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Presented by
M.Sc. Aysu Başak Kök
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**Addressing the impact of sphingoid base chain length
on cell signaling and function**

Referees: Prof. Dr. Ana Martin-Villalba

PD. Dr. Roger Sandhoff

To My Family

„Sizleri birer kıvılcım olarak gönderiyorum, alevler olarak geri dönmelisiniz“

"I am sending you as sparks, you must return as flames"

Mustafa Kemal Atatürk- The Founder of the Turkish Republic

I. Summary

Sphingolipids are essential lipid constituents of cellular membranes and their complex metabolic pathways interconnect amino acid, fatty acid, phosphoglycerolipid and carbohydrate metabolism. They influence the structure of cellular membranes, participate in signal transduction cascades and orchestrate cell-cell communication. Therefore, they contribute to the regulation of numerous cellular processes including proliferation, apoptosis and cellular differentiation. The core of sphingolipids is a sphingoid base, a long chain amino-alcohol, whose synthesis is initiated by the action of the enzyme serine palmitoyltransferase (SPT). SPT is a complex of 3 polypeptides and catalyzes the rate-limiting step of the sphingolipid biosynthesis. Therefore SPT activity is regulated, e.g. by inhibiting ORMDL peptides and by feedback regulation through ceramide concentrations in the membrane of the endoplasmic reticulum. Opposite to that the small subunit of the SPT complex (SPTSSA or SPTSSB) enhances the activity of the enzyme and determines the chain length of the final sphingoid bases. The length of the sphingoid bases in ceramides has been shown to have an impact on the properties of the bilayer, especially in influencing the order and fluidity of membranes, which can result in inducing phase separation for the formation of microdomains. As microdomains act as a hub for cell signaling by selectively recruiting or excluding proteins and facilitating the interaction between interaction partners, they are essential for modulating signaling pathways and associated signaling events. It has been shown that the sphingoid base length of ceramide has a greater impact on initiating microdomain formation and intermolecular interactions within the ceramide-rich gel phase as compared to the length of the acyl chain. As longer sphingoid bases tend to enhance stability of the microdomains, this possibly influences the duration and amplitude of associated signaling events. As of now, the latter had not been studied and the physiological roles and biological importance of sphingoid bases with carbon chains exceeding 18 carbons remain unclear.

To fill this gap, I successfully generated CRISPR-Cas9 edited SPTSSA knock out (KO) monoclonal neuroblastoma cells (SH-SY5Y). SPTSSA KO caused a decrease in SPT activity and an more than 7-fold increase in the amount of ceramides containing C20-sphingosine as compared to corresponding control cell clones, in line with recent reports in other cellular systems. However, steady state levels of total ceramides were not significantly affected. Compared to ceramides with C18-sphingosine, ceramides with C20-sphingosine were enriched

in C22- and C24-acyl chains, while C16-acyl chains were strongly decreased. To study the impact of this shift in ceramide composition on cell homeostasis and function, I monitored the cellular changes in SPTSSA KO cells both at steady state and upon induction of differentiation into neuron-like cells.

Through multiomics studies, I revealed that the changes in distribution of ceramide species altered a small subset of the proteome, phospho-proteome and the lipidome of the cell at a steady state. Most pronounced changes were observed in concentrations of soluble metabolites. Comparison of the proteome after generation of the SPTSSA KO clones and after more than 30 passages, reveal a few proteins, which had persistent differential expression between KO and control groups (AHNAK, SLC27A6, MVP, CNTNAP1, SH3PXD2A). However, those proteins associated with cell cycle and proliferation lost differential expression over time. This was paralleled by an initially reduced proliferation rate in KO clones, which vanished over time. Apparently, KO cells adapted to the genetic modification and transitioned back towards a more proliferative phenotype.

Analysis of EGF receptor signaling by evaluating the alternations in the phosphorylation status of tyrosine 992 (Y992), tyrosine 1173 (Y1173) and tyrosine 1069 (Y1069) following 10 min of EGF stimulation did not reveal noticeable differences in the intensity of induced phosphorylation patterns at the specified EGFR phosphorylation sites and their respective downstream targets.

Finally, I investigated the effect of SPTSSA KO on neuronal differentiation. Induction of differentiation reduced cell viability by inducing cell death specifically in SPTSSA KO. Furthermore, the SPTSSA KO caused a significant increase in the mitochondrial membrane potential as compared to that of control cells while not leading to significant differences in mitochondrial mass. The proteome analysis at steady state already indicated potential modifications in mitochondrial function, which were attributed to altered expression of the mitochondrial respiratory chain complex (I, III, and IV) components. Specifically, I confirmed the decreased levels of MT-CO2, the core catalytic subunit of complex IV, and the increased level of UQCRC2, a core subunit of complex III, in KO cells by Western blot analysis.

In conclusion, this study revealed that alterations in the composition of the SPT complex and corresponding ratio of sphingolipids containing either C18- or C20-sphingosine could lead to changes in the proteome, phosphoproteome, metabolome, and lipidome of the cells as well as to differential fitness for cell transitions. These findings underscored the importance

of maintaining ceramide homeostasis for fundamental cellular physiology, as exemplified here for cell differentiation and mitochondrial homeostasis.

