al., 2022; Mina et al., 2017). Besides the capacity to synthesize SLs, tachyzoites have also been shown to take up fluorescently labeled Cer and other SLs from exogenous sources (de Melo and de Souza, 1996). Uptake is presumably facilitated by recruitment of the host Golgi apparatus close to the PVM and re-routing of Rab14-, Rab30-, and Rab43-coated vesicles to the vacuolar space (de Melo and de Souza, 1996; Romano et al., 2013). Thus, it is assumed that T. gondii is capable of de novo synthesis of SLs while also salvaging these lipids from the host. However, to what extent each pathway contributes to the parasite lipidome remains unknown.

This study aimed to disentangle the contribution of SL salvaging versus biosynthesis during T. gondii development. To this end, parasite mutants lacking the putative SPT-coding (TgSPT1 and TgSPT2) or TgDES-coding genes were generated. The effect on the parasite’s lipidome was characterized by liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS). Lack of TgSPT1 resulted in a marked decrease in 7 short-chain dhCer species, causing a selective deficiency in secretion of rhoptry membranous content, which impeded parasite invasion. In contrast, TgSPT2 showed specificity for generation of long-chain (C44) dhCers, but its deletion caused no fitness defect in vitro or in vivo. TgDES was found to act on products of TgSPT1 and TgSPT2 as well as salvaged dhCers. Lack of TgDES had no fitness cost in tachyzoites but was associated with reduced cyst formation during chronic infection, likely because of a decrease in PE-Cer, the major output of SL synthesis in T. gondii.

RESULTS

Cer biosynthesis enzymes localize to the ER of T. gondii

In T. gondii, Cer biosynthesis occurs in 4 catalytic steps as in model organisms (Futerman and Riezman, 2005). The initial condensation of serine and acyl-CoA is catalyzed by SPTs. Mining of the T. gondii genome database (ToxoDB.org) revealed two genes, TGGT1_290980 (TgSPT1) and TGGT1_290970 (TgSPT2), predicted to code for proteins containing one putative transmembrane (TM) domain and the canonical SPT catalytic domain, an amino transferase class I/II family domain with a pyridoxal phosphate (PLP)-binding motif embedded (Figure 1A). TgSPT1 and TgSPT2 share 66% sequence identity with each other and 24.5% and 24.3% with the human SPT2 sequence, respectively (Table S1; Figure S1A). TgSPT1 and TgSPT2 possess the conserved lysine to which the co-factor PLP binds, distinguishing the regulatory from the catalytically active SPT enzyme (Harison et al., 2018; Figure S1B). For the next step in the pathway, TGME49_304470 encodes a putative 3-ketosphinganine reductase enzyme (Tg3KDHFR) that carries a short-chain dehydrogenase domain and shares 23.4% sequence identity with the human enzyme (Figures 1A and S1C; Table S1). Two genes, TGME49_283710 and TGME49_316450, encode isoforms that likely constitute the putative Cer synthases (CERS; TgCERS1 and TgCERS2), as proposed recently (Koutsogiannis et al., 2022). They share only 15% sequence identity with each other and ~19% with the human CERS1 (Figures 1A and S1D; Table S1). For the last step, TGME49_237200 encodes a putative DES composed of five putative TM domains, an SL delta-4 desaturase domain, and a delta-4 SL fatty acid (FA) desaturase-like domain. The TgDES sequence is 36.2% identical to the human DES1 (Figures 1A and S1E; Table S1), and multiple sequences alignment revealed conservation of the histidine box motifs HX(3–4)H, HX(2–3)HH, and H/OX(2–3)HH, characteristic for membrane bound desaturases and hydroxylases essential for catalytic activity (Figure S1F; Fabrias et al., 2012).

Within the Apicomplexa, the Cer biosynthesis pathway is retained in its entirety in Coccidia and Chromerida but is incomplete in Haemosporida and apparently absent in Piroplasmida and Gregarines (Figure 1B; Table S1). The genome-wide

(C) Immunofluorescence assays (IFAs) of TgSPT1, TgSPT2, and TgDES (all -Myc, magenta, 1:3,000) and the ER marker pTub8-AT1-Ty (α-Ty, green).
(D) IFA of TgSPT1-Myc (α-Myc, magenta) and Golgi reassembly stacking protein-yellow fluorescent protein (GRASP-YFP, green).
(E) IFAs with α-TgSPT1 antibody (1:100) and α-Myc (magenta) in WT RH parasites and in TgSPT1-Myc parasites. Scale bars, 2 μm.
(F) WB of parasite lysates showing TgSPT1-Myc and TgSPT2-Myc, revealed using α-Myc and α-catalase (Cat) antibodies (Ding et al., 2000) as a loading control.

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Figure 2. *TgSPT1* contributes to parasite fitness in vitro

(A) Plaque assays of *Tgsp1-ko, Tgsp2-ko, Tgsp1-ko/TgSPT1-Myc, Tgspt1-ko/Tgsp2-ko,* and *Tgdes-ko* parasites (all type I RH) 7 days after infection on confluent HFFs.

(B) Plaque size quantifications corresponding to representative images shown in (A).

(C) Plaque assays of *Tgsp1-ko, Tgsp2-ko,* and *Tgdes-ko* parasites (all type II ME49) 12 days after infection.

(D) Plaque size quantifications corresponding to representative images shown in (C).

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CRISPR-based fitness screen in *T. gondii* tachyzoites in *vitro* suggests dispensability or a modest fitness cost for all genes encoding Cer biosynthesis enzymes (Sidik et al., 2016; Figure 1B). TgSPT2 shows a 1.68-fold up-regulation of mRNA expression, whereas other transcripts in the pathway are downregulated in bradyzoites compared with tachyzoites (Figure 1B; Table S1). TySPT1, of bacterial origin, has been reported previously to be catalytically active and localized to the parasite ER (Mina et al., 2017). Hyperplexed localization of organellae proteins by isotope tagging (hyperLOPIT) indicated that the entire Cer biosynthesis pathway localizes to the ER, except TyCERS2, for which information on its localization was missing (Barylyuk et al., 2020; Figure 1B; Table S1). However, recently, TyCERS2 has also been shown to localize to the ER through epitope-tagged expression of a second copy of the gene (Koutsogiannis et al., 2022).

To substantiate the subcellular localization of Cer synthesis in *T. gondii*, TySPT1 and TySPT2 were localized by introducing a C-terminal 4-Myc epitope tag to the endogenous loci via homologous recombination (Figure S2A). Transgenic parasite clones were confirmed by genomic PCR (Figure S2B). TgDES possesses a di-lysine ER retention motif, KKAQ, unique to integral membrane proteins, at the C terminus. To avoid interference with this targeting motif, a second copy of TgDES with an N-terminal Myc epitope tag was expressed in parasites. In immunofluorescence assays (IFAs), TgSPT2-Myc and TyMPyc-DES colocalized with the previously characterized ER polytopic membrane protein acetyl-CoA transporter (AT1-Ty, expressed transiently) (Barylyuk et al., 2020; Tymoshenko et al., 2015; Figure 1C). In contrast, TgSPT1-Myc partially colocalized with AT1-Ty but showed a predominant post-nucleus localization (Figure 1C) that colocalized with a transiently expressed marker of the secretory pathway (Golgi reassembly stacking protein—yellow fluorescent protein GRASP-YFP, cis–Golgi) (Pfluger et al., 2005; Figure 1D). This unexpected localization in the secretory pathway did not affect parasite development in *vitro*, as assessed by plaque assay and measurement of plaque sizes on confluent human foreskin fibroblasts (HFFs) 7 days after infection (Figures S2C and S2D). Given the discrepancy with the previously published ER localization of TySPT1 (Mina et al., 2017), we additionally employed a primary rat polyclonal antibody raised against SPT1 (α-SPT1) (Figure 1E; Mina et al., 2017). As described by Mina et al. (2017), the antibody-mediated staining was consistent with ER localization for TySPT1 in wild-type (WT) RH parasites. In contrast, α-SPT1 antibodies confirmed the Golgi apparatus localization of SPT1 in the endogenously Myc-tagged strain. Hence, it appears that the C-terminal Myc tag alters TySPT1 localization. However, the data shown below demonstrate that lack of TySPT1 is associated with a clear phenotype affecting growth, invasion, and lipid synthesis. These defects are not observed in the Myc-tagged knockin (KI) strain and are partially or fully rescued through complementation of the Myc-tagged second copy, which also exhibits predominant Golgi apparatus localization. Thus, sufficient levels of tagged TySPT1-Myc (endogenous and second copy) appear to localize to the ER to fulfill its physiological function. Western blots (WBs) of TySPT1-Myc and TySPT2-Myc revealed migration at band sizes between 55 and 70 kDa, as predicted by their molecular weight of 63 kDa and 64 kDa, respectively (Figures 1F, S2E, and S2F). *T. gondii* possesses a complete set of putative Cer biosynthesis enzymes expressed in tachyzoites and localized to the ER.

**Lack of SPT1 impairs parasite development in *vitro***

To investigate the importance of Cer biosynthesis, the TySPT1, TySPT2, and TyDES genes were deleted in type I RH and type II ME49 by CRISPR-Cas9-mediated gene replacement with a resistance cassette (Figure S2G; Shen et al., 2014). The resulting parasite mutant clones were confirmed by genomic PCR (Figure S2H). Additionally, whole-genome sequencing (WGS) (Figure S3A) was employed to confirm successful deletion of the TySPT1 and TySPT2 loci (Figure S3B) and to screen the mutants’ genome for other modifications (Figures S3C–S3E). Deletion of TySPT1 was validated by employing the α-TgSPT1 antibodies by IFA (Figure S2I). Tgspt2-ko and Tgdes-ko mutants grew normally compared to the corresponding parental strain, as shown by similarly sized plaques formed 7 and 12 days after infection in RH and ME49, respectively (Figures 2A–2D). In contrast, Tgspt1-ko parasites in the RH and ME49 strains formed significantly smaller plaques, indicating a defect in one or more steps of the lytic cycle (Figures 2A–2D). To explore possible redundancy between TySPT1 and TySPT2, a double knockout (KO) mutant was generated in RH and validated by genomic PCR (Figure S2H) and confirmed by WGS (Figures S3A–S3E). Tgspt1-ko/Tgspt2-ko exhibited smaller plaque sizes compared with Tgspt1-ko, revealing that both enzyme contribute non-redundantly to SL synthesis (Figures 2A and 2B). To confirm that lack of TySPT1 is responsible for the observed phenotype, Tgspt1-ko RH parasites were complemented by introduction of a second copy of TySPT1 with a C-terminal 4-Myc tag (Tgspt1-ko/TgSPT1-Myc), controlled by the RON5 promoter and targeted to the UPRT locus (Suarez et al., 2019; Figure S4A). Successful integration in clonal parasites was confirmed by genomic PCR, and expression was confirmed by IFA and WB (Figures S4B–S4D). Second-copy TgSPT1-Myc displayed localization highly similar to the endogenously Myc-tagged SPT1, as assessed by relatively weak colocalization with the ER marker AT1-Ty but strong colocalization with the Golgi apparatus marker GRASP-YFP (Figure S4C). Predominant Golgi apparatus and partial ER localization of the second-copy Myc-tagged SPT1
Figure 3. TgSPT1 is essential for invasion and discharge of evacuoles

(A) Schematic of contributions of microneme proteins and rhoptry contents to steps of the parasite’s lytic cycle in a host cell preceding and including invasion (Carruthers and Sibley, 1997). Cytochalasin D (CytD) inhibits invasion and enriches evacuole formation (Hakansson et al., 2001).

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was validated by employing α-SPT1 antibodies (Figure S4C), WB analyses confirmed migration at the expected molecular weight and up-regulation of Tgspt1 in the Tgspt1-ko/TgSPT1-Myc strain compared with endogenously tagged TgSPT1-Myc parasites (Figure S4D). Higher expression levels of second-copy SPT1 were also confirmed by IFAs, comparing the fluorescence intensity in IFAs between TgSPT1-Myc and Tgspt1-ko/TgSPT1-Myc (Figures S4E and S4F). Plaques of lysis of Tgspt1-ko/TgSPT1-Myc were modestly smaller compared with the WT parental RH strain but significantly larger than Tgspt1-ko (p = 0.0001), indicating that Tgspt1 function had been partially restored (Figures 2A and 2B). Consistent with the plaque assays, no defect in intracellular growth was observed for Tgspt2-ko and Tgdes-ko in RH and ME49 parasites, whereas Tgspt1-ko grew slower, presenting more vacuoles with only 2 parasites in the RH strain (p = 0.0286) and in ME49 (p = 0.1, non-significant) and a dramatic reduction in vacuoles in 16 parasites in RH and ME49 (Figures 2E and 2F). In RH parasites, the defect of Tgspt1-ko parasites was rescued in the complemented Tgspt1-ko/TgSPT1-Myc strain (Figure 2E), whereas the double KO (Tgspt1-ko/Tgspt2-ko) presented a growth defect like Tgspt1-ko (Figure 2E). The aggravated phenotype of the double KO in the plaque assay stands in contrast to a comparable defect in intracellular growth. This might be due to the different duration of the assays, where the longer-lasting plaque assay is better suited to reveal subtle differences. The importance of each gene for parasite fitness was additionally assessed in growth competition assays by growing the mutant strains in competition with a green fluorescent protein (GFP)-expressing RH parental strain (Nyonda et al., 2020; Tosetti et al., 2019). Tgspt2-ko and Tgdes-ko parasites grew normally compared with the WT control, consistent with the plaque assay and intracellular growth assay (p = 0.1 and p > 0.999, respectively; all p values correspond to unpaired t test comparison at passage 4 with the WT control). In contrast, Tgspt1-ko parasites were rapidly out-competed by the WT RH (p < 0.0001) and ME49 control (p = 0.0023), whereas Tgspt1-ko/TgSPT1-Myc grew normally compared with the control (p = 0.2547) (Figure 2G).

