1-Deoxysphingolipid synthesis compromises anchorage-independent growth and plasma membrane endocytosis in cancer cells

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Abstract Serine palmitoyltransferase (SPT) predominantly incorporates serine and fatty acyl-CoAs into diverse sphingolipids (SLs) that serve as structural components of membranes and signaling molecules within or amongst cells. However, SPT also uses alanine as a substrate in the contexts of low serine availability, alanine accumulation, or disease-causing mutations in hereditary sensory neuropathy type I, resulting in the synthesis and accumulation of 1-deoxysphingolipids (deoxySLs). These species promote cytotoxicity in neurons and impact diverse cellular phenotypes, including suppression of anchorage-independent cancer cell growth. While altered serine and alanine levels can promote 1-deoxySL synthesis, they impact numerous other metabolic pathways important for cancer cells. Here, we combined isotope tracing, quantitative metabolomics, and functional studies to better understand the mechanistic drivers of 1-deoxySL toxicity in cancer cells. We determined that both alanine treatment and SPTLC1C133W expression induce 1-deoxy(dihydro)ceramide synthesis and accumulation but fail to broadly impact intermediary metabolism, abundances of other lipids, or growth of adherent cells. However, we found that spheroid culture and soft agar colony formation were compromised when endogenous 1-deoxySL synthesis was induced via SPTLC1C133W expression. Consistent with these impacts on anchorage-independent cell growth, we observed that 1-deoxySL synthesis reduced plasma membrane endocytosis. These results highlight a potential role for SPT promiscuity in linking altered amino acid metabolism to plasma membrane endocytosis.

Supplementary key words metabolism • soft agar • 1-deoxysphingolipid accumulation • RAB5 • serine palmitoyltransferase • mitochondrial stress • alanine • serine • SPT promiscuity • 1-deoxy(dihydro)ceramide

Sphingolipids (SLs) are bioactive molecules and structural components of the cell membrane influencing essential cellular processes (1). Serine palmitoyltransferase (SPT) catalyzes the condensation of palmitate and serine in de novo biosynthesis of SLs but can also produce noncanonical cytotoxic 1-deoxysphingolipids (deoxySLs) when using alanine or glycine as a substrate (2–4). Alterations in serine and alanine levels impact substrate availability for the promiscuous SPT reaction driving the accumulation of noncanonical SLs (5, 6). DeoxySLs differ from canonical SL in that they lack the C1-hydroxyl group of sphinganine, preventing further modification or degradation via canonical SL pathways (3). DeoxySLs are cytotoxic to cells, though specific mechanisms remain unclear (7–9). Accumulation of deoxySLs in the contexts of extracellular matrix detachment and/or serine deprivation compromises anchorage-independent growth and the establishment of xenograft tumors (10). However, serine and glycine restriction impact numerous downstream pathways, including nucleotide synthesis/one carbon metabolism (11–14), NAD(P)H (15–17), and lipid metabolism (10, 18). On the other hand, exogenous addition of deoxySLs impacts diverse cellular processes, including mitochondrial function (8), autophagy (19), migration (20), sphingosine kinase 1 proteolysis (21, 22), and protein folding (23), but this approach does not recapitulate the location and trafficking of deoxySLs actively synthesized on the endoplasmic reticulum (ER). We therefore aimed to determine deoxySL-specific toxic effects independent of the broader metabolic impacts of serine and glycine restriction.

Alanine is abundant in the plasma and accumulates in diseases associated with mitochondrial dysfunction (24), metabolic syndrome (25, 26), or anoxia (25). Transaminases expressed in cancer cells readily metabolize alanine and alpha-ketoglutarate to pyruvate and glutamate, but a reduction in pyruvate oxidation...
will cause alanine accumulation and subsequent conversion to deoxySphinganine (deoxySA) (10). Alanine supplementation therefore offers a means of driving deoxySL accumulation without restricting serine. Furthermore, deoxySLs accumulate in the context of point mutations in SPTLC1 or SPTLC2, which encode subunits of the SPT complex, occur in patients with hereditary sensory neuropathy type I (HSAN1), and increase the complex’s affinity for alanine (2, 4, 27, 28).

Ectopic expression of such SPTLC variants effectively drives deoxySL accumulation in cells (27, 29–31) offering an additional means of probing deoxySL-specific cellular effects.

Here, we investigate how deoxySL biosynthesis alters membrane lipid metabolism and cellular processes, comparing 2D cell growth to anchorage-independent conditions. By combining high-resolution MS, fluorescence imaging, and anchorage-independent growth studies, we highlight key aspects of deoxySLs on cancer cell metabolism and growth. These results demonstrate the impact of deoxySL on soft agar colony growth and endocytosis at the plasma membrane, illustrating key functional impacts relevant for cancer and neuropathy.

RESULTS

Alanine promotes deoxySL synthesis and compromises early spheroid formation

To better understand how cytotoxic deoxySLs impact cell function, we altered alanine availability to drive endogenous deoxySL biosynthesis in 2D cultures. Alanine is highly synthesized in proliferating mammalian cells cultured in vitro but absent from most media. Supplementation of 0.5–1 mM alanine increased deoxy-dihydroceramide (deoxyDHCer) and deoxyceramide (deoxyCer) pools by ∼50%. At the same time, levels of canonical dihydroceramides (DHCer) and ceramides (Cer) were not significantly affected (Fig. 1A). We also observed that UK5099, an inhibitor of the mitochondrial pyruvate carrier (MPC), decreased alanine levels by promoting its oxidation (Fig. 1B and supplemental Fig. S1A). In turn, modulation of MPC and alanine levels had a strong impact on free deoxySA m18:0 in HCT116 cells (Fig. 1C). Furthermore, numerous deoxyDHCer and deoxyCer species increased in response to alanine, whereas UK5099 buffered their accumulation (Fig. 1D and supplemental Fig. S1B).