II. Zusammenfassung

Sphingolipide sind essenzielle Lipidbestandteile von Zellmembranen, und ihre komplexen Stoffwechselwege verbinden den Aminosäure-, Fettsäure-, Phosphoglycerolipid- und Kohlenhydratstoffwechsel miteinander. Sie beeinflussen die Struktur zellulärer Membranen, nehmen an Signaltransduktionskaskaden teil und orchestrieren die Zell-Zell-Kommunikation. Daher tragen sie zur Regulation zahlreicher zellulärer Prozesse wie Proliferation, Apoptose und zellulärer Differenzierung bei. Der Kern der Sphingolipide ist eine Sphingoidbase, deren Synthese durch die Wirkung des Enzyms Serin-Palmitoyltransferase (SPT) initiiert wird. SPT ist ein Komplex aus 3 Polypeptiden und katalysiert den geschwindigkeitsbestimmenden Schritt der Sphingolipid-Biosynthese. Daher wird die SPT-Aktivität reguliert, z.B. durch inhibierende ORMDL-Peptide und durch Rückkopplungsregulation über Ceramidkonzentrationen in der Membran des endoplasmatischen Retikulums. Im Gegensatz dazu verstärkt die kleine Untereinheit des SPT-Komplexes (SPTSSA oder SPTSSB) die Aktivität des Enzyms und reguliert darüber hinaus die Kettenlänge der produzierten Sphingoidbasen. Es wurde gezeigt, dass die Länge der Sphingoidbasen von Ceramiden Einfluss auf die Eigenschaften der Lipidschicht hat, insbesondere durch Beeinflussung der Ordnung und Fluidität von Membranen, was zur Induktion von Phasentrennung und zur Bildung von Mikrodomänen führen kann. Da Mikrodomänen als Knotenpunkte für die Zellsignalgebung fungieren, indem sie selektiv Proteine rekrutieren oder ausschließen und die Interaktion zwischen Interaktionspartnern erleichtern, sind sie wesentlich für die Modulation von Signalwegen und zugehörigen Signalereignissen. Es wurde gezeigt, dass die Länge der Sphingoidbase von Ceramiden einen größeren Einfluss auf die Initiierung der Mikrodomänenbildung und intermolekulare Wechselwirkungen innerhalb der ceramidreichen Gelphase hat als die Länge der Acylkette. Da längere Sphingoidbasen dazu neigen, die Stabilität der Mikrodomänen zu erhöhen, beeinflusst dies möglicherweise die Dauer und Amplitude der zugehörigen Signalereignisse. Bisher wurde dies nicht untersucht, und die physiologischen Rollen und die biologische Bedeutung von Sphingoidbasen mit Kohlenstoffketten über 18 Kohlenstoffen bleiben unklar.

Um diese Lücke zu schließen, habe ich erfolgreich CRISPR-Cas9-editierte SPTSSA-Knockout (KO)-monoklonale Neuroblastomzellen (SH-SY5Y) generiert. SPTSSA-KO führte im Vergleich zu entsprechenden Kontrollzellklonen zu einer Abnahme der SPT-Aktivität und

einer mehr 7-fachen Zunahme der Menge von Ceramiden, die C20-Sphingosin enthalten. Letzteres ist in Übereinstimmung mit Publicationen in anderen zellulären Systemen. Gesamtceramidspiegel wurden durch den KO jedoch nicht signifikant beeinflusst. Im Vergleich zu Ceramiden mit C18-Sphingosin waren Ceramide mit C20-Sphingosin angereichert in C22- und C24-Acyلكetten, während C16-Acyلكetten stark abnahmen. Um den Einfluss dieser Verschiebung auf die Zellhomöostase und -funktion zu untersuchen, habe ich die zellulären Veränderungen in SPTSSA-KO-Zellen sowohl im Gleichgewichtszustand als auch nach Induktion der Differenzierung zu neuronartigen Zellen untersucht.

Durch Multiomics-Studien habe ich festgestellt, dass die Veränderungen in der Verteilung von Ceramidspezies einen kleinen Teil des Proteoms, des Phosphoproteoms und des Lipidoms der Zelle im Gleichgewichtszustand veränderten. Die meisten Veränderungen wurden in den Konzentrationen löslicher Metabolite beobachtet. Der Vergleich des Proteoms nach der Generierung der SPTSSA-KO-Klone und nach mehr als 30 Passagen zeigte einige Proteine, die zwischen KO- und Kontrollgruppen eine persistent differentielle Expression aufwiesen (AHNAK, SLC27A6, MVP, CNTNAP1 SH3PXD2A). Proteine, die jedoch mit dem Zellzyklus und der Proliferation verbunden sind, verloren im Laufe der Zeit ihre differentielle Expression. Dies wurde von einer anfänglich reduzierten Proliferationsrate in KO-Klonen begleitet, die im Laufe der Zeit verschwand. Offensichtlich passten sich KO-Zellen der genetischen Veränderung an und gingen wieder zu einem proliferativeren Phänotyp über.

Eine Analyse der EGF-Rezeptor-Signalgebung durch Auswertung der Veränderungen im Phosphorylierungsstatus von Tyrosin 992 (Y992), Tyrosin 1173 (Y1173) und Tyrosin 1069 (Y1069) nach 10 Minuten EGF-Stimulation ergab keine bemerkenswerten Unterschiede in der Intensität der induzierten Phosphorylierungsmuster an den spezifischen EGFR-Phosphorylierungsstellen und ihren jeweiligen nachgeschalteten Zielproteinen.