Next, because it has been reported that T. gondii can take up exogenous SLs (de Melo and de Souza, 1996; Romano et al., 2013), supplementation with a downstream product of the biosynthesis pathway, sphinganine d18:0, was carried out. This allowed testing whether salvage could rescue the fitness defect observed in the Tgspt1-ko, as shown in Saccharomyces cerevisiae lacking SPT1 (ycbΔ) (Hannich et al., 2017). However, plaque assays in the presence of sphinganine d18:0 led to a reduced number of plaques in a dose-dependent manner 7 days after infection of RH and Tgspt1-ko. The few plaques formed were of the same size as those without supplementation (Figures S4G and S4H). These results point to a toxic effect of sphinganine d18:0 on extracellular parasites and an inability to overcome loss of TgSPT1 through its salvage. Presumably, the metabolite is toxic when taken up prior to the initial invasion, while its instability or metabolization through the host might protect the parasite during the later extracellular periods. KO of 3 genes encoding enzymes that catalyze the first and final step of the lytic cycle (Nyonda et al., 2020) in CytD-treated (dot at parasite tip) or untreated parasites (ring shaped), highlighted by arrowheads; scale bar, 5 μm in the top 3 rows and 1 μm in the bottom row (TgAS3P6KO + aTc).

Tgspt1-ko parasites are deficient in rhoptry “empty vacuole” discharge and invasion

Active host cell entry by T. gondii involves the sequential discharge of micronemes and rhoptries to ensure gliding motility, host cell attachment, and invasion (Carruthers and Sibley, 1997; Figure 3A). Several assays were performed to determine whether Tgspt1-ko parasites display defects in these steps of the lytic cycle.

(B) Assessment of the calcium ionophore A23187 inducing egress of RH and Tgspt1-ko parasites from human foreskin fibroblasts (HFFs) 36 h after infection. Results are means ± SD of 3 independent experiments as a percentage. Unpaired t test was applied; *p < 0.05.
(C) Gliding motility assay of RH WT and Tgspt1-ko parasites, revealing gliding trails of extracellular parasites adhered to poly-L-lysine-coated cover slips stimulated by A23187. Parasites were stained using an α-SAG1 antibody; Images are representative of three biologically independent experiments.
(D) Attachment to (E) and invasion of (F) HFFs by RH WT and Tgspt1-ko parasites. Results are means ± SD of 3 independent experiments presented as a percentage. Unpaired t test was applied; *p < 0.05.
(F) IFAs using α-RON4 (magenta), α-ROP2/3/4 (green) antibodies to visualize the neck and bulb of the parasite rhoptries, respectively; scale bars, 2 μm. Images are representative of three biologically independent experiments.
(G) Schematic of rhoptry content secretion and contribution of rhoptry neck proteins (RONs) to moving junction (MJ) formation during invasion. CytD inhibits invasion but not rhoptry content discharge.
(H) MJ formation assessed by labeling of secreted RON4 using an α-RON4 antibody (green) and parasite periphery marker α-GAP45 antibodies (magenta; Plattner et al., 2008) in CytD-treated (dot at parasite tip) or untreated parasites (ring shaped), highlighted by arrowheads; scale bar, 5 μm. Images are representative of three biologically independent experiments.
(I) Quantification of secreted RON4 at the tip of CytD-treated parasites. Results are means ± SD of 3 independent experiments. 100 parasites were counted per replicate. Unpaired t test was applied; *p < 0.05.
(J) Representative image of evacuole formation when invasion is blocked in the presence of CytD in Tgspt1-ko parasites. IFAs used α-GAP45 antibodies, a parasite pellicle marker, and α-ROP1 antibodies to assess evacuole formation (arrowheads) in RH WT, Tgspt1-ko, and the Tgspt1-ko/TgSPT1-Myc complemented strain and an TgAS3P6KO + anhydrotetracycline (+aTc) control. Images are representative of three biologically independent experiments; scale bar, 5 μm in the top 3 rows and 1 μm in the bottom row (TgAS3P6KO + aTc).
(K) Quantification of ROP1-associated evacuoles. Results are means ± SD of 3 independent experiments presented as a percentage. Unpaired t test was applied; *p < 0.05.
(L) Quantification of secretion of other rhoptry content by phospho-STAT6 assays assessing the ability of Tgspt1-ko parasites to secrete the rhoptry protein ROP16 into the host cell. TgAS3P6-IXD served as a negative control. ROP16 phosphorylates host STAT6 in the nucleus. Results are means ± SD of 3 independent experiments. Unpaired t test was applied; *p < 0.05.
(M) β-Lactamase (BLA) assay to evaluate secretion of toxofilin-BLA in Tgspt1-koToxoF-BLA parasites with coumarin and cells represented as a percentage. Results are means ± SD of 3 independent experiments. Statistical analysis, one-way ANOVA significance with Tukey’s multiple comparisons. All experiments were carried out with type I RH parasites.
cycle, aside from intracellular growth (Figure 2C). Egress is triggered by calcium fluxes and can be induced through calcium ionophores (Arrizabalaga and Boothroyd, 2004). Tgspt1-ko parasites (type I, RH) exhibited no defect in egress induced with the calcium ionophore A23187 (Figure 3B). Gliding motility was also normal, as shown by similar deposits in trails of major surface antigen 1 (SAG1) (Figure 3C) as well as attachment to HFFs (Figure 3D). Induced microneme secretion, which is involved in these three steps of the lytic cycle, was not affected (Figures S5A and S5B). In contrast, Tgspt1-ko parasites exhibited impaired invasion (p = 0.0015), which was rescued upon complementation in the Tgspt1-ko/TgSPT1-Myc strain (Figure 3E). Rhoptries, which critically participate in invasion, are partitioned into the neck region, which contains RON proteins, and the bulb, which holds ROP proteins. IFAs performed on intracellular Tgspt1-ko parasites using RON4, ROP7, and ROPP2, ROP3, and ROP4 antibodies as markers of the two rhoptry sub-compartments revealed no morphological alteration of the organelle (Figure 3F), as confirmed by electron microscopy (Figure S5C). The RON complex (RON2/4/5) is involved in formation of the portal of entry into host cells participating in moving junction (MJ) formation (Shen and Sibley, 2012; Figure 3G). To assess discharge of rhoptry proteins, secreted RON4 can be detected at the point of contact between parasite and host cells, in parasites blocked in invasion through cytochalasin D treatment (+CytD), or at the MJ in invading parasites (–CytD) (Figure 3H). Tgspt1-ko and WT parental RH parasites secreted RON4 normally upon CytD treatment, in contrast to the inducible knockdown (iKD), tet-repressible promoter of ASP3 (TgASP3-iKD), known to be defective in rhoptry content discharge (Dogga et al., 2017), that was used as a control here (Figure 3I). In sharp contrast, the discharge of rhoptry membranous materials in a form of vesicles known as evacuoles (Hakansson et al., 2001), observed in the presence of CytD using anti-ROP1 antibodies (Figure 3G), was impaired in TgASP3-iKD and Tgspt1-ko parasites (Figure 3J). The severe defect of Tgspt1-ko parasites in evacuole formation was rescued in the Tgspt1-ko/TgSPT1-Myc strain (Figures 3J and 3K).

This discrepancy between ROP1-positive evacuole and RON4 secretion assays highlights a remarkable role of the parasite’s lipid content. ROP16 secretion into the host cell leads to phosphorylation of host signal transducer and activator of transcription 3 (STAT3) and STAT6 and serves as an alternative and sensitive readout for rhoptry protein content discharge (Ong et al., 2010; Yamamoto et al., 2009). Using antibodies specific to phosphorylated STAT6 (STAT6-P), its detection in infected host nuclei confirmed the results obtained with RON4 (Figures 3L and S5D). Finally, the rhoptry protein toxofillin, fused to β-lactamase (BLA) and a hemaggulitin (HA) epitope tag, was expressed in Tgspt1-ko and in the RH parental strain to assess rhoptry discharge via fluorescence resonance energy transfer (FRET)-based BLA assay, as described previously (Corti et al., 2021; Lodoen et al., 2010). The transgenic parasites were characterized by WB and IFA to confirm expression of toxofillin BLA-HA (Figures S5E and S5F). In this assay, Tgspt1-ko showed a modest but significant defect in BLA secretion and cleavage of the substrates cephalosporin core linking B7-hydroxycoumarin to fluorescein (CCF2) in the host cytosol compared with the WT control (p = 0.005) (Figure 3M). These results uncover an unexpected evacuole assay defect hampering invasion in parasites lacking TgSPT1. It can be speculated that an altered lipidome could directly affect the integrity of the membrane forming the evacuoles.

**TgSPT1, TgSPT2, and TgDES contribute to chronic infection**

The dispensability (TgSPT2, TgDES) and modest fitness defect (TgSPT1) associated with loss of Cer biosynthesis enzymes might result from the nutrient-rich conditions in vitro, which permit uptake of exogenous SLs from the host cell or serum (de Melo and de Souza, 1996; Romano et al., 2013). The relevance of synthesis versus uptake in vivo has yet to be scrutinized. To determine the importance of Cer biosynthesis during acute infection, five C57Bl/6 mice per group were infected intraperitoneally with 100 WT RH, Tgspt1-ko, Tgspt2-ko, or Tgdes-ko tachyzoites. Mice infected with Tgspt2-ko and Tgspt1-ko were euthanized 1 and 2 days later, respectively, than the parental line, based on clinical symptoms (weight loss, ruffled fur, lack of motility, and reduced responsiveness to stimuli) (Figure 4A). Statistical analyses of survival curve patterns between mutants and controls revealed a notable difference for Tgspt1-ko (p = 0.0177) and Tgspt2-ko (p = 0.0078). In contrast, Tgdes-ko parasites exhibited equal virulence compared with the parental line (p > 0.9999) (Figure 4A).

Next, the effect of Cer biosynthesis was assessed during the chronic stage of infection using the mutant strains generated in type II ME49 parasites. The slight differences in survival patterns of Tgspt1-ko (p > 0.9999), Tgspt2-ko (p = 0.3548), and Tgdes-ko (p = 0.8889) compared to the WT ME49 control were statistically insignificant (Figure 4B). Surviving mice were sacrificed 5 weeks after infection, and brain tissue cysts were counted. The cyst burden was significantly reduced in Tgspt1-ko (median 541, p = 0.0303) and Tgdes-ko (median 580, p = 0.0303) compared with the WT control (median 1804) (Figure 4C). In contrast, although Tgspt2-ko parasites presented markedly reduced cyst formation (median 913) compared with the control (median 7406), the difference was statistically insignificant (p = 0.0556) (Figure 4C). These findings reveal an important role of Cer synthesis during chronic infection in the cyst-prone type II strain.

**T. gondii is enriched in PE-Cer derived from de novo synthesis**

To investigate the proportion of SLs in T. gondii and its host, their Cer lipid content was examined by LC-HRMS/MS. Total lipids were extracted from purified parasites or uninfected HFFs, and lipid species were separated according to their headgroups and identified based on their accurate mass. Phospholipids were the most abundant class and made up 89.9% and 92.9% of the RH and HFF total lipid content, respectively. Among the phospholipids, 66 phosphatidylethanolamine (PE) species were identified. Among the phospholipids, 66 phosphatidylethanolamine (PE) species were identified. Among the phospholipids, 66 phosphatidylethanolamine (PE) species were identified. Among the phospholipids, 66 phosphatidylethanolamine (PE) species were identified. Among the phospholipids, 66 phosphatidylethanolamine (PE) species were identified.
were detected, including 12 dhCer, 27 Cer, 33 SM, and 9 PE-Cer molecular species (Table S4).

To dissect the SL content, the sum of analyte area/internal standard area for each SL class was divided by the total sum of the analyte area/internal standard area for all SL classes and expressed as a percentage. *T. gondii* was found to be markedly enriched in PE-Cer, which made up 89.7% of all SLs in RH compared with only 0.36% in HFFs. In contrast, SMs were the most abundant SL class in HFFs with 96.8% versus 8.2% in RH (Figure 5B; Table S3). Relative Cer and dhCer levels were comparable between HFFs and parasites (Figure 5B; Table S3).