Fig. 1. Alanine promotes deoxySL synthesis and compromises early spheroid formation. A: Total deoxyDHCer, deoxyCer, DHCer, and Cer levels in HCT116 cells cultured with increasing alanine for 3 days relative to 0 mM alanine. B: Alanine levels in HCT116 cells cultured for 3 days with 1 mM alanine and 5 μM UK5099. Schematic depicting alanine involved in mitochondrial and sphingolipid metabolism. C: DeoxySA m18:0 levels in HCT116 cells cultured for 3 days with 1 mM alanine and 5 μM UK5099. D: Levels of deoxyDHCer species in HCT116 cells cultured for 3 days with 1 mM alanine and 5 μM UK5099. E: Adherent cell growth of HCT116 cells cultured with increasing alanine concentrations for 3 days. F: Schematic depicting spheroid formation assay with inhibitors targeting MPC (UK5099), SPT (Myr), and ceramide synthase (CERS, FuB1). G: HCT116 spheroid growth with DMSO, 1 mM alanine, 5 μM UK5099, 10 nM Myr, 10 μM FuB1 (FeB1) for 3–8 days as indicated in the figure. Cells were grown in adherent (A–E) or spheroid cultures (G). Data are presented as means ± SEM. A–D, G: with three cellular replicates or box (25th–75th percentile with median line) and whiskers (minimum to maximum values) (E) with five cellular replicates. One-way ANOVA (B, C) or two-way ANOVA (A, D, G). Significance in A is compared with 0 mM alanine. Significance for D and G is compared with DMSO. Results are depicted from one representative experiment, and each experiment was repeated independently three times with similar results. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
These data highlight the direct impact of alanine and MPC function on deoxySL accumulation. Notably, exogenous alanine did not impact cell growth in 2D adherent culture, despite increasing noncanonical deoxySL species (Fig. 1E). We recapitulated our findings using A549 non-small lung cancer cells, further indicating that different cell types can accumulate deoxySL without exhibiting cytotoxicity (supplemental Fig. S1C, D).

In contrast to adherent culture models, we have previously demonstrated that alanine supplementation and deoxySL accumulation specifically compromise spheroid growth (10). Spheroid growth requires cells to initially survive upon trypsinization and subculture into anchorage-independent conditions; this process has a profound influence on glycans and downstream metabolic processes important for rebuilding these membrane structures (32). Subsequently, cells in spheroid culture must sustain proliferation in larger 3D structures, which establish nutrient and hypoxic gradients and associated imbalances in redox pathways (33–35). To better understand how alanine and deoxySL metabolism impact spheroid growth, we plated cells in low attachment plates and modulated deoxySL synthesis by supplementing alanine or inhibiting the MPC (UK5099), the SPT complex (myriocin [Myr]), or ceramide synthases (CERS) (fumonisin B1 [FuB1]). We monitored changes in sphingoid bases to those confirmed after treatments and associated imbalances in redox pathways (supplemental Fig. S1C, D). Intriguingly, we observed an impact on spheroid biomass only when cells were treated in the early phase (i.e., early and continuous), whereas treatment during later stages of spheroid growth had no effect (Fig. 1G). Remarkably, these changes were recapitulated by all biochemical treatments, including alanine, UK5099, Myr, and FuB1. Presumably the 3–8 day “late” treatments drive similar changes in sphingoid bases to those confirmed after 24 h and 8 day continuous treatments (10); however, these data highlight that alanine and other modulators of deoxySL accumulation compromise growth during the early phase of spheroid formation rather than modulating growth/survival in later-stage larger spheroid structures. Notably, each of these compounds also impacts other pathways (e.g., canonical sphingolipids, tricarboxylic acid, and amino acid metabolism), limiting our ability to confirm promiscuity-linked mechanism using such treatments (and rescues).

Impact of endogenous deoxySL synthesis on metabolic fluxes

To better elucidate the biochemical and functional impacts of endogenously synthesized deoxySLs independent of alanine supplementation, serine restriction, or canonical sphingolipid synthesis, we established a controllable means of accumulating deoxySLs in isogenic cells. Indeed, ectopic expression of mutant SPTLC1 protein variants or fusion proteins robustly drives deoxySL accumulation in cultured cells (27, 29, 30). Here, we expressed doxycycline-inducible constructs containing either WT (gray: SPTLC1WT) or mutant (red: SPTLC1C133W) SPTLC1 in HCT116 cells via lentiviral delivery. SPTLC1C133W has an increased affinity for alanine, driving the synthesis of deoxySLs compared with SPTLC1WT (Fig. 2A), as observed in patients with HSAN1 (2, 4, 27). We first confirmed that SPTLC1 protein variants were consistently expressed in cells (Fig. 2B). As expected, levels of deoxyDHCer and deoxyCer accumulated significantly in doxycycline-treated cells expressing SPTLC1C133W compared with

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those infected with the SPTLC1WT construct, whereas levels of DHCer and Cer were less affected (Fig. 2C). Furthermore, all deoxySL species detected, including free deoxySA (supplemental Fig. S2A), deoxyDHCer (Fig. 2D), and deoxyCer (Fig. 2E), were robustly increased in SPTLC1C133W compared with SPTLC1WT-expressing cells. On the other hand, levels of canonical DHCer and Cer species were less impacted in both polycytoplial cell lines (supplemental Fig. S2B, C).