Abschließend untersuchte ich die Auswirkungen von SPTSSA-KO auf die neuronale Differenzierung. Die Induktion der Differenzierung reduzierte die Lebensfähigkeit der Zellen, indem sie den Zelltod bei SPTSSA-KO induzierte. Darüber hinaus führte der SPTSSA-KO zu einer signifikanten Zunahme des mitochondrialen Membranpotenzials im Vergleich zu dem der Kontrollzellen, ohne signifikante Unterschiede in der mitochondrialen Masse zu verursachen. Die Proteomanalyse im Gleichgewichtszustand deutete bereits auf potenzielle Modifikationen in der mitochondrialen Funktion hin, die auf eine veränderte Expression der Komponenten des mitochondrialen Atmungskettenkomplexes (I, III und IV) zurückzuführen

waren. Speziell bestätigte ich durch Western-Blot-Analyse die Abnahme von MT-CO2, der kernkatalytischen Untereinheit von Komplex IV, und die Zunahme von UQCRC2, einer Kernuntereinheit des Komplex III, in KO-Zellen. Zusammenfassend zeigt diese Studie, dass Veränderungen in der Zusammensetzung des SPT-Komplexes und des entsprechenden Verhältnisses von Sphingolipiden, die entweder C18- oder C20-Sphingosin enthalten, zu Veränderungen im Proteom, Phosphoproteom, Metabolom und Lipidom der Zellen sowie zu unterschiedlicher Fitness in der Zelltransformation führen können. Diese Ergebnisse unterstreichen die Bedeutung der Aufrechterhaltung der Ceramidhomöostase für die grundlegende zelluläre Physiologie, wie hier am Beispiel der Zelldifferenzierung und der mitochondrialen Homöostase aufgezeigt ist.

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As I put the final touches on this thesis and reach this incredible academic milestone, my heart overflows with love and gratitude for my amazing family. To my beloved parents - you are truly my heroes. You walked every step of this journey with me, listened to me for hours when the experiments didn't work, and celebrated every small victory with as much enthusiasm as I did. Whenever I stumbled, you held my hand. Your unconditional love gave me strength, encouraged me to persevere, work harder, and stay motivated. I am forever grateful for the countless sacrifices you made to provide me with every opportunity to pursue my dreams. To my dear brother - words cannot express how lucky I am to have such an amazing sibling. This accomplishment would not have been possible without having the best brother in the world cheering me on every step of the way. You were source of joy and truly my charger when my batteries were running low. Thank you for making me laugh even in the most stressful situations and providing me with the fresh perspectives when I desperately needed. You were not only my brother but also my intellectual companion throughout this process. I could discuss complex scientific concepts, theories, and experimental methodologies with you. You asked insightful questions that helped me view my research from new angles. My dear family- from the bottom of my heart, thank you very much for everything. I am truly blessed to have you as my family. Sizi çok seviyorum, iyi ki varsınız.

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Figure 1. From (Rabionet et al., 2023) “Sphingolipids are structurally defined by the long-chain (2S,3R)-2-amino-1,3-dihydroxy motif. R” describes a hydrophobic organic residue. Most common in mammals is an unbranched C13H27 residue, but variations in length between 16 and 28 carbons (total) are possible (blue box). Modifications of this long-chain base result in different sphingoid bases including sphinganine (Sa), sphingosine (So), (2S,3S,4R)-phytosphingosine (Phy), (4E,14Z)-sphingadienes (Sd), 6-hydroxy-sphingosine (6OH), 3-ketodihydrosphingosine (KDS), and the branched-chain anteiso 16-methyl-sphingosine (meC18So). In addition to these physiological bases (green box), mutations of serine-palmitoyl-CoA transferase (SPT) can result in the formation of pathological 1-deoxy(methyl)-sphinganine (dO(M)-Sa) and (14Z)-1-deoxy(methyl)-sphingosine (dO(M)-So) (red box). Sphingoid bases can be acylated at the 2-amino group. The variety of acyl residues covers saturated, mono and poly-unsaturated, hydroxylated, and ω -hydroxy-esterified fatty acyls, often found in lengths between 14 and 26 carbons. Mono-unsaturated species are rather very long (e.g., C24) and poly-unsaturated species are very to ultra-long (>C25). Longer species (up to C36) can be observed in specialized tissues like the skin or testis. The structural variety of sphingolipids is further expanded by modifications of the 1-hydroxy group (right box). This can involve glycosidic linkage of monosaccharides (e.g., Glucosylceramide (GlcCer) and galactosylceramide (GalCer)) or complex glycans (glycosphingolipids), and esterification with a phosphocholine group (sphingomyelin), a phosphate group (e.g., ceramide-1-phosphate; sphingosine-1-phosphate) or an acyl group (1-O-acylceramides)“.....1

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List of Abbreviations

μmol	Micromol
AGC	Automatic Gain Control
Akt	Protein Kinase B
ATRA	All-Trans Retinoic Acid.
bp	Base Pair
CAR	Ceramide analog
CD49b	Integrin subunit alpha 2
CE	Cholesteryl ester
Cer	Ceramide
Cholesterol-d7	Deuterated cholesterol
CL	Cardiolipin
CoQ6	Coenzyme Q6
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside Triphosphate.
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence Activated Cell Sorting
FCS/FBS	Fetal Calf/Bovine Serum
GAP43	Growth associated protein 43
GC-vial	1.8mL glass vial with screw cap containing a pre-slit Silicon/PTFE-septum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HexaN	Hexanucleotide Mix
HexCer	Hexosylceramide