To specifically examine the synthesis and uptake capabilities of *T. gondii*, U-13C palmitate (C16:0 FA) labelling was carried out in infected and uninfected host cells, followed by purification and extraction of lipids from purified parasites and uninfected host cells. During active Cer synthesis, labeled palmitate is incorporated into palmitoyl-CoA to form 3-ketoshpinganine and can additionally be incorporated as a fatty acyl-CoA to form dhCer, depending on the carbon chain length of the dhCer species. Higher labeling in parasite lipids compared with the host indicates parasite synthesis, whereas salvaged lipids are expected to be labeled equally or less in parasites compared with the host. Examination of U-13C-palmitate labeling revealed higher...
$^{13}$C labeling in parasite PE-Cer compared with HFFs, suggesting a contribution of parasite synthesis (Figure 5C). Labeling in other SL classes was low (SMs) or pronounced (dhCers, Cers) but comparable between the host and parasite (Figure 5C).

To avoid interference of host lipid synthesis, extracellular parasites were labeled with $^{15}$N/D$_3$-serine (Figure S6A). Continued lipid synthesis in extracellular parasites has been shown previously for phosphatidylinositols (Pis) (Ren et al., 2020), whereas FA synthesis is inactive in extracellular parasites (MacRae et al., 2012). During condensation of serine and palmitoyl-CoA, one deuterium atom at the $\alpha$-carbon is lost, resulting in labeling of the SLs with $^{15}$ND$_2$ (+3 mass units) (Figures S6A–S6C; Wigger et al., 2019). In the case of lipid class separation, this creates a risk of signal interference between the labeled analog and natural-abundance isotopologs of a lipid of the same class and chain length with a difference in saturation (Figures S6C–S6E). Thus, reverse-phase LC (RPLC) was used to separate lipid sum composition by acyl side-chain length (Figure S6E). To avoid coelution of other lipid classes with a similar acyl chain, methylamine-based hydrolysis of the phospholipids and glycerolipid acyl groups was performed, separating hydrolyzed products (retention time [RT], 7–10 min) from SLs (RT, 11–20 min) (Table S5). Using this approach, only a fraction of dhCer and Cer was reliably detected. After labeling with $^{15}$N/D$_3$-serine, between 30% and 80% incorporation of labeling was observed in RH WT, Tgspt1-ko, Tgspt2-ko, and Tgdes-ko (Figure 5D; Table S3). Instead, other species, such as some Cers and SMs, were readily detected in Tgspt1-ko parasites (Figure 5D; Table S3).
### A

|              | RH | ME49 |
|--------------|----|------|
|              | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko |
| dhCer(d52:0) | 0.27* 1.39 1.81 4.41* | 0.29 1.29 2.48* |
| dhCer(d53:0) | 0.53* 2.10* 1.67 4.37* | 0.46 1.28 2.23* |
| dhCer(d54:0) | 0.10* 1.04 0.97 0.25* | 0.29 1.72 0.68 |
| dhCer(d54:1) | 0.23* 1.35 1.28 4.07* | 0.30 1.36 2.05* |
| dhCer(d55:0) | 0.41* 2.77 1.79 4.74* | 0.23 1.55 2.59* |
| dhCer(d56:0) | 0.25* 2.57 1.71 6.38* | 0.32 1.38 2.42* |
| dhCer(d56:1) | 0.30* 1.17 1.11 0.82 | 0.69 1.56 1.48 |
| dhCer(d42:1) | 2.56 2.65 1.86 0.89 | 0.86 1.23 2.05* |
| dhCer(d44:1) | 7.50 8.37 0.60 2.24* | 1.05 0.22 4.14* |

### B

|              | RH | ME49 |
|--------------|----|------|
|              | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko |
| Cer(d32:1)   | 0.52 1.22 1.14 0.39* | 0.60 1.86 0.59* |
| Cer(d32:2(0H)) | 0.90 1.34 1.08 0.27* | 0.58 1.03 0.44 |
| Cer(d34:1)   | 0.32* 1.14 0.86 0.21* | 0.38 1.64 0.30* |
| Cer(d34:2)   | 1.04 1.49 1.21 2.21* | 0.81 1.69 0.46 |
| Cer(d35:1(0H)) | 0.80 0.98 0.98 0.41* | 0.63 1.14 1.08 |
| Cer(d35:1)   | 0.66* 1.77 1.16 0.35* | 0.57 2.07 0.73 |
| Cer(d36:1)   | 0.31* 1.45 1.11 0.88 | 0.40 1.87 1.38 |
| Cer(d36:2)   | 0.22* 0.63 0.46 0.39* | 0.52 1.58 0.34* |
| Cer(d40:1)   | 2.30* 3.13* 1.72 0.30* | 0.78 1.50 1.17 |
| Cer(d41:2)   | 1.96 2.59 1.77 0.14* | 0.76 1.36 1.31 |
| Cer(d42:1)   | 2.00* 3.02 1.75 0.26* | 0.71 1.44 1.76 |
| Cer(d42:2)   | 2.52* 3.14 1.99 0.32* | 0.65 1.48 0.78 |
| Cer(d44:1)   | 5.81* 9.47 0.55 0.59 | 0.87 0.26 3.79 |
| Cer(d44:2)   | 7.77* 7.65 0.71 0.94* | 1.66 0.41 0.51 |

### C

|              | RH | ME49 |
|--------------|----|------|
|              | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko |
| PE-Cer(d32:1) | 0.34* 1.56 1.18 0.28* | 0.36 1.43 0.29* |
| PE-Cer(d33:1) | 0.60* 1.59 1.14 0.27* | 0.26 1.33 0.37* |
| PE-Cer(d34:2) | 1.11 1.43 1.33 0.59* | 0.50 1.37 0.55* |
| PE-Cer(d33:2(0H)) | 0.15* 0.95 0.83 0.20* | 0.28 1.15 0.20* |
| PE-Cer(d34:1) | 0.12 1.09 0.96 0.05* | 0.25 1.23 0.07* |
| PE-Cer(d36:3) | 0.03* 1.31 1.08 2.87* | 0.18 1.31 3.20* |
| PE-Cer(d36:2) | 0.19* 1.49 1.05 0.46* | 0.21 1.41 0.39* |
| PE-Cer(d36:1) | 0.07* 1.57 1.10 0.16* | 0.22 1.47 0.32* |

### D

|              | RH | ME49 |
|--------------|----|------|
|              | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko | spf1-ko | spf1-ko/ SPT1 | spf2-ko | des-ko |
| SM(d32:1)    | 1.94* 1.97* 1.30 0.48* | 0.86 1.15 0.99 |
| SM(d33:1)    | 2.06* 2.02* 1.29 0.51* | 1.21 1.11 1.06 |
| SM(d34:2)    | 1.78* 1.80* 0.85 0.50* | 1.41 1.27 0.94 |
| SM(d34:1)    | 2.00* 2.09* 1.26 0.43* | 1.14 1.15 0.99 |
| SM(d35:1)    | 2.06* 2.13* 1.34 0.43* | 1.35 1.17 1.09 |
| SM(d36:2)    | 1.07 1.25 0.76 0.47 | 1.27 1.26 0.97 |
| SM(d36:1)    | 2.00* 2.18* 1.30 0.45 * | 1.27 1.25 0.97 |
| SM(d38:1)    | 1.09 1.31 1.10 0.66* | 1.03 1.12 1.07 |
| SM(d40:1)    | 1.78* 1.89* 0.85 0.49* | 1.11 1.27 1.00 |
| SM(d42:2)    | 3.54* 3.68* 1.58 0.41* | 1.14 1.35 0.90 |

(legend on next page)
subtle increase in most dhCers, whereas only dhCer and Cer hinges dhCers. In sharp contrast, sites (Figure 6A; Table S3). These findings further strengthen our complemented depleted species were restored to higher than WT levels in the Tg strain, points to the existence of another DES catalyzing this reaction, whereas absence of the other labeled Cer species suggests inactivity of TgDES in extracellular parasites. Labelling was insignificant (below 10%) in all SM species in the examined parasite strains, providing evidence that SMs are largely derived from salvage (Figure S6B; Table S3).

**T. gondii Cer biosynthesis mutants display distinct SL profiles**

Next, the effect of deletion of Cer biosynthesis genes on the parasite SL content was investigated. Lipids extracted from type I (RH, Tgspt1-ko, Tgspt2-ko, Tgspt1-ko/TgSPT1-Myc, and Tgdes-ko; all type I RH) and type II (ME49, Tgspt1-ko, Tgspt2-ko, and Tgdes-ko; all type II ME49) parasites were measured and compared. The abundance of each SL species was measured, normalized to a standard of the respective SL class, and expressed relative to the level in the corresponding control strain (RH or ME49 WT; abundance = 1). A marked and significant (all p < 0.0004) decrease in nearly all dhCer species was observed in Tgspt1-ko parasites (RH and ME49) compared with the WT control, consistent with the observed lack of synthesis of these species in extracellular Tgspt1-ko parasites, as described above (Figure 6A; Table S3). A decrease in these species was accompanied by statistically significant upregulation of a couple of long-chain species (dhCer(d42:1, p = 0.0003; d44:1, p < 0.0001) (Figure 6A; Table S3). These long-chain species likely compensate for the drop in other dhCers and must be derived from salvage or synthesis through TgSPT2. The levels of depleted species were restored to higher than WT levels in the complemented Tgspt1-ko/TgSPT1-Myc strain in type I RH parasites (Figure 6A; Table S3). These findings further strengthen our hypothesis that TgSPT1 is critical for formation of various prominent dhCers. In sharp contrast, Tgspt2-ko mutants display a subtle increase in most dhCers, whereas only dhCer and Cer of 44-carbon chain length were decreased in RH and ME49 parasites (Figure 6A; Table S3). These findings indicate that both SPTS are active, with TgSPT1 generating the majority of dhCer (32–36 carbon chains), whereas dhCer42:1 is likely salvaged (unaltered in Tgspt1-ko and Tgspt2-ko), and dhCers with 44-carbon FA chain length appear to be synthesized by TgSPT2.

Deletion of TgDES in both strains (RH and ME49) led to significant accumulation of dhCer in nearly all species synthesized by TgSPT1 and TgSPT2 (Figure 6A; Table S3). The levels of other synthesized dhCers (dhCer(d34:0(2OH), dhCer(d36:0(2OH)), dhCer(d36:1), dhCer(d40:0), and dhCer(d42:0)) remained unchanged or were lower in Tgdes-ko parasites (Table S3) compared with the control, which may point to the existence of a second unidentified DES gene in T. gondii, as hypothesized above.

For several SL species, T. gondii SPTS and DES appear to function in a concerted action, as indicated by a drop in a dhCer species in Tgspt1-ko and its accumulation in Tgdes-ko, concomitant with a drop in the respective Cer species in both strains. Other commonly detected Cer species (in particular Cer(d32:2(2OH)), Cer(d34:2), Cer(d35:1(2OH)), Cer(d40:1), Cer(d41:2), Cer(d42:1), and Cer(d42:2)) were not products of TgSPT1 or TgSPT2 activity, based on their unaltered abundance in the respective KO strains but decreased abundance in the absence of TgDES. This suggests uptake of the precursor dhCer species, which is subsequently desaturated by TgDES (Figure 6B; Table S3), highlighting a complex mode of acquisition of Cers through de novo synthesis as well as uptake of dhCer precursors or Cer species from host sources. A significant drop in almost all PE-Cer species (all p < 0.003) was observed upon deletion of TgSPT1 and TgDES, whereas TgSPT2 does not appear to contribute to PE-Cer synthesis (Figure 6C; Table S3). PE-Cer(d33:2(2OH)) may also be a product of synthesis even though the precursor Cer was not detected in any of our analyses. PE-Cer(d34:2) levels were unaltered in Tgspt1-ko parasites and approximately halved in Tgdes-ko parasites compared with WT parasites. Cer(d34:2) and PE-Cer(d34:2) were detectable in HFFs (Figure 6D; Table S3), suggesting possible uptake of its precursor or this species directly by these mutants. The SM levels remained unchanged in most of the mutants except in Tgdes-ko parasites, which exhibited a reduction of all species detected (Figure 6D; Table S3). This points to predominant salvage of SMs, which is consistent with the stable isotope labeling data. This was validated by generating parasites lacking TgSLS (TGME49_246290) and a putative SM synthase (TgSMs, TGME49_247360), orthologs of the *Plasmodium falciparum* SMS. Lipidomics analyses of these T. gondii mutants did not reveal any decrease in parasite SM levels (Table S3), consistent with the putative inactivity of these enzymes. Tgspt1-ko, Tgspt2-ko, and Tgdes-ko parasites display a significantly altered lipidome, highlighting a vital role of these enzymes and a considerable contribution of de novo SL synthesis in T. gondii, which is complemented by uptake of species that can be further processed into more complex SLs.

**DISCUSSION**

Cer biosynthesis has only been retained in part during adaptation to parasitism across the phylum of Apicomplexa. In T. gondii...
from other organisms (Fabrias et al., 2012). The phenotype investigation.

leading to impairment in physical properties of the membranous branes in discharge of rhoptry proteins, as measured by several independent modes of SL acquisition and host niches occupied.