To further understand the turnover and metabolism of sphingolipid intermediates in the context of deoxySL synthesis, we cultured cells in the presence of [U-13C16]palmitate and quantified 13C incorporation into sphingoid bases and Cer species using high-resolution MS (Fig. 3A). Although this approach is limited by the nonphysiological concentration and localization of the administered [U-13C16]palmitate tracer, simultaneous quantitation of SL and deoxySL labeling kinetics is informative of the relative flux through each pathway. Focusing on the more abundant canonical Cer pools (i.e., SA d18:0, DHCer d18:0/24:0, and Cer d18:1/24:0) that achieved either steady-state isotopic labeling or >20% turnover, we observed that SPTLC1C133W cells exhibited reduced labeling kinetics compared with SPTLC1WT cells (Fig. 3B). Since Cer pools (including 24:0 n-acyl species) were generally not altered by expression of SPTLC1 constructs (supplemental Fig. S3A, B), these results suggest that SPTLC1C133W expression or deoxySL accumulation modestly reduced d18:0/24:0 DHCer biosynthesis, but the larger Cer metabolic network mitigates this impact at this level of SPTLC1C133W expression (Fig. 2A). On the other hand, turnover of the lower abundance deoxySA m18:0, deoxyDHCer m18:0/24:0, and deoxyCer m18:1/24:0 pools was significantly lower than that observed for canonical sphingolipids (Fig. 3C), which highlights that biosynthetic flux of deoxySL species generally occurs at much slower rates compared with the production of canonical Cers in SPTLC1C133W-expressing cells. Similar trends were observed for 24:1 n-acyl (supplemental Fig. S3A–D) as well as 16:0 n-acyl (supplemental Fig. S3A, B, E, F) Cers, in the latter case labeling on both sphingoid bases and n-acyl chains was evident from M+32 isotopologues (supplemental Fig. S3G, H). However, labeling kinetics were not as consistently impacted, suggesting that deoxySL synthesis and accumulation have diverse impacts on downstream sphingolipid metabolism depending on the specific molecular species.

To determine how deoxySL accumulation influenced other metabolic pathways, we quantified broader labeling and flux changes occurring in SPTLC1WT and SPTLC1C133W-expressing cells. For example, palmitate oxidation and enrichment of citrate via acetyl-coenzyme A and citrate synthase were unaltered in both cell lines (Fig. 3D). Isotope enrichment from [U-13C6]glucose was similarly unchanged when deoxySL synthesis was active (supplemental Fig. S3I). In addition, doxycycline treatment and deoxySL accumulation over 6 days failed to impact mitochondrial respiration measured by Seahorse (supplemental Fig. S3J). These results suggest that endogenous deoxySL synthesis is not sufficient to significantly alter central carbon metabolism or mitochondrial respiration. Indeed, adherent growth was not impacted by doxycycline-induced expression with increased deoxySL biosynthesis by expression of SPTLC1C133W, suggesting that cells can accommodate a certain level of de novo synthesized deoxySLs (or canonical SLs) without experiencing deleterious effects on growth (Fig. 3E). Consistent with prior results (10) and those presented above (Fig. 1G), we observed a slight but significant reduction in spherosid growth in SPTLC1C133W compared with SPTLC1WT HCT116 cells suggesting that the stress from initiating nonadherent growth sensitizes cells to deoxySL accumulation-induced toxicity (Fig. 3F).

We and others have also administered exogenous deoxySA to culture media for analysis of downstream effects (8, 10, 29), observing significant toxicity in some cell types. However, administration of free deoxySA significantly impacts its localization and thus local concentrations within cells compared with processing of newly synthesized deoxySLs on the ER membrane. In fact, we observed that deoxySA treatments, even at relatively moderate concentrations of 100 nM, resulted in a much greater accumulation of deoxyDHCer and deoxyCer species (~20 fold) compared with alanine supplementation or SPTLC1C133W expression (2- to 5-fold) (Fig. 3G and supplemental Fig. S3K). These increases with deoxySA treatment were most pronounced in abundant deoxyDHCer species containing very-long chain fatty acids (supplemental Fig. S3L), highlighting that exogenous deoxysphingoid bases drive much higher deoxyDHCer biosynthetic flux compared with expression of SPTLC1C133W. Notably, our adherent cell models achieved moderate, but more physiologically relevant, increases in deoxySL levels without showing overt cytotoxicity. Exogenous deoxySA administration may elicit distinct biological impacts because of aberrant localization of deoxySLs compared with that occurring via synthesis on the ER membrane. Indeed, CERS are expressed in the mitochondria and can drive cellular toxicity (36–38).

### Alanine and deoxySL synthesis influence colony formation in soft agar

Single-cell growth, migration, and invasive growth are hallmarks of transformed cells and correlate with anchorage-independent growth. As noted above, metabolically altering deoxySL synthesis consistently influenced “3D” anchorage-independent cell growth but not 2D adherent cell growth. To better understand the impact of deoxySL synthesis on proliferation in these contexts, we next examined its impact on soft agar colony formation. Notably, we observed a significant growth defect in cells ectopically expressing SPTLC1C133W compared with SPTLC1WT (Fig. 4A)
effects on colony size and number (supplemental Fig. S4A, B). We also rescued colony formation of *SPTLC1<sup>C133W</sup>*-expressing cells using 10 nM Myr, which had little impact on colony formation in cells expressing *SPTLC1<sup>WT</sup>* (Fig. 4B). We also engineered the A549 non-small cell lung cancer cell line to express doxycycline-inducible *SPTLC1<sup>WT</sup>* or *SPTLC1<sup>C133W</sup>* (supplemental Fig. S4C). As in HCT116 cells, expression of *SPTLC1<sup>C133W</sup>* potently increased deoxySLs (supplemental Fig. S4D) and suppressed soft agar colony formation in these cells (supplemental Fig. S4E). On the other hand, Myr treatment blocked growth...
Intriguingly, normalizing deoxySL synthesis in A549 cells with alanine and agar colony formation were observed when modulating this supplementation (Fig. 4C). Similar trends in soft deoxySL accumulation, mitigated the negative effects of inhibition of SPT or MPC, both of which prevent deoxySL accumulation specifically impacts soft agar growth, or more generally, cellular processes important for anchorage-independent growth.