IBAQ	Intensity Based Absolute Quantification
ITGA2	Integrin Subunit Alpha 2
KCl	Potassium Chloride
kDa	Kilodalton
LB	Lysogeny Broth
LC-MS/MS	Liquid Chromatography-Coupled Tandem Mass Spectrometry
LFQ	Label free quantification
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
M	Molar
MAP2	Microtubule associated protein 2
MG	Monoacylglycerol
ml	Milliliter
MM	Mitochondrial Mass
mM	Milimolar
MMP	Mitochondrial Membrane Potential
Na ₂ HPO ₄	Disodium Hydrogen Phosphate
NaCl	Sodium Chloride
NaF	Sodium Fluoride
NCE	Normalized Collision Energy
nmol	Nanomol
NSE	Enolase 2
ORMDL	Sphingolipid Biosynthesis Regulator
P/S	Penicillin and Streptomycin
PA	Phosphatidic acid
PBS	Phosphate-Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol

pmol	Picomol
PP	Polypropylene
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPL13	Ribosomal Protein L13
RT	Room Temperature
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SM	Sphingomyelin
SPTLC1	Serine palmitoyltransferase long chain base subunit 1
SPTLC2	Serine palmitoyltransferase long chain base subunit 2
SPTLC3	Serine palmitoyltransferase long chain base subunit 3
SPTSSA	Serine palmitoyltransferase small subunit A
SPTSSB	Serine palmitoyltransferase small subunit B
TBS	Tris-Buffered Saline
TBS-T	Tris-Buffered Saline with Tween-20
TFA	Trifluoroacetic acid
TFB1	Transformation Buffer 1
TFB2	Transformation Buffer 2
TG	Triacylglycerol

1. INTRODUCTION

1.1 De novo Sphingolipid Biosynthesis

Sphingolipids are class of lipids that contain a sphingoid base as core building block (Figure 1) and have a complex set of metabolically connected pathways in biological systems. These pathways involve the synthesis, breakdown, recycling, and conversion of sphingolipids (Figure 2) (Hannun & Obeid, 2018). De novo sphingolipid biosynthesis starts with a condensation of amino acid L-serine and acyl-CoA thioester substrate (generally, palmitoyl CoA) and this reaction is catalyzed by serine palmitoyltransferase (SPT). SPT is proposed to be a critical node for the regulation of sphingolipid metabolism since it facilitates the rate limiting step of the sphingolipid biosynthesis and accumulation of metabolic sphingolipid intermediates can be ceased by the regulation of this enzyme (Hanada, 2003).

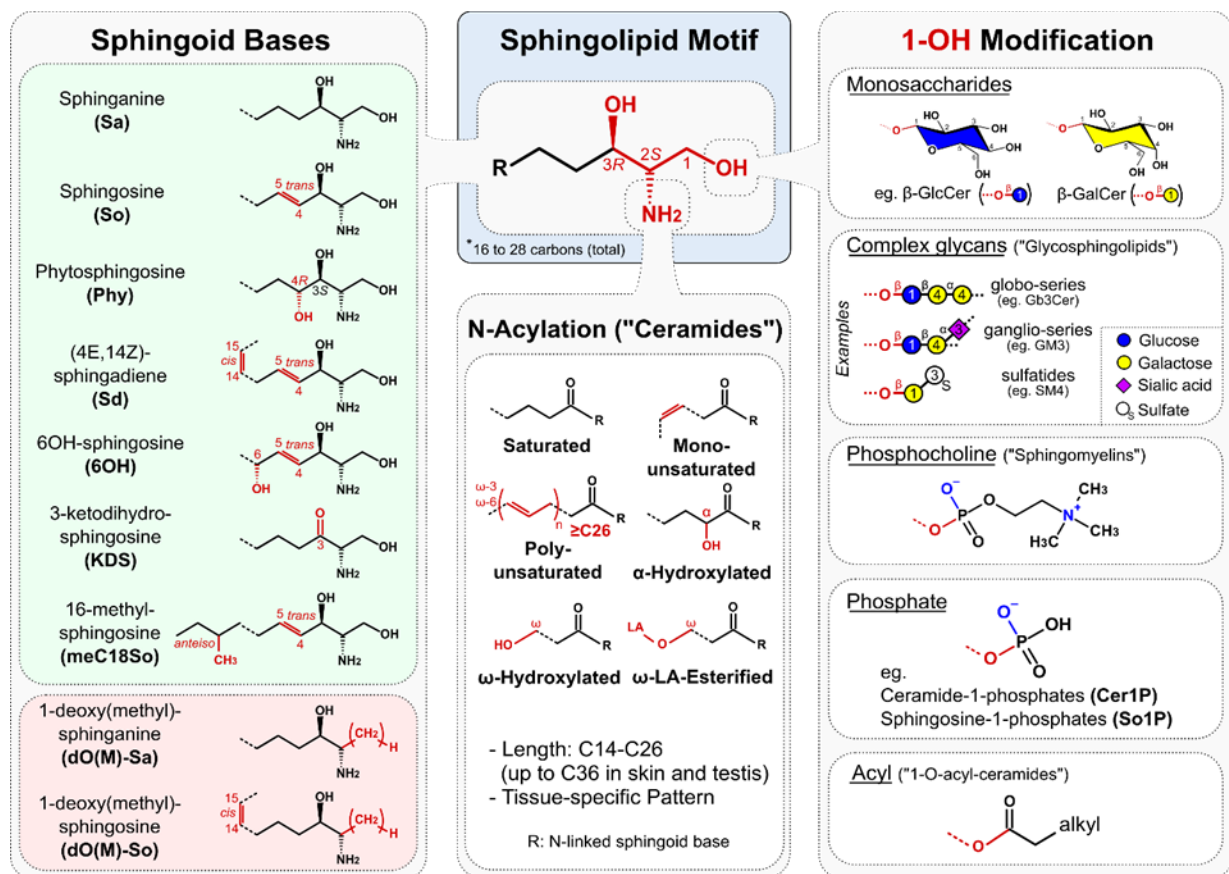


Figure 1. From (Rabionet et al., 2023) "Sphingolipids are structurally defined by the long-chain (2S,3R)-2-amino-1,3-dihydroxy motif. R" describes a hydrophobic organic residue. Most common in mammals is an unbranched C13H27 residue, but variations in length between 16 and 28 carbons (total) are possible (blue box). Modifications of this long-chain base result in different sphingoid bases