The comprehensive lipidomics analysis performed on T. gondii Cer biosynthesis mutants revealed striking differences in the products of TgSPT1 and TgSPT2. This suggests use of distinct acyl-CoA substrates of different chain lengths by these two enzymes, resulting in increased diversity of formed Cers. Besides the most abundant FAs (C16:0, C18:0), the parasite also contains considerable levels of odd-chain FAs (C17:0) and unusually long monounsaturated FAs, including C20:1, C26:1, and C28:1 (Kloehn et al., 2020; Ramakrishnan et al., 2012, 2015). Although SPTs typically show a preference for palmitoyl-CoA (C16-CoA), some isoenzymes have a distinct affinity for different saturated or unsaturated acyl-CoA substrates ranging from C12–C20 (Han et al., 2009). CERSs also exhibit specificity for fatty acyl-CoAs of different chain lengths (Mullen et al., 2012). T. gondii encodes two putative CERS (CERS1, TMG49_316450 and CERS2, TMG49_283710) that may contribute further to this diversity by distinct amide linkage of the short- or long-chain FAs to the different long-chain bases (LCBs) synthesized by TgSPTs. A recent study demonstrated that TgCERS1 utilizes C16:0 acyl-CoA and sphinganine as substrates, whereas TgCERS2 did not exhibit any activity in vitro with the tested substrates (Koutsogiannis et al., 2022). However, it appears that a broad range of substrate specificity of TgSPTs and/or TgCERSs is needed to generate the parasite’s diverse spectrum of Cers.

In Tgsp1-ko parasites, several dhCers were drastically reduced. This mutant exhibits a significant invasion defect and selective impairment in evacuole formation, whereas the discharge of rhoptry proteins, as measured by several independent assays, was not significantly affected. Evacuole membranes in Plasmodium species and T. gondii are observed as multilamellae sheets (Bannister et al., 1986; Hakansson et al., 2001; Stewart et al., 1986). The reduction of seven dhCers species results in a clear defect in release of membranous materials from rhoptries, suggesting that these lipids are critical for generation of these membranes, either directly as building blocks or through a signaling role. The content of the evacuoles and parasite-derived lipids presumably participates in formation of the nascent PVM, allowing extension of this membrane to form a replication-permissive niche (Hakansson et al., 2001). The rhoptry organelles contain cholesterol and are enriched in choline-containing phospholipids, including SM (Besteiro et al., 2008; Foussard et al., 1991b). Depletion of cholesterol does not impede evacuole formation (Besteiro et al., 2008) or the invasion process (Besteiro et al., 2008; Coppens and Joiner, 2003). Plasmodium-derived membranes have been proposed to contribute to the energetic portion during invasion (Dasqupta et al., 2014). In consequence, the invasion defect of Tgsp1-ko might be due to the distinct SL perturbations presumably leading to impairment in physical properties of the membranous material and affecting PVM formation, which warrants further investigation.

The last enzyme in Cer synthesis, TgDES, resembles DES1 from other organisms (Fabrias et al., 2012). The phenotype observed in parasites lacking TgDES aligns with studies showing that inhibited human DES1 functions using small interfering RNA (siRNA), resulting in accumulation of endogenous dhCers (Kraveka et al., 2007). Accumulation of dhCers has been implicated in a broad range of biological processes, including inhibition of cell growth and cell cycle arrest (Gagliostro et al., 2012; Kraveka et al., 2007). However, we did not observe any growth defect in Tgdes-ko parasites, and accumulation of dhCer was not toxic to the rapidly dividing tachyzoite forms in vitro and in vivo. Synthesis of some Cers was still observed in Tgdes-ko, pointing to the existence of a potential second unidentified enzyme. The large superfamily of membrane-bound desaturases is defined by the presence of the catalytic active motifs referred to as histidine boxes and act on several substrates, including FAs and lipids (Shanklin and Cahoon, 1998). These desaturases exist as paralogs with desaturation or hydroxylation activity on sphingoid bases, but bifunctionality has been described for murine DES1 (Spering et al., 2003). Bioinformatics searches using the histidine sequence motif on ToxoDB yielded 18 genes, including the investigated TgDES, of which 9 encode hypothetical proteins with no data available on localization from the hyperLOPIT screen (Table S1, sheet 3; Barylyuk et al., 2020). Whether any of these proteins have DES activity awaits further experimental investigation.

PE-Cer are scarce in the host but enriched in the parasite, as observed in this study and by others (Welti et al., 2007), necessitating parasite de novo synthesis as demonstrated here. PE-Cer are likely the product of a PE-Cer synthase (TgCEPS, TMG49_276190), a homolog of the Drosophila melanogaster enzyme (Gene ID: FBgn0025335; Vacaru et al., 2013). Compared with the other enzymes in the pathway, TgCEPS has a strikingly low fitness score (−3.94) in the CRISPR genome-wide screen (Sidik et al., 2016), highlighting an important role of these unusually abundant lipids. Although a dramatic reduction in PE-Cer was observed in parasites lacking TgSPT1 or TgDES, residual PE-Cer indicate that low levels of dhCers and Cers can be salvaged and utilized as substrate by TgCEPS. This residual PE-Cer synthesis might be vital, given the expected essentiality of TgCEPS.

The perturbations in lipid content in the Cer biosynthesis mutants have important consequences for the biology of the parasites. Depletion of dhCers observed in Tgsp1-ko and Tgsp2-ko led to moderate attenuation of acute virulence. Instead, the decrease of Cer and PE-Cer in Tgsp1-ko and Tgdes-ko affects the chronic stage of infection, causing a decrease in cyst burden. Relevance of Cer and PE-Cer synthesis for chronic-stage development arises when salvage might be inefficient, given the cyst wall barrier and the large size of the cysts. Preferred uptake in tachyzoites versus reliance on de novo synthesis in encysted bradyzoites was also observed for acquisition of other metabolites, such as pantothenate (vitamin B5) (Lunghi et al., 2022).

Our results uncover a remarkable plasticity of T. gondii for SL acquisition, ranging from de novo synthesis to salvage of SLs, including uptake and processing of salvaged intermediates. This plasticity likely contributes to the adaptability of T. gondii to a broad host cell spectrum that is unmatched by other apicomplexans.
Limitations of the study
It remains unclear how the sphingolipidome affects evacuole formation. This could be partially addressed by determining the lipid content of rhoptries in WT and Tgspf1-ko parasites. However, purification of sufficient and pure rhoptry material is highly challenging. Similarly, it remains ambiguous why Cer synthesis is relevant for encysted bradyzoites. Lipidomics studies of this stage in vivo are currently not feasible, given the low yield of parasites and the difficulty of purification. Last, the role of several enzymes in SL synthesis remains to be investigated. Of particular interest is TgCEPS because of its assumed essentiality. The selective effect of some enzymes during the chronic stage of infection deserves to be addressed genetically in a stage-specific fashion.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit anti-GAP45   | Plattner et al., 2008 | N/A |
| Rabbit anti-Catalase| Ding et al., 2000 | N/A |
| Mouse anti-TY       | Clone BB2 | N/A |
| Rabbit anti-HA      | Sigma-Aldrich | Cat# H6908; RRID:AB_260070 |
| Mouse anti-RON4     | kind gift from Dr. Maryse Lebrun | N/A |
| Rabbit anti-RON4    | kind gift from Dr. Maryse Lebrun | N/A |
| Mouse anti-ROP1     | kind gift from Dr. JF. Dubremetz | N/A |
| Mouse anti-ROP7     | kind gift from Dr. JF. Dubremetz | N/A |
| Mouse anti-ROP2,3,4 | kind gift from Dr. JF. Dubremetz | N/A |
| Mouse anti-MIC2     | T34A1, Achbarou et al., 1991 | N/A |
| Mouse anti-MYC      | mAb9E10 | N/A |
| Mouse anti-GRA3     | kind gift from Dr. JF. Dubremetz | N/A |
| Rabbit anti-STAT6-P | Cells signaling | Cat# 56554S; RRID:AB_2799514 |
| Mouse anti-SAG1     | T4-1E5 | N/A |
| Rat anti-TgSPT1     | Kindly provided by Dr. Paul W. Denny, Mina et al., 2017 | NA |
| Mouse-HRP, horseradish peroxidase | Sigma-Aldrich | Cat# A5278; RRID:AB_258232 |
| Rabbit-HRP, horseradish peroxidase | Sigma-Aldrich | Cat# A8275; RRID:AB_258382 |
| Alexa-488/ Alexa-594 | Invitrogen |          |
| Alexa-Fluor-680     | Invitrogen | Cat# A21057; RRID:AB_2535723 |
| **Bacterial and virus strains** |        |            |
| **Escherichia coli** XL-10 Gold | Stratagene | Cat# 200314 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| Pyrimethamine       | Sigma-Aldrich | Cat# P7771 |
| D-erythro-sphinganine sphinganine (d18:0)) | Avanti® Polar Lipids | Cat# 860498P |
| Mycophenolic Acid   | Sigma-Aldrich | Cat# M5255 |
| Bovine Serum Albumin (BSA) | Sigma-Aldrich | Cat# A7030 |
| CCF4-AM             | ThermoFisher Scientific | Cat# K1029 |
| Xanthine            | Sigma-Aldrich | Cat# x0626 |
| Fluromount-G®       | Southern Biotech | Cat# 0100-01 |
| Gentamicin          | Gibco | Cat# 15750-045 |
| 5-Fluoro-2'-deoxyuridine (FUDR) | Sigma-Aldrich | Cat# F0503 |
| DAPI                | Southern Biotech | Cat# 0100-20 |
| Crystal violet      | Sigma-Aldrich | Cat# C3886 |
| Amersham Nitrocellulose | GE Healthcare | Cat# 10600003 |
| Restriction enzymes | New England Biolabs | N/A |
| U-13C palmitic acid | Sigma-Aldrich | Cat# 605573 |
| L-serine (2,3,3-D3, 98%, 15N 98%) | Cambridge Stable Isotope Laboratories | Cat# DNL-6863-PK |
| **Critical commercial assays** |        |            |
| Q5 Mutagenesis Kit  | New England Biolabs | Cat# E0552-5 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| KOD DNA Polymerase  | Novagen| Cat# 71085-3|
| GoTaq DNA polymerase| Promega| Cat# M7848 |
| Pangea HF polymerase| Canvax | Cat# P0033 |
| Wizard SV genomic DNA purification | Promega | Cat# A2361 |
| Gibson Assembly Cloning Kit (NEB Builder) | New England Biolabs | Cat# M5520AA |
| DNA purification Mini | EurogenTec | Cat# SK-PLPU-100 |
| NucleoBond Xtra Midi | Macherey Nagel | Cat# 740410 |
| Amersham ECL Prime | GE Healthcare | Cat# RPN2232 |

Deposited data

Lipidomics LC-MS/MS quantification This study Database: Yareta Archive Portal: https://doi.org/10.26037/yareta:jy7xnljd6vdv5dl4zqtvly2oj4

Experimental models: Cell lines

| Human foreskin fibroblasts | ATCC | SCRC-1041 |

Experimental models: Organisms/strains

| C57BL/6mice, female, 6 weeks old | Charles River | GE41-15 and GE150-16 |
| T. gondii: Strain RH/ΔHX/ΔKU80 | ATCC | Cat# 50174 |
| T. gondii: Strain ME49/ΔHX/ΔKU80 | ATCC | Cat# 50611 |
| T. gondii: Strain RHsp1t-ko | This study | N/A |
| T. gondii: Strain RHsp2t-ko | This study | N/A |
| T. gondii: Strain RHsp1t-ko/sp2t-ko | This study | N/A |
| T. gondii: Strain RHsp1t-ko/SPT1-myc | This study | N/A |
| T. gondii: Strain RHdes-ko | This study | N/A |
| T. gondii: Strain RHtoxofilin-Blam-HA | This study | N/A |
| T. gondii: Strain RHsp1t-ko-toxofilin-Blam-HA | This study | N/A |
| T. gondii: Strain RHsp1t-myc | This study | N/A |
| T. gondii: Strain RHsp2t-myc | This study | N/A |
| T. gondii: Strain ME49sp1t-ko | This study | N/A |
| T. gondii: Strain ME49sp2t-ko | This study | N/A |
| T. gondii: Strain ME49des-ko | This study | N/A |

Oligonucleotides

For primers and oligonucleotides, see Table S2

Recombinant DNA

| Ct-SPT1-4Myc_HXGPRT (Plasmid) | This study | N/A |
| SPT1_gRNA_Crispr/Cas9 (Plasmid) | This study | N/A |
| pSAG1::CAS9-GFP-U6::sgGOI(#gRNA.1/gRNA.2) (Plasmid) | This study | N/A |
| TgSPT1-HXGPRT.1 (Plasmid) | This study | N/A |
| UPRT::SPT1 (Plasmid) | This study | N/A |
| Ct-SPT2-4Myc_HXGPRT (Plasmid) | This study | N/A |
| SPT2_gRNA_Crispr/Cas9 (Plasmid) | This study | N/A |
| TgSPT2-HXGPRT.1 (Plasmid) | This study | N/A |
| TgSPT2-DHFR.1 (Plasmid) | This study | N/A |
| pTub8-Myc-DES-HXGPRT (Plasmid) | This study | N/A |
| DES_gRNA_Crispr/Cas9 (Plasmid) | This study | N/A |
| TgDES-HXGPRT.1 (Plasmid) | This study | N/A |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dominique Soldati-Favre (dominique.soldati-favre@unige.ch).

Materials availability
Toxoplasma gondii transgenic strains and unique reagents including plasmids are available upon request from the lead contact without restrictions.