DeoxySLs compromise plasma membrane endocytosis

The above studies demonstrate that deoxySL accumulation inhibits spheroid cell growth and soft agar colony formation. The temporal studies outlined in Fig. 1F, G also highlighted the sensitivity of this process to cell seeding just after passaging, which induces membrane turnover facilitated by endocytosis. Soft agar growth also requires endocytic flux to catabolize matrix and establish multicellular colony formation. We therefore hypothesized that metabolic induction of deoxySL compromises plasma membrane endocytosis, which is mediated by GTPases such as RAB5. Since quantification and visualization of endocytosis and vesicular trafficking in 3D cultures are technically challenging, we determined the impact of deoxySL synthesis on endocytic events at the plasma membrane in adherent cell culture models. To directly examine whether this process was altered by deoxySL accumulation, we quantified expression of the early endosomal marker RAB5, which regulates endosomal biogenesis and trafficking, in cells expressing SPTLC1WT or SPTLC1C133W. RAB5-positive endosomes were significantly decreased in SPTLC1C133W-expressing HCT116 cells compared with control conditions, indicating decreased endocytic activity (Fig. 5A). We also observed similar changes in A549 cells expressing SPTLC1C133W (Fig. 5B). Ectopic SPTLC1C133W expression induced reductions in RAB5 similar to that occurring with dynamin inhibition (Fig. 5A, B). Furthermore, we also observed decreased RAB5-positive endosomes in HCT116 cells induced to accumulate deoxySLs with alanine treatment, providing an additional link to defects in endocytosis (Fig. 5C). In fact, supplementation of both serine and alanine prevented this decrease in RAB5-positive endosomes from occurring (Fig. 5C).

To further visualize alterations in membrane events, we imaged uptake of the styryl membrane-impermeable lipid fluorescence probe FM1-43 and quantified internalized FM1-43 stained vesicles as a measurement of endocytosis at the plasma membrane (39). Cells with high deoxySL synthesis, either induced by SPTLC1C133W or alanine addition, had significantly decreased FM1-43 loading compared with control conditions (Fig. 5D), providing additional functional evidence that deoxySL synthesis influences endocytic events at the plasma membrane.

**DISCUSSION**

Here, we investigated the effects of endogenous deoxySL accumulation in cancer cells, using alanine supplementation or ectopic expression of SPTLC1C133W. Our engineered cell systems allowed us to determine deoxySL-specific cellular effects independent of
broader metabolic changes caused by serine and glycine deprivation. We observed that synthesis and accumulation of noncanonical deoxySLs specifically compromised anchorage-independent growth without influencing central carbon metabolism or respiration. Rather, these amino acid-driven metabolic changes influence plasma membrane endocytosis, highlighting an important functional role for SPT promiscuity in coordinating such processes (Fig. 6). While deoxySA synthesis has negligible effects on adherent cancer cell growth, we observed reduced proliferation in soft agar colony assays, an environment that places significant load on the plasma membrane. Therefore, in the context of dietary serine/glycine restriction, elevated deoxySLs may compromise tumor seeding and combine with additional downstream metabolic effects to reduce tumor progression (40). On the other hand, deoxySL synthesis also influences T-cell function (41), which also must be considered in the context of cancer therapy. Moving forward, these results clarify how deoxySL accumulation impacts cell metabolism and further highlight the impact of these species on membrane processes relevant for cancer cell, immune cell, and neuronal function.

We also observed differences in cells that synthesized deoxySLs endogenously versus those treated with exogenous deoxySA. Notably, SPT, CERS, dihydroceramide desaturase, and fatty acid desaturase 3 are predominantly localized to the ER membrane, catalyzing the synthesis and processing of (deoxy)DHCer and (deoxy)Cer species that are further trafficked to membranes throughout the cell. Therefore, proteins and organelles exposed to deoxySA via exogenous treatments versus endogenous synthesis will differ, which could account for lack of any pronounced effect observed with SPTLC1\text{C133W} expression on intermediary metabolism.

Diverse phenotypes have been proposed for cells and tissues exposed to deoxySA, including mitochondrial dysfunction and apoptosis (8, 20). However, HSAN1

Fig. 5. DeoxySLs compromise plasma membrane endocytosis. A: RAB5 immunostaining with SPTLC1\text{WT}-and SPTLC1\text{C133W}-expressing HCT116 cultured for 4 days with 0.1 \( \mu \)g/ml doxycycline. Dynasore (dyn) was given with 20 \( \mu \)M for 3 h. B: RAB5 immunostaining with SPTLC1\text{WT}- and SPTLC1\text{C133W}-expressing A549 cultured for 4 days with 1 \( \mu \)g/ml doxycycline. Dynasore (dyn) was given with 20 \( \mu \)M for 3 h. C: RAB5 immunostaining with HCT116 cells cultured with 1 mM alanine or 1 mM serine. D: FM1-43 staining in HCT116 and A549 cells cultured for 4 days with 0 or 1 mM alanine or expressing WT or mutant (C133W) SPTLC1. Cells were grown in adherent cultures, and violin plots depict relative RAB5 or FM1-43 intensity obtained from \( n \) = cell number. Data are representative of two independent experiments. White scale bar indicates 10 \( \mu \)m. One-way ANOVA (A–C) or Student’s t-test (D) with \(*P* < 0.05, **P* < 0.01, ***P* < 0.001, and ****P* < 0.0001.