including sphinganine (Sa), sphingosine (So), (2S,3S,4R)-phytosphingosine (Phy), (4E,14Z)-sphingadienes (Sd), 6-hydroxy-sphingosine (6OH), 3-ketodihydrosphingosine (KDS), and the branched-chain anteiso 16-methyl-sphingosine (meC18So). In addition to these physiological bases (green box), mutations of serine-palmitoyl-CoA transferase (SPT) can result in the formation of pathological 1-deoxy(methyl)-sphinganine (dO(M)-Sa) and (14Z)-1-deoxy(methyl)-sphingosine (dO(M)-So) (red box). Sphingoid bases can be acylated at the 2-amino group. The variety of acyl residues covers saturated, mono and poly-unsaturated, hydroxylated, and ω -hydroxy-esterified fatty acyls, often found in lengths between 14 and 26 carbons. Mono-unsaturated species are rather very long (e.g., C24) and poly-unsaturated species are very to ultra-long (>C25). Longer species (up to C36) can be observed in specialized tissues like the skin or testis. The structural variety of sphingolipids is further expanded by modifications of the 1-hydroxy group (right box). This can involve glycosidic linkage of monosaccharides (e.g., Glucosylceramide (GlcCer) and galactosylceramide (GalCer)) or complex glycans (glycosphingolipids), and esterification with a phosphocholine group (sphingomyelin), a phosphate group (e.g., ceramide-1-phosphate; sphingosine1-phosphate) or an acyl group (1-O-acylceramides)“

Human SPT is a heterotrimeric protein residing in the endoplasmic reticulum membrane. It has two large catalytic subunits and one small regulatory subunit. The obligatory subunit for the SPT complex is SPTLC1. However, SPTLC1 lacks of the pyridoxal 5'-phosphate (PLP) binding motif which helps SPT complex to bind PLP for its activity. Therefore, SPTLC1 exists as a complex with either SPTLC2 or SPTLC3 which provides a lysine residue for PLP binding (Harmon et al., 2013). These pairings, SPTLC1/SPTLC2 and SPTLC1/SPTLC3 exhibit relatively low intrinsic catalytic activity and the basal activity of large subunits dimer is significantly enhanced (nearly 100-fold) when either of the two small subunits (SPTSSA or SPTSSB) is present in the complex (Han et al., 2009; Harmon et al., 2013; Li et al., 2021). The activity of SPT complex is regulated by three ORMDL family members to maintain homeostatic level of sphingolipid synthesis (Wattenberg, 2021).