Data and code availability
- Lipidomics data was archived and is accessible under the Yareta Archive Portal under the name "LC-HRMS lipidomics analysis of Toxoplasma gondii samples" or following the link: https://doi.org/10.26037/yareta:jy7xnljd6vdv5dl4zqtvly2oj4. All data is available from the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Toxoplasma gondii strains
The tachyzoite forms of the parental (RHΔku80Δhxgprt, ME49Δku80Δhxgprt) and derived modified strains of T. gondii were propagated on confluent human foreskin fibroblast (HFF-1 from ATCC SCRC®-1041) using Dulbecco’s Modified Eagles Medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS), 25 mg/mL gentamicin and 2 mM glutamine in incubators at 37°C and 5% CO₂.

Host cell lines
HFF-1 from ATCC SCRC®-1041 were cultivated in ATCC-formulated DMEM medium (Cat#30–2002) supplemented with 10% FCS in incubators at 37°C and 5% CO₂.

Bacteria
Escherichia coli XL-10 Gold chemo-competent bacteria were used to carry out all recombinant DNA experiments and were maintained in liquid or on solid LB agar plates. Transformed bacteria carrying ampicillin resistance genes were cultured in presence of 100 µg/mL ampicillin.
Animals
Female, 6-weeks old C57BL/6 mice from Charles River Laboratories were used for all experiments. Mice were housed in clean filter-top cages with 12-h day/night cycle and free access to food (standard chow diet) and water. The well-being of infected mice was monitored daily. Animal experiments were conducted under the authorization number GE150-16 and GE411-15 according to the guidelines and regulations issued by the Swiss Federal Veterinary Office. No human samples were used for these experiments.

METHOD DETAILS

DNA cloning and generation of constructs
The Wizard SV genomic DNA purification system (Promega) was used to extract genomic DNA (gDNA) from all strains.

Gene tagging
The 3’-end of TgSPT1 and TgSPT2 genes of accession number TGME49_290980 and TGME49_290970, respectively, on ToxoDB.org were amplified using primers 7411/7412 and 7409/7410 which contain either ApaI (7411, 7409) or NsiI (7412, 7410) restriction sites for TgSPT1 and TgSPT2 respectively. The purified PCR product was digested with appropriate enzymes and cloned into a pT8-TgMIC13-4Myc-HXGPRT plasmid (Friedrich et al., 2010) between ApaI and NsiI to obtain Ct-TgSPT1-Myc-HXGPRT and Ct-TgSPT2-Myc-HXGPRT. The clones were confirmed using primers sets 7652/3980 and 7650/3980 for Ct-TgSPT1-Myc-HXGPRT and Ct-TgSPT2-Myc-HXGPRT respectively. All oligonucleotide primers sequences used for gene tagging are listed in (Table S2).

DES second copy expression construct
Primers 8376/8377 containing sites NsiI and PacI respectively were used for Thermococcus kodakaraensis (KOD, ultrahigh fidelity DNA polymerase) PCR (Novagen) on cDNA of RH. The product was ligated to plasmid pT8-Myc-GFP-PfMyotail-Ty-HXGPRT (Jacot et al., 2013; Santos et al., 2011) to generate pT8-Myc-DES-HXGPRT of which 40 μg was transfected into RH parasites. All oligonucleotide primers sequences used for gene tagging are listed in (Table S2).

CRISPR cas9 and gRNA plasmid mediated gene deletions
CRISPR/Cas9 directed KO strains were produced using pSAG1-CAS9-GFP-U6::sgUPRT plasmid (Shen et al., 2014) specific dgRNA plasmids were produced by PCR on the CAS9 template using primers sets (4883/7436, 4883/7437) for TgSPT1, (4883/7432, 4883/7433) for TgSPT2 and (4883/7373, 4883/7374) for TgDES. Amplification was achieved using the Q5 site directed mutagenesis kit (NEB) according to manufacturer’s instructions. Then, a fragment of pSAG1::CAS9-GFP-U6::sgSPT1 (#7437), pSAG1::CAS9-GFP-U6::sgSPT2 (#7433) and pSAG1::CAS9-GFP-U6::sgDES (#7374) containing the specific sgRNA sequence was amplified using the primers 6147/6148 and sub-cloned into pSAG1::CAS9-GFP-U6::sgSPT1(#7436), pSAG1::CAS9-GFP-U6::sgSPT2 (#7432) and pSAG1::CAS9-GFP-U6::sgDES (#7373) between the KpnI and Xhol restriction sites. KOD DNA polymerase (Novagen) was used to amplify HXGPRT cassettes with primer sets 7438/7439, 7434/7435 and 7375/7376 respectively for TgSPT1, TgSPT2 and TgDES genes. These primers carry 3’ and 5’ homology sequences to genes of interest, 30 base pairs long each, downstream of the gRNA sequence and just upstream of the stop codon, respectively. The KOD PCR product was precipitated using sodium acetate and ethanol, re-suspended in 100 μL of water prior to co-transfection with 30 μg of the gRNA. To generate the TgSpt1-kotgsp2-ko, KOD PCR to amplify DHFR cassette with primer set (9001/9002) for TgSPT2 gene and transfected in the spt1-ko strain. Integration of the HXGPRT selection cassette was examined by GOtaq PCR (Promega) using primers sets (7497/5360, 7508/5370) for TgSPT1, (7495/5369, 7496/5370) for TgSPT2 and (7440/5369, 7441/5370) for TgDES. Integration of DHFR cassette in spt1-ko/spt2-ko was analyzed using (7495/2017, 7496/2018) for SPT2. Loss of the exons of interest was examined using primer sets (7497/7508) for TgSPT1, (7496/7497) for TgSPT2 and (7440/7441) for TgDES. All oligonucleotide primers sequences used for gene KO are listed in (Table S2).

Complementation of spt1-ko
Full length TgSPT1 gDNA was amplified by KOD PCR using primer pair 7513/7514 which have PacI and EcoRV restriction sites respectively. The purified product was cloned into plasmid pUPRT-promRON5-G13-4Myc between PacI and EcoRV sites to generate pUPRT-promRON5-SPT1-4Myc. This plasmid was co-transfected with pgRNA-UPRT-Cas9-/CRISPR a donation from Maryse Lebrun, followed by selection using FUDR. Integration of TgSPT1 sequence was confirmed by PCR primer pairs 8656/7652 and modification to the UPRT locus with primer pair 8655/8656. All oligonucleotide primers sequences used for gene tagging are listed in (Table S2).

Parasite transfection and selection of stable transgenic parasites
Transfections on tachyzoite forms of T. gondii were executed by electroporation as previously described (Soldati and Boothroyd, 1993). Transfectants were progressively put through selective pressure using mycophenolic acid and xanthine for HXGPRT selection (Donald et al., 1996), pyrimethamine (Pyr) for DHFR selection (Donald and Roos, 1993) or 5-fluorodeoxyuridine (FUDR) (Donald and...
Roos, 1995). All strains stably expressing the drug selection markers were cloned by limited dilution in 96-well plates. PCR and IFAs were performed on single clone transgenic parasites to confirm genomic integration of constructs and expression of integrated DNA.

**Genome sequencing**

**High molecular weight DNA extraction**

Freshly egressed parasites from RH_Jku80, Tgspt1-ko, Tgspt2-ko and Tgspt1-ko/Tgspt2-ko were purified using a 3 μm polycarbonate membrane filter (Millipore) and washed twice in phosphate buffered saline (PBS). The pellets containing 10^8 to 10^9 parasites were kept at −80°C until DNA extraction. High molecular weight DNA (HMW DNA) was obtained using the Nanobind CBB Big DNA kit (Circulomics) following the manufacturer’s instruction. DNA quality control and quantification were determined via Nanodrop (Thermo Scientific) and Qubit dsDNA broad range assay kit (Thermo Scientific).

**Library preparation and ONT sequencing**

DNA libraries were prepared using 500 ng to 2.8 μg of HMW DNA according to the manufacturer’s Nanopore Protocol. Rapid Sequencing (SQK-RAD004) (Oxford Nanopore technologies). Each strain was whole-genome sequenced on one flow cell (FLO-MIN106, R.9.4.1 chemistry) (Oxford Nanopore Technologies) for 16 h using a MiniON Mk-1c device and the MinKNOW operating software (v21.11.7) (Oxford Nanopore Technologies, Oxford, UK).

**Qualitative analysis of genome sequencing**

Sequencing data were base called using Guppy v.5.1.13. The analysis of the fastq pass files was performed using the web-based platform VEuPathDB Galaxy (https://veupathdb.globusgenomics.org/). Input reads were aligned to the reference genome ToxoDB-29_TgondiiGT1 with minimap2 (v.2.20) using the preset options PacBio/Oxford Nanopore reads to reference mapping (-Hk19) (Li and Durbin, 2010). BAM files were converted into a BigWig format for display in VEuPathDB genome browser as a custom track. Gap in alignments were identified from scanning the track.

**Lysis plaque assay**

Confluent monolayers of HFFs were infected with approximately 50 freshly egressed parasites and allowed to sit for 7 days or 12 to 14 days for type I and type II strains respectively. The infected cells were then fixed using 4% paraformaldehyde/0.05% glutaraldehyde (PFA/GA) and plaque sizes revealed by staining with 0.1% crystal violet (Sigma).

**Sphinganine d18:0 supplementation**

D-erythro-sphinganine powder (sphinganine (d18:0)) product number 860498P was purchased from Avanti Polar Lipids and dissolved in ethanol and then conjugated to FA free bovine serum albumin (BSA) catalogue number A7030 from Sigma to make a 20 mM stock solution. For supplementation assay, the stock solution was diluted to 1 μM and 3 μM sphinganine d18:0 concentrations in DMEM and plaque assays conducted.

**Intracellular growth assay**

Confluent monolayer of HFFs seeded on coverslips were infected with WT RH (type I) parasites and its respective mutants or WT ME49 parasites (type II) and its respective mutants and left to grow for 24 h (type I) or 40 h (type II). IFAs were performed as outlined below (indirect immunofluorescence assay) using αGAP45 antibody (1:10,000) to stain the parasite pellicle and parasites per vacuole were counted across several fields of vision using a Nikon eclipse Ti microscope. Experiments were repeated three independent times, 100 vacuoles counted for each replicate; data is presented as mean ± standard deviation.

**Indirect immunofluorescence assay**

Confluent monolayer of HFFs seeded on coverslips were infected. The infected cells were then fixed with PFA/GA for 10 min or 20 min for GRAs PV and PVM localization followed by a quenching step in 0.1 M glycine/PBS. Infected cells were then permeabilized with 0.2% Triton X-100/PBS (PBS/Triton) succeeded by a blocking step with 3% BSA in PBS. An incubation step for 1 h with primary antibodies diluted in 1% BSA/PBS was carried out followed by (3 × 5 min) PBS washes. Next, coverslips were incubated for 1 h with the secondary antibodies described above diluted in 1% BSA/PBS solution. Parasite and host cell nuclei were then stained by incubation in DAPI (dilution 1:1,000, 4’,6-diamidino-2-phenylindole; 50 μg/mL in PBS) for 7 min. Final (3 × 5 min) PBS washes preceded mounting of coverslips on slides using Fluoromount G (Southern Biotech) and slides stored at 4°C in the dark. Confocal images were taken using Zeiss microscopes (LSM700 or LSM800 Airyscan objective apochromat 63x/1.4 oil) found at the Bioimaging core facility of the Faculty of Medicine, University of Geneva. Z-stack sections were processed using the ImageJ software.

**Evacuole assay**

Discharge of rhoptry contents was assessed by evacuole detection assay as described before (Hakansson et al., 2001). Freshly egressed parasites were incubated on ice in serum free DMEM media containing 1 μM CytD for 10 min. The parasites were then added to pre chilled HFFs monolayer with serum free DMEM media with 1 μM CytD, centrifuged at 2000g for 30 sec, allowed to attach to host cells on ice for 20 min. The cells were washed with ice cold 1X PBS, then media was replaced with complete DMEM with or without 1 μM CytD and incubated in 37°C water bath for 20 min. The cells were the fixed for 15 min PFA, and IFAs performed using
α-ROP1 antibody, (dilution 1:10) as a rhoptry bulb marker to identify evacuoles and α-GAP45 antibody (dilution 1:10,000) to stain the parasites.

To distinctly assess the discharge of RON4, the same procedure as above was used and then the cells were permeabilized with 0.1% saponin. IFAs were performed using α-RON4 (dilution 1:10) and α-GAP45 antibodies (dilution 1:10,000) in 3% BSA/PBS. 100 parasites per experiment were counted and RON4 secretion quantified based on the presence of staining at the apical tip of the parasite. Results are a mean ± standard deviation of three independent biological replicates.

**STAT6-P base rhoptry secretion assay**

2 x 10^6 parasites/ml of freshly egressed parasites were resuspended in cold DMEM without serum. 250 μL of the parasite suspension were added to pre-chilled confluent HFFs on coverslips with DMEM without serum. The parasites were left to settle and attached by centrifugation at 1100 g for 30 s and then incubated on ice for 20 min. The wells containing parasites were subsequently incubated in water bath at 37°C for 20 min. As negative control, 250 μL of dimethyl sulfoxide (DMSO) was added to 2 wells and incubated at 37°C for 15 min. Both DMSO and parasites treated wells were fixed with ice-cold methanol for 8 min at -20°C, the methanol was removed and 1xPBS added to the wells. Subsequent IFAs were carried out as follows: blocking step with 3% BSA/PBS for 30 min followed by overnight incubation with STAT6-P antibodies (dilution 1:400). The wells were washed 3x using PBS and subsequent IFA steps followed as described in the STAR Methods. >200 host nuclei were counted for each replicate.