Metabolic consequences of 1-deoxysphingolipid biosynthesis
patients acquire specific sensory defects with age and do not present with broad metabolic dysfunction (42), suggesting that the effects of deoxySL accumulation are more subtle or specific to neurons. Rather, we observed specific effects of endogenously produced deoxySL on membrane processes that are critically important for anchorage-independent cancer cell growth and colony formation as well as neuronal function. As growth factor receptor-mediated signaling is intimately tied to plasma membrane trafficking, the impact on distinct signaling pathways (e.g., epidermal growth factor or nerve growth factor) warrants further investigation (43, 44). Furthermore, deoxySA regulation of sphingosine kinase could impact signaling through S1P receptors (22). Karsai et al. (20) also recently observed that deoxySLs influence cell migration, which requires membrane dynamics for movement. Collectively, these studies highlight key cellular phenotypes associated with deoxySL accumulation and membrane processes. One key question that remains is why the SPT complex retains this promiscuity and capacity for deoxySL synthesis. By integrating metabolic signals from amino acid metabolism into the ER and plasma membrane, SPT promiscuity could act as a regulatory node linking changes in amino acid or central carbon metabolism to lipid processing machinery.

MATERIALS AND METHODS

Reagents

Media and sera were purchased from Life Technologies. Isotope tracers were purchased from Cambridge Isotopes. Sphingolipid standards were purchased from Avanti Polar Lipids. All other reagents were purchased from Sigma unless otherwise noted.

Cells were treated at a concentration of 5 μM UK5099 (catalog no.: PZ0160; MilliporeSigma), 10 nM Myr (catalog no.: MI177; MilliporeSigma), 10 μM FuB1 (catalog no.: F1147; MilliporeSigma), or 20 μM Fenofibrate (catalog no.: PHR1246; Sigma) with stock solutions prepared in DMSO. DeoxySA m18:0 (catalog no.: 860493P; Avanti) and amino acids were supplemented to growth media with concentrations as indicated in the text.

Cell culture

All cell lines were purchased from ATCC and tested negative for mycoplasma contamination: A549 (ATCC; catalog no.: CCL-185), HCT116 (ATCC; catalog no.: CCL-247), and human embryonic kidney 293FT (HEK293FT) (ATCC; catalog no.: CRL-3216). Cells were cultured in DMEM (catalog no.: 11965-092; Gibco) containing 25 mM glucose, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified cell culture incubator at 37°C and 5% CO2. HCT116, A549, and HEK293T cells were cultured in growth medium containing 10% FBS (catalog no.: 16000-044; Gibco) and
engineered SPTLC1-expressing cell lines containing 10% tetracycline (TET)-free FBS (catalog no.: FB15; Omega, Scientific, Inc). Cells were detached with 0.05% trypsin-EDTA.

Engineering SPTLC1-expressing cell lines

SPTLC1 gene complementary DNA ORF nucleotide sequence (NM_006415) was purchased from GenScript. The ORF was subjected to point mutation to generate mutant SPTLC1 WT ORF and cloned into pCW57.1 vector (Addgene plasmid 41393). Briefly, the ORF and the plasmid were digested with NheI and AgeI, gel purified, and ligated. The engineered pCW57.1 plasmids containing SPTLC1 WT or SPTLC1 WT were delivered into HCT116 and A549 cells using lentivirus delivery strategy.

Lentivirus were produced using HEK293T cells in high-glucose DMEM supplemented with 10% FBS. One 10 cm cell culture dish of HEK293T cells at 60% confluence were transfected with 1.3 μg VSV.G/pMD2.G (Addgene plasmid 12259), 5.5 μg lenti-gag/pol/pCMVR8.2 (Addgene plasmid 12263), and 4 μg of the plasmid using 16 μl Lipofectamine 3000 diluted in 0.66 ml of OPTI-MEM (Life Technologies). Medium containing viral particles was harvested after 48 and 72 h, filtered with 0.45 μm filters, and further concentrated using Amicon Ultra15 centrifugal ultrafilters with a 100,000 nominal molecular weight limit cutoff (catalog no.: UFC910024; Merck Millipore), and stored at −80°C until use.

Cells were infected in 6-well tissue culture plates with 6 μl concentrated virus particles in 0.5 ml growth medium containing 6 μg/ml polybrene for 4 h before addition of 2 ml virus-free growth medium containing polybrene. After 24 h, medium was changed to growth medium containing 2 μg/ml puromycin. After puromycin selection, immunoblotting and RT-PCR analyses were carried out to confirm the expression of SPTLC1 in response to doxycycline as indicated in the figure. For metabolic and functional experiments, HCT116 cells were induced with 0.1 μg/ml doxycycline and A549 cells using lentivirus delivery strategy.

Adherent cell growth assay using PrestoBlue

Cells were cultured in 96-well plates for 3 days with treatments as indicated in each figure. Cell viability was determined using PrestoBlue Cell Viability Reagent (Invitrogen) per the manufacturer’s instructions.