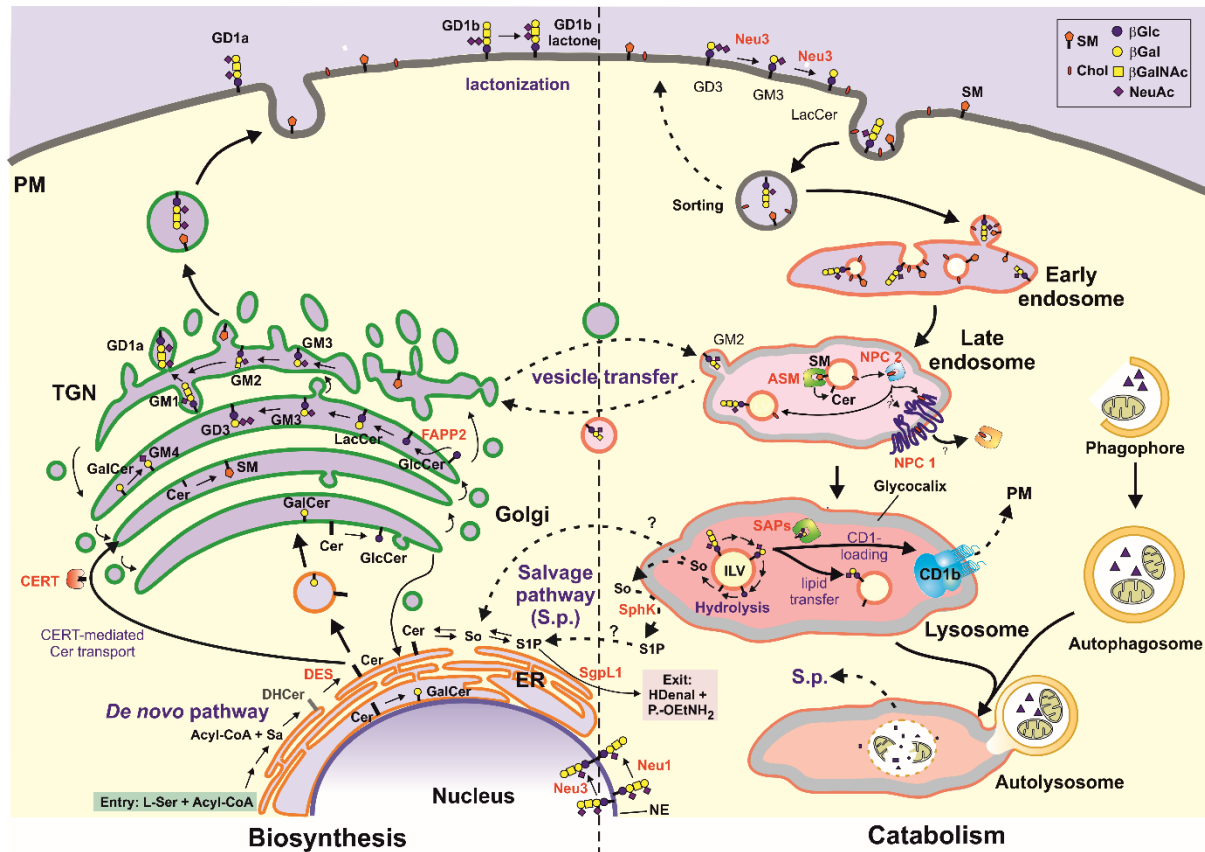


Figure 2. From (Sandhoff & Sandhoff, 2023) “Ganglioside biosynthesis and secretion starts at the endoplasmic reticulum (ER) and finalizes in the lumen of the Golgi system before gangliosides are carried to the plasma membrane by vesicular transport (anabolism, left side). Besides vesicular transport, some sphingolipids such as Cer and GlcCer are also transported between the ER and the Golgi system by lipid transfer proteins like CERT and FAPP2, respectively. Upon endocytosis and incorporation into intraendolysosomal luminal vesicles (ILVs), GG are degraded into monosaccharides, free fatty acids, and sphingoid bases (catabolism, right side), which are recycled for sphingolipid synthesis by the salvage pathway. Functional defects of any catabolic step cause an accumulation of the undegradable substrates in the lysosomes. The increasing lysosomal storage can trigger a reduced ability of lysosomes to fuse with autophagosomes, attenuating autophagy. ASM acid sphingomyelinase, Cer ceramide, CERT ceramide transfer protein, DES dihydroceramide desaturase, DHCer dihydroceramide, FA fatty acid, GlcCer glucosylceramide, ER endoplasmic reticulum, FAPP2 (PLEKHA8) four-phosphate adaptor protein 2 (pleckstrin homology domain containing A8), LacCer lactosylceramide, NE nuclear envelope, NEU neuraminidase, NPC Niemann–Pick disease type C protein, PM plasma membrane, Sa sphinganine, SAP sphingolipid activator protein, Sgpl1 S1P lyase, SM sphingomyelin, So sphingosine, S1P sphingosine-1-phosphate, SphK sphingosine kinase, TGN trans Golgi network, HDenal hexadecenal, P.-OEtNH₂ phosphoryletanolamine”

1.2 Variations in Sphingoid Backbone Chain Length

Sphingoid bases which are also known as long chain bases (LCBs) are the precursors of complex sphingolipids. Sphingosines and sphinganine, which are the most commonly found sphingoid bases in mammals, are generated through different pathways. Sphingosines are typically produced via the salvage pathway, while sphinganine is synthesized as part of the de novo sphingolipid synthesis (Quinville et al., 2021).

More than 60% of mammalian sphingolipids contains a sphingoid base backbone composed of a canonical 18-carbon and dihydroxy, monounsaturated acyl chain (d18:1) (Lam et al., 2021). However, there is a significant variation in the backbone structures. In mammals, the length of the sphingoid bases typically falls within the range of 14 to 22 carbons. However, there have been reports of sphingoid bases with a length ranging from 12 to 26 carbons (Pons et al., 2002; Stewart & Downing, 1995). The diversity in sphingoid bases can stem from various factors. One factor contributing to this diversity is mutations in SPT complex, which affects the selection of amino acids during the condensation reaction catalyzed by SPT (see deoxycompound in Figure 1). Variations in sphingoid bases can also arise from the incorporation of the type of condensing acyl-CoA (Tidhar & Futerman, 2013). This is determined by the configuration of the SPT enzyme complex. The SPT complex may be formed by four possible combinations where each configuration gives a unique preference for the length of the acyl-CoA substrate (Figure 3). In general, SPTSSA is highly selective for 16C-CoA and shows little activity with shorter or longer acyl-CoA substrates. However, SPTSSB enables the use of 18C-CoA or even longer acyl-CoAs as substrate for SPT to change the acyl-CoA preference of SPT in mammalian cells (Han et al., 2009). Therefore, the combination of SPTLC1/SPTLC2/SPTSSA favors the condensation of L-serine with palmitoyl-CoA (16 carbon) and thus leads to generation of 18 carbon sphingoid bases. On the other hand, presence of SPTSSB instead of SPTSSA in the complex prefers stearoyl-CoA (18 carbon) next to palmitoyl-CoA, which results in the additional production of 20 carbon sphingoid bases besides of 18 carbon (Harmon et al., 2013). Another possible combination of SPTLC1/SPTLC3/SPTSSA promotes the use of myristoyl-CoA (14 carbon) as a substrate and this results in synthesis of 16 carbon sphingoid bases besides of 18 carbon (Hornemann et al., 2009). The final combination of the SPT complex (SPTLC1/SPTLC3/SPTSSB) can utilize diverse acyl-CoAs as a substrate to produce 16-24 carbon sphingoid bases (Suzuki et al., 2022).