**Toxofilin β-lactamase based rhoptry secretion assay**

CRISPR/Cas9 directed endogenous C-terminus tagging of Toxofilin gene was done using pSAG1-CAS9-GFP-U6:sgUPRT plasmid (Shen et al., 2014) to produce specific gRNA plasmid by PCR on the CAS9 template using primers 4883 and 8833. Primers 8834 and 8835 bearing 30 bp homology to the 5' and 3' end of the C-terminus of toxofilin gene were used to perform KOD PCR on the vector SP3-Toxofilin-BLA-HA a kind donation from Dr. Lodoen MB (Lodoen et al., 2010). The secretion of Toxofilin protein by RH and Tgspt1-ko parasites was assessed by co-transfecting the two plasmids in RH and Tgspt1-ko-Transgenic parasites were selected by flow cytometry based on GFP expression. All oligonucleotide primers sequences used for gene KOs are listed in (Table S2).

Analysis of rhoptry protein secretion into the host cells was assessed by flow cytometry as previously described (Lodoen et al., 2010). Briefly, HFF monolayers were infected with extracellular parasites from RH-Toxofilin-BLA-HA and Tgspt1-ko-Toxofilin-BLA-HA-parasites at a multiplicity of infection (MOI) of 30. After one hour, cells were washed and incubated with the BLA substrate CCF4-AM (K1029, ThermoFisher Scientific) or DMSO (control) for two hours in the dark at room temperature. Cells were washed 3 times with PBS, trypsinized and analyzed by flow cytometry on a Gallios flow cytometer (Beckman Coulter) at the Flow Cytometry cell reports platform (University of Geneva, Switzerland). Samples were excited at 405 nm and coumarin and fluorescein were detected with the 450/50nm laser and the 550/40 nm laser respectively. FlowJo (Becton, Dickinson & Company) and Kaluza (Beckman Coulter) software was used for analysis. Data represents mean ± SD of 3 independent assays. Statistical significance was assessed by a paired t test.

**Induced egress assay**

Freshly egressed parasites were inoculated on confluent HFF monolayers and grown for 30 h at 37°C. Parasite egress was then stimulated as follows; infected cover slips were washed with serum-free medium followed by incubation with serum-free medium containing 3 μM of the Ca²⁺ ionophore A23187 from Streptomyces chartreusensis (Calbiochem) or DMSO as a control at 37°C for 7 min before fixation with PFA/GA. IFAs were performed using α-GAP45 (dilution 1:10,000) and α-GRA3 antibodies (dilution 1:10). 100 vacuoles per test strain were counted and proportion in percentage of egressed versus non-egressed parasites determined, DMSO treated parasites showed no egress. Results are a mean ± standard deviation of three independent biological replicates.

**Host cell attachment assay**

The ability of Tgspt1-ko versus RH WT parasites to attach to host cells was assessed as previously described (Dogga et al., 2017; Mueller et al., 2013). Extracellular GFP expressing parasites and test strains were mixed at 50/50 ratio and used to infect HFF monolayer on coverslips. The cells were centrifuged for 1 min at 1000g, washed with PBS and then fixed with PFA/GA for 10 min. IFAs were performed using α-GAP45 antibody (dilution 1:10,000). 100 parasites were counted, and ratio of attached parasites (green/red) determined. Results are a mean ± standard deviation of three independent biological replicates.

**Gilding motility assay**

Freshly egressed parasites were washed in serum free DMEM and then added to poly-L-lysine coated cover slips in 24 well plate. The cells were centrifuged at 1100 g for 1 min to allow them to settle. The media on the plate was replaced with serum free DMEM plus 3 μM of the Ca²⁺ ionophore A23187 or DMSO and then incubated at 37°C for 30 min. The cells were fixed with PFA/GA for 10 min followed by IFA without permeabilization using α-SAG1 antibody, (dilution 1:10). Images presented are representatives of 3 independent experiments.
Invasion assay
Freshly egressed parasites were allowed to invade confluent host cell monolayers on coverslips for 30 min before fixing with PFA/GA for 5 min. Non-permeabilized cells were incubated with α-SAG1 antibody, (dilution 1:10) in 2% BSA/PBS for 20 min and washed three times with PBS. Cells were then fixed with 1% formaldehyde/PBS for 7 min, washed with PBS and subsequently permeabilized using 0.2% Triton X-100/PBS. Parasites were subsequently labelled using α-GAP45 antibody (dilution 1:10,000) followed by secondary antibodies as described in the IFA section. 100 parasites were counted for each strain and experiment; percentage of intracellular parasites was calculated. Data shown are mean ± standard deviation from three independent experiments.

Microneme secretion assay
Freshly egressed parasites were washed in egress buffer (DMEM without serum) and resuspended in an equal volume of warm egress buffer. The samples were pelleted at 2000 g, for 5 min at room temperature followed by treatment with 100 µL of 2% ethanol in warm egress buffer or DMSO for 30 min at 37°C. Subsequent steps were carried out on ice or at 4°C. Parasites were centrifuged at 1200g for 5 min at 4°C and supernatant was transferred to new Eppendorf tubes and re-centrifuged at 2000g for 5 min at 4°C. The pellets were also washed in 1 mL of PBS. The final supernatant containing secreted microneme proteins and pellet fractions were resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2 mM EDTA, 2% SDS, 0.05% bromophenol blue, 100 mM dithiothreitol (DTT)) and boiled for 5 min before analysis by immunoblotting, antibodies used α-MIC2 (dilution 1:10) (Achbarou et al., 1991) and α-catalase (dilution 1:1,000).

Western blot analysis
Pelleted extracellular parasites were resuspended in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) containing protease inhibitor and incubated on ice for 15 min and then centrifuged at 14000 g at 4°C for 15 min. The supernatant was collected in a separate tube and labelled with SDS–PAGE loading buffer under reducing conditions. The proteins were then transferred to nitrocellulose membranes and probed with appropriate antibodies in 5% non-fat milk powder dissolved in PBS-0.05% Tween20. Bound secondary peroxidase-conjugated antibodies were revealed using the ECL systems (Amersham). Antibodies used α-Myc (dilution 1:200) and α-catalase (dilution 1:1,000).

Parasite growth competition assay
Non-GFP-expressing type I RH and II ME49 WT or mutant parasites were mixed with the respective GFP-expressing WT parasites in an estimated ratio of about 80:20 respectively; control (WT 80% and WT -GFP 20%); test (80%mutant and WT -GFP 20%). HFFs were infected with these preparations and GFP expressing versus non GFP expressing parasite proportions measured progressively over five passages by Flow cytometry. Briefly, at each passage 200 µL of freshly egressed parasites were collected in an Eppendorf tube and incubated with Hoechst DNA stain; (dilution 1:1,000). A fixation step with 200 µL PFA/GA for 10 min ensued. Next the parasites were centrifuged at 2000g for 5 min, the fixative removed and re-suspended in 500 µL 0.1 M glycine/PBS solution. Samples were excited at 488 nm and 350 nm and detected with the 530/30 nm laser and the 460/30 nm laser for GFP and Hoechst, respectively. 10,000 cells were gated bases on fluorescence emission using the Analyser 3 Laser Gallios 4 instrument and analysis done using the Kaluza software provided at the Flow Cytometry Facility Platform at University of Geneva. Data are presented as mean ± SD of three independent experiments.

Transmission electron microscopy
Confluent monolayer of HFFs on coverslips were infected with parasites and incubated for 24 h. Infected cells were washed one time with 0.1 M phosphate buffer pH 7.2 before fixation with 2.5% GA (Electron Microscopy Science) and 2% PFA (Electron Microscopy Science) in 0.1 M phosphate buffer pH 7.4 at room temperature for 1 h. The subsequent steps were as described before (Hammoudi et al., 2018) and examined using a Technai 20 electron microscope (FEI Company). Images shown are a representative of three independent analyzed samples.

Acute virulence in mice
Five female C57BL/6 mice from Charles River Laboratories were used per test group. Intraperitoneal injections with 100 tachyzoites forms of appropriate test strains and control were done. Infection progression was monitored daily and appearance symptoms characteristic of acute toxoplasmosis namely, bristled hair, inability to eat or drink and complete prostration were used as cues to humanely euthanize animals upon presentation.

Cyst counting from processed brain infected tissue
Eight female C57BL/6 mice per test group were infected with 250 tachyzoites and mice monitored. Mice were sacrificed 5 weeks post infection. The brains were collected and homogenized in 1 mL of 1% Tween in PBS by sequential passing 5X through a 18G needle, 10X through a 20G needle and finally 10X through the 23G. Cyst burden was determined by counting cysts in 5 portions of 10 µL volumes per sample using the 20X objective lens of an inverted microscope.
Ethics statement
All experiments were carried out under license number GE150-16 and GE41-15 governed by the Swiss Federal Veterinary Office rules and regulations.

Lipidomics analyses
Sample preparation
RH and ME49 controls alongside the derived spt1-ko, spt2-ko, spt1-ko/SPT1-Myc and des-ko mutants were analyzed. Parasite and host metabolism was quenched through addition of excess ice-cold PBS. All subsequent steps were carried out at 4 °C or on ice. Freshly egressed parasites were lysed by repeated passage through a syringe needle (3 x, 26G) and subsequently purified from host cell material by filtration (3 µm pore size, Millipore/Merck). The parasites were pelleted by centrifugation (2800g, 20 min, 4 °C) in acid washed, rinsed and baked-out conical glass vials (Pyrex) and pellets were washed with ice-cold PBS (3 x), and lipids extracted as outlined below. Three or more technical replicates per strain were used for analyses.

U-13C-palmitate labelling
Intracellular parasites were incubated for 30 h prior to egress in medium containing 0.1 mM U-13C-palmitate (Cambridge Isotope Laboratories) coupled to FA free BSA (Sigma-Aldrich). Parasites were harvested as outlined above and lipids extracted and analyzed as described below.

15N/D3-serine labelling
Per sample, 10⁸ freshly egressed parasites were harvested and freed from host cell material through filtration as described above. Parasite pellets were resuspended in serine-free minimal essential medium (MEM, Gibco), supplemented with 5% dialyzed FBS (Pan Biotech) and 1 mM natural abundance serine or 15N/D3-serine. Following 5 h incubation at 37 °C, parasites were pelleted and washed (3 x) with ice-cold PBS before lipids were extracted as described below.

Lipid extraction
Pellets of 10⁸ parasites were harvested as described above and metabolites extracted in chloroform/methanol/water (C/M/W) (1:1:0.9). Briefly, pellets of 10⁸ parasites were lysed through addition of 134 µL chloroform and vigorous vortexing. After addition of 120 µL water (de-ionised, filtered, MilliQ) and 134 µL methanol, samples were vigorously vortexed again and phases separated through centrifugation (500g, 10 min, 4 °C). The lower apolar phase was transferred to a mass spectrometry vial equipped with a 250 µL capacity insert (Macherey and Nagel) using a gas-tight glass syringe (Hamilton).

Glycerolipids and phosphoglycerolipids hydrolysis
500 µL of the reagent mixture consisting of methanol, water, n-butanol and 40% methylamine solution in water at a ratio of 4:3:1:5 (v/v) were added to the dried lipid extracts. After sonication for five min and vortex mixing, the mixture was incubated for one hour at 53 °C. Lipid samples were then dried in the SpeedVac at 50 °C. 150 µL of water were added to the dried samples and extracted with 300 µL of water saturated n-butanol. Phase separation was achieved by centrifugation for 10 min at 3200g. The combined butanol layers of the three extractions were dried in the SpeedVac at 50 °C. Samples were reconstituted in 100 µL of MeOH.

Lipidomics materials
LC-MS grade water was provided by Huberlab; methanol, acetonitrile (both HPLC grade) by VWR; ammonium acetate by Sigma-Aldrich; acetic acid by Biosolve; n-butanol by Acros Organics (Thermo Fisher Scientific); 40% methylamine solution in water by Sigma-Aldrich. Splash Lipidomix and 18:0 Cer(d7) were purchased from Avanti Polar Lipids.

Lipidomics class separation
Lipid extracts were spiked with internal standards and class separated using hydrophilic interaction liquid chromatography (HILIC). A PAL RTC autosampler (CTC Analytics) was used for the introduction of 2 µL of sample. An LC-30AD pump (Shimadzu) was run in gradient mode with the gradient being as follows: 0–1 min 80% B, 1–5 min from 80-20% and 5–30 min from 20-5% B. C was raised linearly from 0-35% from 0-30 min. The mobile phases were A) MeOH, B) water and C) isopropanol with 10 mM of ammonium acetate contained in each of them. An Xbridge BEH C8 column (2.1 x 150 mm, 2.6 µm, HILIC) was used for lipid separation (Phenomenex) and maintained at 40 °C with a total flow rate of 300 µL/min.