Spheroid growth assay

For spheroid assays, 20,000 singularized cells were plated in 2 ml growth medium on 6-well ultra-low attachment plates (catalog no.: 3471; Corning) with treatments as indicated in the text (1 μM alanine, 5 μM UK5099, 10 nM Myr, and 20 μM FuB1). HCT116-expressing cell lines were induced with 0.1 μg/ml doxycycline and A549 cells with 1 μg/ml doxycycline.

Soft agar colony formation assay

Soft agar colony formation assay was conducted in 6-well tissue culture plates. A 3% low melting temperature agarose (catalog no.: A4018; MilliporeSigma) stock solution was prepared in water, autoclaved, and kept at 55°C in a water bath until use. About 2 ml of the lower hard layer containing 0.6% in growth medium was added per well and allowed to solidify for 20 min at room temperature. About 3,000 cells were mixed with 1 ml of 0.3% agarose in growth medium supplemented with treatments of choice (DMSO, 5 μM UK5099, 10 nM Myr, 1 mM serine, and 0.5 mM or 1 mM alanine) as indicated in figure legends and overlayed on the lower hard layer. The plates were placed at 4°C for 5 min to allow for a fast solidification improving uniform cell distribution in the soft agarose layer. The plates were then transferred into a humidified cell culture incubator at 37°C with 5% CO2 to allow colony formation. HCT116 colonies were counted on day 7 using a brightfield microscope. Pictures of soft agar colonies were taken with a Leica DMi8 Microsystem with 5×, 10×, or 20× objective as indicated in each figure legend. Colonies were stained by addition of 0.3 ml of 0.1% crystal violet solution per well and incubated for 15 min at room temperature. The wells were washed five times with 1 ml water and incubated for 5 min each at room temperature. Pictures were taken with the Bio-Rad ChemiDoc XRS+ imaging station.

Protein extraction and immunoblotting

Cells were plated on 6-well plates in growth medium containing 10% TET-free FBS and doxycycline for 4 days as indicated in the figure legend. Medium was changed on day 2. Cells were lysed in ice-cold RIPA buffer (catalog no.: BP-115-5x; Boston Bioproducts) supplemented with 1x HALT protease inhibitor cocktail (catalog no.: 78430; Thermo Fisher Scientific), vortexed for 20 min at 4°C, and centrifuged at 20,000 g for 10 min at 4°C. Supernatant was used to quantify protein using BCA protein assay kit and prediluted Protein Assay Standards (catalog no.: 23208; Thermo Fisher Scientific) as per the manufacturer’s instruction. Samples were incubated for 5 min at 95°C in 4x Laemmli buffer. About 40 μg of total protein was separated on a 4–20% SDS-PAGE gel (mini-PROTEAN TGX gels; Bio-Rad) along with PageRuler Prestained protein ladder (catalog no.: 26619; Thermo Fisher Scientific), and proteins were transferred onto a PVDF membrane (Millipore; catalog no.: ISEQ00010). The membrane was blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween-20 for 1 h and immunoblotted with primary antibody at 4°C overnight diluted in 5% nonfat milk, anti-SPTLC1 (1:1,000 dilution; catalog no: 15376-1-AP; Proteintech), and anti-β-actin (1:1,000 dilution; catalog no: 5700; Cell Signaling Technology). The immunoblots were then incubated with secondary antibody for 1 h at room temperature (1:1,000 dilution, anti-rabbit, catalog no: 7074, lot no: 28; anti-mouse HRP conjugate, catalog no: 7076S, lot no: 32, Cell Signaling Technology). Specific signal was detected using SuperSignal West Pico Chemiluminescent Substrate (catalog no: 1705061; Bio-Rad) and imaged with a Bio-Rad ChemiDoc XRS+ imaging station. Band signal from Western blots was determined using Image Lab Software from Bio-Rad and normalized to signal of β-actin. Antibodies for Western blotting were validated for human reactivity by the manufacturer and used per their instructions. Uncropped raw blots are provided in Supplemental Data S1.
Respirometry

Respiration was measured in adherent monolayers of cells using an Agilent Seahorse XF96 Analyzer with a minimum of five cellular replicates per condition. Engineered SPTLC1G131W expression cell lines were cultured in the presence of doxycycline 4 days prior and 2 days after seeding on 96-well plates in the presence of doxycycline. Cells were assayed in DMEM (catalog no: 5030; Sigma-Aldrich) supplemented with 8 mM glucose, 3 mM glutamine, 3 mM pyruvate, and 2 mM Hepes. Cells were washed twice with 100 μl assay medium and cultured in 150 μl assay medium for 1 h before measurement. Respiration was measured under basal conditions as well as after injection of 2 μM oligomycin (port A), sequential addition of 200 nM FCCP (port C, D), and addition of 0.5 μM rotenone and 1 μM antimycin (port D). Oxygen consumption rates were normalized to protein content using BCA protein assay kit.

Isotopic tracing and analysis

Cells were cultured in medium containing stable isotope tracers of choice as indicated in the text. Tracers were purchased from Cambridge Isotopes, Inc or Sigma: [U-35,36,38-13C6] glucose (catalog no: CLM-1396-25), [U-13C16]palmitate (catalog no: CLM-409), and [2,3,13C2]alanine (catalog no: 604682). For alanine tracing studies, HCT116 cells were cultured in growth medium supplemented with 1 mM [13C16] alanine for 24 h in the presence of DMSO or 5 μM UK5099. For glucose isotopic labeling experiments, HCT116 SPTLC1-expressing cells were induced with 0.1 μg/ml doxycycline for 5 days before starting tracing for 24 h. Tracing was performed with DMEM (catalog no: 5030; Sigma-Aldrich) where 13C glucose was replaced with 25 mM [U-13C6] glucose and 10% TET-free FBS was supplemented. Labeling on metabolites from 13C alanine and 13C glucose was quantified using GC-MS technology. Mass isotopomer distributions and total metabolite abundances were computed by integrating mass fragments using a MATLAB-based algorithm with corrections for natural isotope abundances as described previously (45, 46).