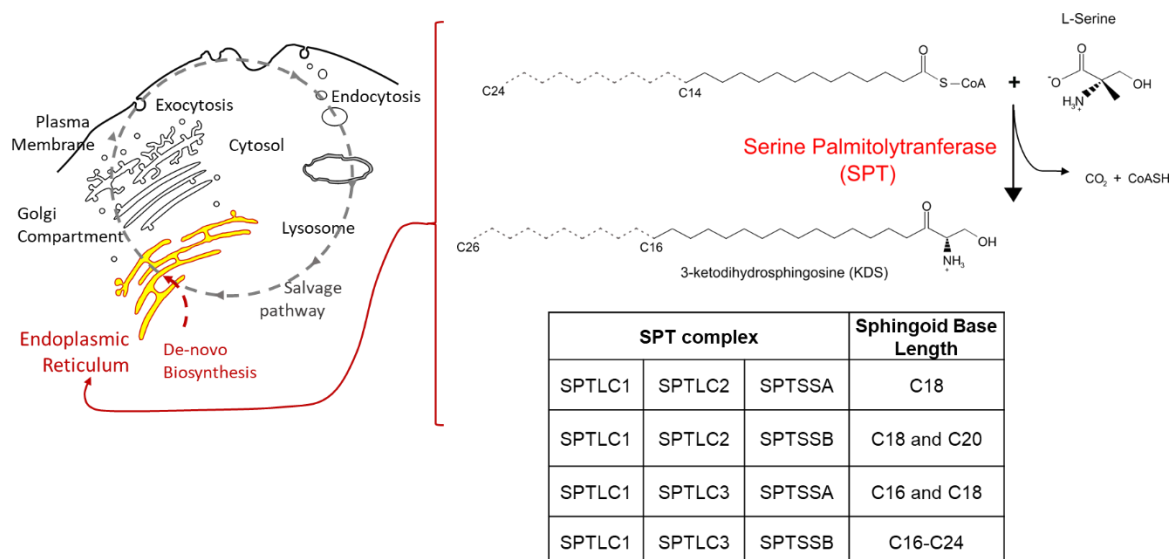


Figure 3. SPT complex subcellular location within the cellular flux of sphingolipids, its catalyzed reaction and its compositional variations with in correlation with preferred length of products

SPTSSA and SPTSSB have been shown to contain ~35 amino acid long conserved core domain which is named as minimal activating domain. This part of the small subunits is highly homologous to each other and is responsible for enhancing the activity of SPT complex. The core domain does not only regulate the activation of the complex but also carry the information of acyl-CoA preference. Surprisingly, this information is hidden in a single residue, Met²⁵ in SPTSSA and Val²⁵ in SPTSSB of core domain (Harmon et al., 2013). The preference for the acyl-substrate due to a change in single amino acid suggested to be caused by the size of the side chain of the amino acid in this position. The structural analysis of SPT complex focusing on SPTSSA and SPTLC2 interactions has revealed that the side chain of Met in SPTSSA protrudes and covers the substrate binding pocket in SPTLC2. Therefore, the binding of substrates longer than 16 carbon such as stearyl-CoA would be energetically disfavored and this limits the length of acyl-CoA that can be used. On the other hand, Met is substituted by Val in SPTSSB and Val contains a much smaller side chain compared to the Met which allows longer acyl-CoA to fit (Wang et al., 2021). Consistent with these findings, the mutant SPTSSA^{M25G}, where Met has been replaced with Gly in residue 25, has been shown to efficiently utilize acyl-CoA with 20- and with lesser extent also 22-carbon chains (Harmon et al., 2013).

1.3 The Concept of Microdomains as Signaling Platforms

Microdomains are assemblies on the cell membrane which are characterized by their high content of cholesterol and sphingolipids and show dynamic movement within the fluid like lipid bilayer of the membrane (Allen et al., 2007). The structure of microdomains varies considerable in terms of size, composition and dynamics. There are four described forms of lipid domains: small regions enriched in fluid lipids, glycosylphosphatidylinositol (GPI)-anchored protein and glycosphingolipid enriched nanodomains (20-30 nm) (Mayor & Maxfield, 1995), small and flask shaped invaginations called caveolae (55 nm) (Kurzchalia & Parton, 1999) and transient confinement zones (100-300 nm) (Chen et al., 2004). The exact relationship or interaction of each lipid domain is not fully understood. However, components of caveolae and nanodomains have been shown to be detergent resistant and are thought to form liquid ordered phase (I_o) domains (Maxfield, 2002).

In general, microdomains are short lived assemblies, typically lasting for 1 millisecond or less. Their composition varies due to dynamic nature of the surrounding environment where molecules move in and out of these microdomains. The smallest microdomain (radius of 5 nm) consist of approximately 30-40 lipids and around 6-10 proteins (Sengupta et al., 2007). This raises the question of how such a small and short lived microdomains influences the cellular processes, particularly the signal transduction. Nicolau et al. used a stochastic spatial model of the plasma membrane to investigate the impact of microdomains on protein dynamics. They purposed that microdomains can concentrate or isolate the proteins which might eventually help or hinder protein interactions. They serve as selective concentrators for specific proteins with which they exhibit high affinity. Consequently, they enhance the collision between proteins, even when the concentration of these proteins of interest is low (Nicolau et al., 2006). Consequently this could impact signaling intensity and duration by that impacting for example signal intensity and duration (Golub et al., 2004)

Microdomains act as a hub by selectively recruiting or excluding proteins including kinases, phosphatases and proteins that are part of the signaling pathways. They adjust their size and composition based on the stimuli to protect proteins from degradation and facilitate the interaction between interaction partners (Li et al., 2022). They help proteins to oligomerize and form large molecular assemblies. This modulates their special arrangement and eventually the accessibility to regulatory or effector molecules. Moreover, microdomains can shield signaling complexes from the inhibitory effect of non-microdomain molecules. Therefore, they

isolate proteins that have the potential to stimulate or inhibit certain pathways by preventing the signal to further move onto the downstream targets. In this manner, microdomains act as signaling platforms and participate in key cellular processes including signal transduction, membrane trafficking and protein sorting (Mollinedo & Gajate, 2020).