Lipid sum composition separation
Lipid extracts were separated by their acyl side chain using reverse-phase chromatography (RPLC). A SIL-30AC autosampler (Shimadzu) was used for the introduction of 20 µL of sample. An LC-30AD pump (Shimadzu) was run in gradient mode with a gradient being as follows: 0–1 min 80% B, 1–5 min from 80-20% and 5–30 min from 20-5% B. C was raised linearly from 0-35% from 0-30 min. The mobile phases were A) MeOH, B) water and C) isopropanol with 10 mM of ammonium acetate contained in each of them. An Xbridge BEH C8 column (2.1 x 150 mm, 2.6 µm) was used (Waters) maintained at 45 °C and with a total flow rate of 600 µL/min.
Mass spectrometry
Data acquisition was performed on a TripleTOF 5600 (Sciex) in SWATH mode with electrospray ionization in both positive and negative ion mode. Consecutive Q1 isolation windows of 25 units were set for a Q1 mass range of 400–1000 Da with an accumulation time of 30 ms for each window. The collision energy was spread from 10 to 70 to 70 eV. The other MS parameters were as follows: DP 80/-80 V, T 500°C, GS1 and GS2 at 30 (arbitrary unit).

Lipid classes were identified based on accurate mass of the precursors, head group specific fragments and retention time information. LipidView 1.2 was used to create precursor quantification methods for MultiQuant 2.1 (both Sciex). For class separation, M+2 deisotoping at MS1 level was performed in Excel (Microsoft) after data extraction.

QUANTIFICATION AND STATISTICAL ANALYSIS
The type of statistical test, number of independent biological replicates (n, depending on the experiment referring to number of animals or number of independent measurements, e.g., separate measurement of parasite lipid extracts derived from a distinct infection event) and p values for statistical significance are given in the figure legends or in the Results text. Means or medians were determined as indicated in the figure legend. Graphs were made and statistical analyses performed using GraphPad Prism 8. Most statistical tests were unpaired t-tests, comparing one factor between two conditions (e.g., WT vs. KO) and assuming normal distribution. The threshold for statistical significance was p < 0.05.
Supplemental information

Ceramide biosynthesis is critical for establishment of the intracellular niche of *Toxoplasma gondii*

Mary Akinyi Nyonda, Joachim Kloehn, Piotr Sosnowski, Aarti Krishnan, Gaëlle Lentini, Bohumil Maco, Jean-Baptiste Marq, J. Thomas Hannich, Gerard Hopfgartner, and Dominique Soldati-Favre
Supplementary Figure 1. Sequence alignments of sphingolipid synthesis enzymes, Related to Figure 1.

Multiple sequence alignments performed in MultAlin, consensus index; Texts in red, high consensus (90%), in blue low consensus (50%). (A) Multiple sequence alignments of *T. gondii* ceramide biosynthesis enzymes to homologous human sequences: SPT, *H. sapiens* SPT2 HGNC: 11278; *Tg*SPT1 TGME49_290980, *Tg*SPT2 TGME49_290970; *Plasmodium falciparum* 3D7 PF3D7_1415700. (B) Multiple sequence alignment of SPT sequences of *S. cerevisiae* SPT1_P25045, *H. sapiens* SPT1_O15269, *S. cerevisiae* SPT2_P40970, *H. sapiens* SPT2_O15270, *H. sapiens* SPT3_Q9NUV7, S. *paucimobilis* SPT_Q93UV0, *T. gondii* SPT1_TGME49_290980, *T. gondii* SPT2_TGME49_290970, *P. falciparum* SPT PF3D7_1415700 to depict conservation of the Lysine (K) residue (C) 3KDHR, *H. sapiens* HGNC: 4021, Tg3KDHR TGME49_304470, PF3D7_040950 (D) CER, *H. sapiens* CERS1 HGNC: 14253 TgCERS1 TGME49_283710, TgCERS2 TGME49_316450 ; PfCERS1 PF3D7_0508200, PfCERS2 PF3D7_0409500 (E) DES *H. sapiens* DES1 HGNC: 13709 ; TgDES TGME49_237200 (F) Multiple sequence alignment of the fatty acid desaturase domain in the DES sequence of *Drosophila melanogaster* GenBank: AAM12535.1, *Mus musculus* GenBank: AAM12532.1, *Solanum lycopersicum* DES AAM12534.1, *H. sapiens* DES1 HGNC: 13709 ; TgDES TGME49_237200 and conservation of the characteristic sequence motifs HX_3–4H, HX_2–3HH, and (H/Q)X_2–3HH referred to as Histidine boxes highlighted in blue.
Supplementary Figure 2. Genome editing strategy to tag and delete Tgspt1, Tgspt2 and Tgdes, Related to Figure 1 and 2.

(A) Illustration of the strategy used to fuse a Myc epitope tag to the C-terminus of the endogenous copy of TgSPT1 and TgSPT2. (B) Agarose gel electrophoresis images of PCR analysis on gDNA of TgSPT1-Myc and TgSPT2-Myc strain shows integration of the Myc tag and clonality. Sequence of primers used are listed in (Table S2). (C and D) Plaque assay and plaque size quantification at 7 days post infection of confluent human foreskin fibroblasts (HFFs) with TgSPT1-Myc and plaque area measurement show Golgi localization of TgSPT1 has no consequences to parasite development. Measurement of 10 plaques per experiment. (E) Uncropped original immunoblot showing the α-catalase (CAT) blot and the ladder. (F) Uncropped original immunoblot showing the α-Myc blot. (G) Schematic illustration of CRISPR/Cas9 and specific gRNAs mediated deletion of the TgSPT1, TgSPT2 and TgDES coding sequences in ME49 and RH allowing for integration of HXGPRT selection cassette. (H) Agarose gel electrophoresis images of PCR analysis on gDNA of transgenics to diagnose 5′ and 3′ integration of cassette as well as excision and replacement of the exon with the selection cassette. Primers used and expected PCR product size indicated, sequence of primers used are listed in (Table S2). (I) IFAs with α-SPT1 antibody confirm lack of TgSPT1 in mutant parasites compared to wildtype, RH. Scale bar: 2 μm.
Supplementary Figure 3. Genome sequencing to validate *T. gondii* genome modulations, Related to Figure 2.

(A) Workflow of genomic DNA long-read sequencing of *T. gondii* RH, Tgspt1-ko, Tgspt2-ko and Tgspt1-ko/Tgspt2-ko tachyzoites. The table summarizes the data obtained for the four strains: reads = total number of reads (10^3); passed bases = total number of sequenced bases that pass filter (gigabase); estimated N50 = median of the read lengths (kilobase). (B-D) Snapshots of the VEupathDB genome browser displaying Bigwig-formatted coverage tracks using the RH GT1 annotated track as the reference genome (bottom). Read density (purple) is shown when reads are aligned to the reference genome. Colored lines show alignment gaps. (B) The absence of anchored sequences for the *TgSPT1* locus (green) and *TgSPT2* locus (orange) confirms proper gene deletion in the corresponding strains. (C) Gaps / divergent sequences are also observed at the *KU80* and *HXGPRT* loci. (D) Whole genome sequencing also revealed two other regions (TGGT1_chrVI:1150781-1185680 and TGGT1_chrXII:5697111-5714560) where the reads do not align with the reference genome. These gaps have been observed in all the strains sequenced including the parental strain (RH) and another laboratory strain (RH Tir1). (E) The genomic region TGGT1_chrVI:1150781-1185680 is highly divergent among *T. gondii* strains.
Supplementary Figure 4. Complementation of TgSPT1 and attempted sphinganine d18:0 rescue, Related to Figure 2.
(A) Illustration of the complementation strategy of spt1 gene under ron5 promoter at the UPRT locus of Tgspt1-ko parasites. (B) PCR diagnostics showing integration of TgSPT1 sequence at the UPRT locus, expected PCR product sizes indicated, sequence of primers used are listed in (Table S2). (C) IFAs to determine the subcellular localization of second copy complemented TgSPT1-Myc in Tgspt1-ko/SPT1-Myc parasites. As observed for the endogenous gene, second copy TgSPT1-Myc showed a predominant post nuclei staining, as shown by use of α-Myc antibody and αSPT1 antibody (both green, pellicle marker GAP45 in magenta). The protein showed relatively weak colocalization with the ER marker (AT1-Ty, green; α-Myc, magenta) but strong colocalization with the Golgi marker (GRASP-YFP, green; α-Myc, magenta). Scale bar: 2 μm. (D) Immunoblot of parasite lysates showing lack of Myc staining in Tgspt1-ko parasites (second last lane) and increased abundance of TgSPT1-Myc in second copy complemented Tgspt1-ko/SPT1-Myc parasites (last lane) compared to endogenously tagged TgSPT1-Myc (third lane). Proteins were separated by SDS-PAGE, revealed using α-Myc antibody and α-catalase (Cat) antibody as a loading control. Scale bar: 2 μm. (E) Representative images showing increased fluorescent intensity of SPT1-Myc in complemented strain under RON5 promoter (Tgspt1-ko/SPT1-Myc) compared to endogenous promoter (TgSPT1-Myc). IFAs carried out using α-Myc antibodies on TgSPT1-Myc and Tgspt1-ko/SPT1-Myc (magenta, Myc-tag), transiently expressed pTub8-GRASP-YFP vector (green) and (blue, DAPI) nuclei. Images representative of three independent experiments. Scale bar: 2 μm. (F) Plots of fluorescence quantification of (magenta, Myc-tag) in TgSPT1-Myc and Tgspt1-ko/SPT1-Myc dividing and non-dividing parasites. Results are means ± SD of 3 independent experiments. Unpaired statistical significance test applied *p < 0.05. p values. (G) Supplementation with sphinganine (d18:0) shows toxicity in a dose dependent manner examined by plaque formation assay 7 days post infection of confluent HFFs RH and spt1-ko strains. (H) Plot showing quantification of plaque numbers of RH and spt1-ko strains grown in media (DMEM +/- 3 μM sphinganine (d18:0)). Results are means ± SD of 3 independent experiments.
Supplementary Figure 5. Investigation of the invasion defect of *Tgspt1-ko* parasites, Related to Figure 3.

(A) Western blot analyses and (B) quantification of microneme excreted/secreted antigens (ESA) and pellets from *Tgspt1-ko* parasites, after stimulation with 2% Ethanol (EtOH). Normal secretion of the microneme proteins MIC2. Catalase was used as cytosolic control. (C) Electron microscopy image of a section through RH and *Tgspt1-ko* tachyzoites show normal structure of the rhoptry organelles (arrow heads). Scale bars, 1 µm. (D) Fluorescent images of rhoptry secretion test using phosphor-STAT6 as a readout with fibroblast nuclei stained with DAPI (blue) and ROP16-injected cells stained with anti-phospho-STAT6 (STAT6-P) antibody (green, arrow heads). Scale bars: 25 µm. (E) The rhoptry protein toxofilin, fused to β-lactamase (BLA) enzyme and a hemagglutinin (HA) epitope tag was expressed in *Tgspt1-ko* and in RH parental strain to assess rhoptry discharge via FRET-based BLA. The gene was inserted into the *uprt* locus of wildtype RH and *Tgspt1-ko* parasites. Expression and correct localization of the construct was assessed by immunofluorescence assay (IFA) using α-HA antibody (green) and a rhoptry marker (armadillo repeats only protein, ARO; magenta). Scale bars: 2 µm. (F) Additionally, expression and correct size of the construct was assessed in an immunoblot, using α-HA antibody and actin (ACT) as a loading control. Images are representative of three biologically independent experiments.
Supplementary Figure 6. Stable isotope labeling with $^{15}$N/D$_3$-serine to trace sphingolipid synthesis, Related to Figure 5.