For tracing studies with [U-35,36,38-13C6] glucose, HCT116 cells were cultured in growth medium in the presence of 0.1 μg/ml doxycycline for 7 days before tracer start. Growth medium was replaced to DMEM containing 1% (v/v) delipidated FBS 24 h prior tracer start and medium exchange again 1 h prior tracer trace. [U-35,36,38-13C6] palmitate was noncovalently bound to fatty acid-free BSA and added to culture medium at 5% of the final volume (50 μM final concentration). Media were prewarmed to 37°C in a cell incubator with 5% CO2, and cells were traced for 15 min, 1 h, and 4 h. Metabolites were extracted, and labeling was quantified on a Q-Exactive GC-MS system. Metabolites were annotated, and labeling was corrected for natural isotope abundance using Maven software, version 2011l.17 (47). Because of [U-13C6] palmitate tracer purity of 98% (catalog no: CLM-409; Cambridge Isotope Laboratories, Inc), we also observed a low fraction of labeled M+15 as well as M+31 on deoxyDHCer m180/160. In supplemental Fig. S3A, B, M+16 depicts the combined label of M+15 and M+16, whereas M+32 depicts the combined label of M+31 and M+32.

GC-MS and sample preparation

Metabolites were extracted, analyzed, and quantified, as previously described in detail (46). Briefly, cells were washed with saline solution and quenched with 0.25 ml at −20°C methanol. After adding 0.1 ml 4°C cold water, cells were collected in tubes containing 0.25 ml at −20°C chloroform. The extracts were vortexed for 10 min at 4°C and centrifuged at 16,000 g for 5 min at 4°C. The upper aqueous phase was evaporated under vacuum at 4°C. Derivatization for polar metabolites was performed using a Gerstel MPS with 15 μl of 2% (w/v) methoxyamine hydrochloride (Thermo Fisher Scientific) in pyridine (incubated for 60 min at 45°C) and 15 μl 2-tert-butyldimethylsilyl-N-methyl trifluoroacetamide with 1% tert-butyldimethylchlororosilane (Regis Technologies) (incubated further for 30 min at 45°C). Derivatives were analyzed by GC-MS using a DB-5MSUI column (30 m × 0.25 i.d. × 0.25 μm) installed in an Agilent 7890B GC interfaced with an Agilent 5977A MS operating under electron impact ionization at 70 eV. The MS source was held at 230°C and the quadrupole at 150°C, and helium was used as carrier gas. The GC oven was held at 100°C for 2 min, increased to 300°C at 10°C/min, and held for 4 min, and held at 325°C for 3 min.

FM1-43 dye uptake assay, RAB5 immunostaining, and fluorescence microscopy

Cells were plated on 12-well plates containing cover glass coverslips for 4 days in the presence of doxycycline or small-molecule treatments as indicated in the text. Medium was changed on day 2. Media were supplemented with 20 μM dynasore (catalog no: D7693; Sigma Millipore) 3 h. To visualize RAB5-positive endosomes, cells were fixed with ice-cold 4% paraformaldehyde solution (catalog no: J19943-K2; Thermo Fisher Scientific), washed three times with 0.1% Tween-20 in PBS (PBST) before incubation with 0.2% Triton X-100 for 10 min. Cells were then washed three times with PBST (each 5 min), incubated with 8% BSA in PBST (catalog no: 700-100P; Gemini Bio-Products) for 20 min, and washed three times with PBST (each 5 min). Cells were then incubated with primary anti-RAB5 antibody at 4°C overnight diluted in 8% BSA in PBST (1500 dilution; catalog no: 3547, lot 7; Cell Signaling Technology). Secondary anti-rabbit Alexa Fluor 568 antibody (1:1000 dilution, catalog no: A-11011; Life Technologies) and Hoechst (1:1000 dilution, catalog no: 4082; Cell Signaling Technology) diluted in 8% BSA in PBST were applied for 2 h at room temperature followed by an incubation of HCS Cell Mask Deep Red Stain (catalog no: H332720; Invitrogen) for 30 min at room temperature. Cells were then washed three times with PBST (5 min each). Antibodies were validated for human reactivity by the manufacturer and used per their instructions. Coverslips were mounted with Fluoromount-G (catalog no: 0100-01; Southern Biotech) on microscopy plates (catalog no: 12-544-7; Cell Signaling Corp). For FM1-43 dye uptake assay, cells were incubated with 5 μg/ml FM1-43FX (catalog no: F35355; Invitrogen) for 10 min at 37°C and fixed with ice-cold 4% paraformaldehyde solution as per the manufacturer’s instruction. Nuclei were visualized with Hoechst as described above.

Cells were imaged using a 50x Plan Apo 1.4 numerical aperture objective on a Nikon Ti2-E microscope with a Yokogawa XI spinning disk confocal system, MLC400B 4-line (405, 488, 561, and 647 nm) dual-fiber laser combiner (Agilent), Prime 95B back-thinned sCMOS camera (Teledyne Photometrics), piezo Z-stage (Mad City Labs), and running NIS Elements software. To separate the emission of individual fluorophores, bandpass emission filters were used for each channel (450/50, 525/36, 605/52, and 705/72). Background fluorescence intensity was corrected for each image. One representative image is depicted per experimental condition.
was quantified using ImageJ L53k with n cell number as depicted in each figure.