(A) Cartoon depiction showing incorporation of $^{15}$N/D$_3$-serine into dhCer through activity of the annotated enzymes. Exemplarily, label incorporation into dhCer C34:0 is shown (here: dhCer d18:0/16:0), which was identified as labeled in the described experiments. Labeled/heavy atoms (nitrogen 15 and deuterium) are highlighted in red and with an asterisk. One deuterium atom is lost during the condensation reaction catalyzed by SPT1, leading to an M+3 mass shift of the analysed dhCer and the downstream Cer. (B) Label (%) in several sphingomyelin species following incubation of extracellular RH parasites (RH wildtype, Tgspt1-ko, Tgspt2-ko and Tgdes-ko) for 5 hours in the presence of 1 mM $^{15}$N/D$_3$-serine or natural abundance serine (Nat. Ab.). Labelling in SMs was negligible and noisy compared to labelling in some Cer and dhCer species (see Figure 5 D and E). (C) (top) High resolution mass spectrometry (HRMS) spectrum of unlabeled RH extract sample. The ion at $m/z$ 540.5355 corresponds to dhCer(34:0). (Bottom) RH extract sample after 5 hours labelling where the signal at $m/z$ 540.5364 corresponds to dhCer(34:0) and the ion at $m/z$ 543.5459 corresponds to labelled lipid dhCer(34:0 $^{15}$ND$_2$). (D) Chromatogram of Total Ion Current (TIC) obtained during HILIC chromatography of RH extract, (middle panel) Extracted Ion Current (XIC) of dhCer(d34:0) and Cer(d34:1) and (bottom panel) mass spectrum, of detected peak of found peak. Coelution of the lipids would potentially interfere with labelling due to the overlapping of the saturated lipid’s M0 mass and the M+2 isotopologue of the unsaturated lipid. (E) Chromatogram of TIC obtained during reverse phase liquid chromatography (RPLC) (top panel). Separation of RH extract, (middle panel). XIC of dhCer(d34:0) RT = 18.6 min and Cer(d34:1) RT= 18.1 presenting resolved peaks and (bottom panel) mass spectrum of detected peak at 18.1 min.
| Primer name | Nr | Enzyme | Sequence (5'-3') | Resulting plasmid |
|-------------|----|--------|------------------|-------------------|
| TgSPT1.1    | 7411 | APal   | GCCGGGGCCCGCAGCGTTGCTCGACCGGAAATCA | TgSPT1-4Myc_HXGPRT |
| TgSPT1.2    | 7412 | Nsil   | GGCATGCAATACGAGCATGTCAGTGGGTTG | TgSPT1-4Myc_HXGPRT |
| TgSPT1.3    | 7652 | -      | GCAAGAGTCCAATCAAACCTG | Primer for screen |
| TgSPT1.4    | 7653 | -      | GAAGAGGAGGAGATGAGGATGTC | Primer for screen |
| P30A        | 3980 | -      | GTGACACCTGCAAGGGACACGGG | Primer for screen |
| M13F        | 4748 | -      | GAAAACACGAGCCGAGT | Primer for screen |
| gRNA.1      | 4883 | -      | AACTTGACATCCCCCATTTAC | CRISPR/Cas9 |
| TgSPT1.gRNA.1 | 7436 | -      | GACGTTTTCGACCCCTGCAGTTAGATAGCTAGGAAATAGC | TgSPT1_gRNA_CRI SPR/Cas9 |
| TgSPT1.gRNA.2 | 7437 | -      | GTAGACACATCCAGGCTGTTATAGGTCCGTCTTCC | TgSPT1_gRNA_CRI SPR/Cas9 |
| CRISPR/Cas9.1 | 6147 | KpnI   | CGAATTGGTTCCCAATGACAAGAAGCACCGCT | pSAG1::CAS9-GFP-U6::sgGOI(#gRNA.1/gRNA.2) |
| CRISPR/Cas9.2 | 6148 | Xho1   | TGCACCTCAGAATTAACCTCTAGACTAAAGG | pSAG1::CAS9-GFP-U6::sgGOI(#gRNA.1/gRNA.2) |
| TgSPT1.HXGPRT.1 | 7438 | -      | CCTCACCACGAGCCCCGACCTTACAGCGGTGCTCGAC | TgSPT1-HXGPRT.1 |
| TgSPT1.HXGPRT.2 | 7439 | -      | CCGGGGAGAAGAGATGAGGATGTCAGTCGACCGGCGCTTTACGCTG | TgSPT1-HXGPRT.1 |
| TgSPT1.5    | 7497 | -      | GCTGCTCTCTTCTACACCACG | Primer for screen |
| TgSPT1.6    | 7508 | -      | ACGCAACGAAACACACATGG | Primer for screen |
| HXGPRT.1    | 5369 | -      | AGATTTCCGGACCTCGACCAC | Primer for screen |
| HXGPRT.2    | 5370 | -      | GCCGATGTCATTCAATTGGGTTTGG | Primer for screen |
| TgDHFR.1    | 2017 | -      | GTCACTTGTGTGCCAGTTCTAC | Primer for screen |
| TgDHFR.2    | 2018 | -      | CTGGGGGTACAGGACGACACCAC | Primer for screen |
| UPRT.gRNA  | -    | -      | - | CRISPR/Cas9 |
| TgSPT1.7    | 7513 | -      | GGCTTAATTAGCCTCAACCATGGCTCTGGTGCAC | UPRT :SPT1 |
| TgSPT1.8    | 7514 | -      | GCCGATATCTCGGAGCATGTCAGTGGGTTG | UPRT :SPT1 |
| TgUPRT.1    | 8655 | -      | CAACGTCTTGTCAGCGACACCA | Primer for screen |
| TgUPRT.2    | 8656 | -      | GTACCTTAGACATCTCTCGAC | Primer for screen |
| TgSPT2.1    | 7409 | APal   | GCCGGGCACCGACGCGGAGTGCAGTGCTTGG | TgSPT2-4Myc_HXGPRT |
| TgSPT2.2    | 7410 | Nsil   | GGCATGCAATACCGGTTAGGCTAGTGGGTTG | TgSPT2-4Myc_HXGPRT |
| TgSPT2.3    | 7650 | -      | CGTTGAAGTCCGATATAC-GC | Primer for screen |
| TgSPT2.4    | 7651 | -      | CTATCTACAGACACATGTCCAC | Primer for screen |
| TgSPT2.gRNA NA.1 | 7432 | -      | GAAGGCAGTATGATACAGATGTTTATAGCTAGAAATAGC | TgSPT2_gRNA_CRI SPR/Cas9 |
| TgSPT2.gRNA NA.2 | 7433 | -      | GATCTATTTTGGCTGAACTTGTTTATAGCTAGAAATAGC | TgSPT2_gRNA_CRI SPR/Cas9 |
| TgSPT2.HXGPRT.1 | 7434 | -      | CCCGTACGCCGTGCTTTTACTCTTATCGCAGTTGCTCAC | TgSPT2-HXGPRT.1 |
| TgSPT2.HXGPRT.2 | 7435 | -      | GATCTCTCGTGCGCTGCTTCTCCTCCTCAAGTCCGCTCTGGCTG | TgSPT2-HXGPRT.2 |
### Supplementary Table 2: List of oligonucleotides used for this study, related to Figure 2 and Figure S2.

Names, sequences and resulting plasmids are listed.

| Oligonucleotide | Sequence | Function |
|-----------------|----------|----------|
| TgSPT2-DHFR.1   | CCCGTAGCCGCGTTGCCTTTTACTTTATCAGCGGCGGCTC TAGAACTAG | TgSPT2-DHFR.1 |
| TgSPT2-DHFR.2   | GATTTCTTCGCGCCGCTGGCTCTTCAAAAGTTCCGGGAGACTCGATCTTG | TgSPT2-DHFR.2 |
| TgSPT2.5        | GACGGGGTGAGTGTATGCG | Primer for screen |
| TgSPT2.6        | GCTCCTCAAGAGACAGAGTG | Primer for screen |
| TgDES.1         | GCTCCTCAAGAGACAGAGTG | pTub8-Myc-DES-HXGPRT |
| TgDES.2         | GCGTTAAATACACTGCCCTTCTTGTGGTTC | pTub8-Myc-DES-HXGPRT |
| TgDES.gRN A.1   | TTCGTCAAGAGACACTGGGTTTATAGCTAGAATAGC | DES_gRNA_CRI SPR/Cas9 |
| TgDES.gRN A.2   | GCAGACGAGTTGAAGCACCAGCTTGGTGGC | DES_gRNA_CRI SPR/Cas9 |
| TgDES-HXGPRT.1  | ACTCGGCCTTTCTCTTTCCCATACCCGCGGTTCAC TGTAGCGTC | TgDES-HXGPRT.1 |
| TgDES-HXGPRT.2  | GGGTTTCCGCGTACTGCGCCCTTCTTGTGCGACTTTGCG TGTGTCGTC | TgDES-HXGPRT.2 |
| TgDES.5         | CGCCTTCTTCCGTGTTTTC | Primer for screen |
| TgDES.6         | CTACTTGCAAGCAGCTAGAC | Primer for screen |
| TgToxofilin. gRNA.1 | GAATCCCGAATCTGTTTGGGAGTTTTAGAGCTAGAAATAGC | Toxofilin_gRNA_Cas9 |
| TgToxofilin-BLA-HA-HXGPRT.1 | TGAAAAAGGTAGGGCGCTATGCCCTTCTTCTGCGTACCC GTACGACGT | SP3-Toxofilin-BLA-HA.1 |
| TgToxofilin-BLA-HA-HXGPRT.2 | CAATGTGTTGCTGAGAAAGCTGTTGTGTTTTACCAATAGC TTAATCAGTG | SP3-Toxofilin-BLA-HA.2 |
**Supplementary Table 4. Detected lipid classes and species in T. gondii extracts by HILIC-SWATH/MS, Related to Figure 5 and 6.**

The sum of the carbon chain tail (sphingosine backbone and fatty acid residue) is given as well as the degree of desaturation. Abbreviations: PC, phosphatidylcholine; PC-O, phosphatidylcholine alkly ether substituent; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; DAG, diacylglycerol; MAG, monoacylglycerol; TAG, triacylglycerol; CE, cholesterol esters; dhCer, dihydroceramide; Cer, ceramide; SM, sphingomyelin; PE-Cer, ceramide phosphoethanolamine.
| Compound       | Molecular formula | RT [min] | [M+H]⁺ calculated | [M+H]⁺ found | Error [ppm] |
|---------------|-------------------|----------|-------------------|---------------|-------------|
| Cer(d32:0)    | C₃₂H₆₅NO₃        | 14.3 - 17.5 | 512.5037        | 512.5044      | 1.4         |
| Cer(d32:0) ¹⁵ND₂ | C₃₂H₆₅D₂¹⁵NO₃   | 14.3 - 17.5 | 515.5133        | 515.5134      | 0.2         |
| Cer(d34:0)    | C₃₄H₇₃NO₃        | 15.8 - 19.1 | 540.5350        | 540.5355      | 0.9         |
| Cer(d34:0) ¹⁵ND₂ | C₃₄H₇₃D₂¹⁵NO₃   | 15.8 - 19.1 | 543.5446        | 543.5459      | 2.4         |
| Cer(d36:0)    | C₃₆H₇₉NO₃        | 17.4 - 20.7 | 568.5663        | 568.5677      | 2.5         |
| Cer(d36:0) ¹⁵ND₂ | C₃₆H₇₉D₂¹⁵NO₃   | 17.4 - 20.7 | 571.5759        | 571.5773      | 2.4         |
| Cer(d34:1)    | C₃₄H₆₇NO₃        | 15.3 - 18.6 | 538.5194        | 538.5202      | 1.5         |
| Cer(d34:1) ¹⁵ND₂ | C₃₄H₆₇D₂¹⁵NO₃   | -        | 541.5290        | -             | -           |
| Cer(d34:2)    | C₃₄H₆₉NO₃        | 14.3 - 17.3 | 536.5037        | 536.5015      | -4.1        |
| Cer(d34:2) ¹⁵ND₂ | C₃₄H₆₉D₂¹⁵NO₃   | -        | 539.5133        | -             | -           |
| Cer(d36:1)    | C₃₆H₇₁NO₃        | 16.4 - 19.8 | 566.5507        | 566.5503      | -0.7        |
| SM(d32:1)     | C₃₇H₇₅N₂O₆P      | 13.2 - 16.3 | 675.5436        | 675.5442      | 0.9         |
| SM(d32:1) ¹⁵ND₂ | C₃₇H₇₅D₂N₁⁵O₆P  | -        | 678.5531        | -             | -           |
| SM(d32:2)     | C₃₇H₇₃N₂O₆P      | 17.0 - 20.4 | 673.5279        | 673.5310      | 4.5         |
| SM(d32:2) ¹⁵ND₂ | C₃₇H₇₃D₂N₁⁵O₆P  | -        | 676.5375        | -             | -           |
| SM(d34:1)     | C₃₉H₈₃N₂O₆P      | 14.7 - 17.9 | 703.5749        | 703.5760      | 1.6         |
| SM(d34:1) ¹⁵ND₂ | C₃₉H₈₃D₂N₁⁵O₆P  | -        | 706.5844        | -             | -           |
| SM(d34:2)     | C₃₉H₈₁N₂O₆P      | 13.7 - 16.8 | 701.5592        | 701.5604      | 1.7         |
| SM(d34:2) ¹⁵ND₂ | C₃₉H₈₁D₂N₁⁵O₆P  | -        | 704.5688        | -             | -           |
| SM(d36:1)     | C₄₁H₈₅N₂O₆P      | 16.3 - 19.5 | 731.6062        | 731.6079      | 2.3         |
| SM(d36:1) ¹⁵ND₂ | C₄₁H₈₅D₂N₁⁵O₆P  | -        | 734.6157        | -             | -           |
| SM(d36:2)     | C₄₁H₈₃N₂O₆P      | 15.2 - 18.5 | 729.5905        | 729.5897      | -1.1        |
| SM(d36:2) ¹⁵ND₂ | C₄₁H₈₃D₂N₁⁵O₆P  | -        | 732.6001        | -             | -           |
| SM(d34:0)     | C₃₀H₆₂N₂O₆P      | 15.3 - 18.5 | 705.5905        | 705.5916      | 1.6         |
| SM(d34:0) ¹⁵ND₂ | C₃₀H₆₂D₂N₁⁵O₆P  | -        | 708.6001        | -             | -           |

**Supplementary Table 5.** Reverse phase liquid chromatography (RPLC) high resolution mass spectrometry (HRMS) data of lipids detected in purified extracellular RH parasites after 5h labelling with ¹⁵N/D₃-serine.

Retention time, accurate mass of the parental M₀ ion and the corresponding labeled M₃ mass isotopologue of lipid species detected in WT RH parasites, related to Figure 5D and E and Figure S6. Abbreviations: Cer, ceramide; SM, sphingomyelin.