**LC-MS, sample preparation, and sphingolipid analysis**

For targeted sphingolipid analysis, cells were washed with 0.9% (w/v) NaCl and extracted with 0.25 ml of −20°C methanol, 0.25 ml of −20°C chloroform, and 0.1 ml of water spiked with deuterated internal standards (20 pmol of D7-sphinganine [Avanti, Croda International Plc; catalog no: 860658]), 2 pmol of D3-deoxysphinganine [Avanti, Croda International Plc; catalog no: 860474], 200 pmol of C15 Cer-d7 [d18:1-d7/15:0] [Avanti, Croda International Plc; catalog no: 860681], 100 pmol of C13-dihydroceramide-d7 [d18:0-d7/13:0] [Avanti, Croda International Plc; catalog no: 330726]. The tubes were vortexed for 5 min, centrifuged at 20,000 g at 4°C for 5 min, and the lower organic phase was collected. The remaining polar phase was re-extracted with 2 μl formic acid and 0.25 ml of −20°C chloroform. The organic phases were combined, dried under air, resuspended in 80 μl buffer B (0.2% formic acid and 1 mM ammonium formate in methanol), sonicated for 10 min, and centrifuged for 10 min at 20,000 g at 4°C. Cer species in the supernatant were quantified using LC-MS (Agilent 6460 QQQ, MassHunter LC/MS Acquisition [v.B.08.02]) equipped with a C8 column (Spectra 3 μm C8SR 150 × 3 mm inner diameter, Pecke Scientific) with mobile phase A (2 mM ammonium formate and 0.2% formic acid in HPLC grade water) and mobile phase B (0.2% formic acid and 1 mM ammonium formate in methanol). The gradient elution program consisted of the following profile: 0 min, 82% B; 3 min, 82% B; 4 min, 90% B, 18 min, 99% B; 25 min, 99%; 27 min, 82% B, and 30 min, 82% B. Column re-equilibration followed each sample and lasted 10 min. The capillary voltage was set to 3.5 kV, the drying gas temperature was 350°C, the drying gas flow rate was 10 l/min, and the nebulizer pressure was 60 psi. Cer species were analyzed by selective reaction monitoring of the transition from precursor to product ions at associated optimized collision energies, and fragmentor voltages are provided elsewhere (10).

SL and deoxySL species were then quantified from spiked internal standards and normalized to protein content.

To quantify labeling on SL and deoxySL species from [U-13C18]palmitate, a Q-Exactive orbitrap mass spectrometer with a Vanquish Flex Binary UHPLC system (Thermo Fisher Scientific) was used with a Kinetex 2.6 μM C8 100 Å 150 × 3 mm LC column (Phenomenex) at 40°C. About 5 μl of sample was injected. Chromatography was performed using a gradient of 2 mM ammonium formate and 0.2% formic acid (mobile phase A) and 1 mM ammonium formate and 0.2% formic acid in methanol (mobile phase B), at a flow rate of 0.5 ml/min. The LC gradient held at 82% B for 0–3 min, then ran from 82 to 90% B in 3–4 min, then 90–99% in 4–18 min, held at 99% B for 7 min, then reduced from 99 to 82% from 25 to 27 min, and then held at 82% for a further 13 min. Lipids were analyzed in positive mode using spray voltage of 3 kV. Sweep gas flow was 5 arbitrary units, auxiliary gas flow was 7 arbitrary units, and sheath gas flow was 50 arbitrary units, with a capillary temperature of 300°C. Full MS (scan range m/z 150–2,000) was used at 70,000 resolution with 1e6 automatic gain control and a maximum injection time of 200 ms. Data-dependent MS2 (top 6) mode at 17,500 resolution with automatic gain control set at 1e5 with a maximum injection time of 50 ms was used for peak identification, combined with known standards where possible. Abundances of SL and deoxySL species were normalized to the spiked internal standards and protein content.

**Statistics**

Data visualization and statistical analysis were performed using GraphPad Prism (version 9.1.0, GraphPad Software, www.graphpad.com) and Adobe Illustrator CS6 (version 16.0.0, Adobe Inc, https://adobe.com/products/illustrator). The type and number of replicates and the statistical test used are described in each figure legends. Data are presented as means ± SEM or box (25th–75th percentile with median line) and whiskers (minimum to maximum values). Tissue culture was conducted in 12-well tissue culture plates for metabolic studies, 6-well tissue culture plates for soft agar colony formation assay, and 96-well culture plates for Seahorse analysis and growth responses (PrestoBlue assay). Each culture well was considered as a cellular replicate for a given preparation. One representative figure per condition is depicted for confocal microscopy analysis. P values were calculated using a two-sided Student’s t-test, one-way ANOVA or two-way ANOVA, and nested analysis was performed as indicated, with *P < 0.05; **P < 0.01; ***P < 0.001, and ****P < 0.0001 as indicated in each figure legend.

**Data availability**

The datasets generated and analyzed during the current study are available in the article and from the corresponding author upon reasonable request. The SPTLC1WT and SPTLC1C133W mammalian expression constructs are available at Addgene (Addgene plasmid 181920 and 181921). Data obtained from high-resolution MS are available at the NIH Common Fund’s National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned the Study ID ST002274. The data can be accessed directly via its Project DOI: http://dx.doi.org/10.21228/M8NH7M, and this work is supported by NIH grant U2C-DK119886.

**Supplemental data**

This article contains supplemental data.

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**Author contributions**

T. C. and C. M. M. methodology; T. C., R. S. K., G. H. M., J. G., and T. M. investigation; T. C. visualization; T. C. and C. M. M. writing—original draft.

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