Localization of the proteins to the microdomains is often determined by structural properties or posttranslational lipid modifications. For example, proteins having hydrophobic tail including GPI linkage (due to phosphoinositide anchors typically with saturated chain), N-myristoylation, or S-palmitoylation may contribute to their localization in rafts as palmitoylation is reversible and can make proteins flexible to move in or out of the microdomains (Levental et al., 2010; Mollinedo & Gajate, 2020). Some proteins have been suggested to have higher tendency to be present in microdomains. Some examples are dually acylated proteins (e.g. Src-family protein tyrosine kinases) (Zaman et al., 2008), palmitoylated type-I transmembrane proteins (e.g. CD44) (Thankamony & Knudson, 2006), transmembrane proteins (e.g. flotillin), and receptor tyrosine kinases with two transmembrane subunits (such as insulin receptor (IR) (Saltiel & Pessin, 2003) and the epidermal growth factor receptor (EGFR) (Hofman et al., 2008)). The length of the proteins transmembrane helices also impacts the presence in microdomains as the latter are thought to be thicker than liquid-disordered phases of the cell membrane.

1.4 Function of Lipids in Microdomains

The plasma membrane of the cell is composed of wide range of lipids including sterols, phosphoglycerolipids, glycolipids and sphingolipids. Each is present in different quantities and proportions. (Kim et al., 2021). The lipid bilayer, regardless of the diversity in lipid content, serves as a barrier that separates subcellular compartments as well as the cell from its surrounding. Diversity in lipid structure from subtle difference (for example, position of the double bonds) to significant difference (for example, change of the backbone) leads to an additional layer of complexity and influences the essential biological functions. However, the diversity in lipids extends beyond their chemical structure. Lipids also vary in how they're arranged in cells, like how many of each type there are in different parts of the cell, such as in different organelles or in different layers of cell membranes, and in different regions of those membranes (Antonny et al., 2015; Sezgin et al., 2017). This diversity in composition influences the collective behaviour of lipids in the membranes, governs interactions between lipids and

proteins, as well as between different lipid species and eventually translates into different functions (Harayama & Riezman, 2018).

The membrane lipid bilayer can be present in two phases: the gel phase or the liquid phase. This is determined by the lipid composition of the membrane including the degree of the saturation of the lipids. Depending on these parameters, each bilayer has its unique melting temperature. Below the melting temperature, the lipid bilayer presents in gel phase. In this state, lipid molecules are organized and have restricted mobility. Above this temperature, the bilayer transitions into fluid state and is disordered. This state also known as liquid-disordered (l_d) phase. However, diversity in the lipid composition of the membrane can lead to a phase separation and formation of liquid-ordered phase (l_o) also called as microdomains (or lipid rafts). Liquid-ordered phase has the property that bridges the gap between the gel and the liquid phase. This phase is similar to the ordered like gel phase but it still maintains the fluid like mobility (Gupta & Surolia, 2010). Therefore, microdomain formation is influenced by interactions between different lipids and these interactions rely on the structure and biophysical features of the lipid components.

The presence of long-chain saturated sphingolipids and phospholipids together with physiological level of cholesterol has been suggested to enhance microdomain formation and help gathering the membrane proteins to collaborate in their function (Barba-Bon et al., 2020; Chakraborty & Jiang, 2013; Sonnino & Prinetti, 2010). Cholesterol plays a major role in modulating the lipid organization within cell membrane and thereby the phase state of the lipid bilayers. It acts as a glue to closely pack the acyl chains of phospholipids which increases the order of the acyl chains and slows down the lateral movement of the lipids (Goh & Tero, 2021; Mollinedo & Gajate, 2020). Moreover, microdomain formation is influenced by cholesterol levels and can respond to both acute and chronic changes in cholesterol levels within the raft (Fessler & Parks, 2011). Therefore, they are crucial for defining the membrane's unique characteristics and sustaining both rigid and flexible structures in the plasma membrane

Sphingolipids are relatively minor but essential components of the eukaryotic cell membranes. They orient themselves in a way that their hydrophilic head group faces towards the extracellular environment and their long hydrophobic chain is embedded into the hydrophobic core of the lipid bilayer (D'Aprile et al., 2021; van Meer et al., 2008). Their hydrophobic core is mostly saturated (or trans-unsaturated), allowing them to pack more tightly and adopt the gel phase. They are composed of sphingoid bases, amide linked fatty acid with

variable chain length (C14-C26) and a polar head group (Figure 1). The sphingoid base linked to a long chain fatty acyl chain form a ceramide (Pinto et al., 2011). Therefore, ceramide is the simplest bilayer forming sphingolipid and serves as the foundational structure for all complex sphingolipids. Ceramide can be modified further by addition of polar head groups, including phosphocholine (resulting in sphingomyelin), carbohydrates (forming glycosphingolipids), or other functional groups (Figure 1). Glycosphingolipids can be transformed into ganglioside by the addition of terminal sialic acids. These modifications lead to the formation of wide variety of complex sphingolipids which together give them their characteristic amphipathic structure (Reynolds et al., 2004; van Meer et al., 2008).

As a central metabolite in the biosynthesis of sphingolipids, ceramides, can be produced through *de novo* synthesis, via the hydrolysis of sphingomyelin by sphingomyelinases or the salvage pathway from long chain sphingoid bases by the action of ceramide synthases (Kitatani et al., 2008). Sphingomyelinases associated with microdomains have been suggested to hydrolyze the sphingomyelin and cause the release of ceramide *in situ* e.g. by converting sphingomyelin at the plasma membrane into local ceramide enriched plasma membrane domains upon activating stimuli. Therefore, ceramides are considered to be part of the microdomains (Holopainen et al., 1998; Schneider & Kennedy, 1967; Xu et al., 2001). Ceramides are naturally hydrophobic molecules because of its long fatty acyl chains and a small single hydroxyl polar head group. Due to their nature, they increase the acyl chain order and stabilizing the liquid-ordered state of phospholipid bilayer (Massey, 2001; Quinville et al., 2021). In more complex bilayers, ceramide has the capacity to bring sphingomyelin to the microdomains to form sphingolipid-enriched microdomains. In microdomains, cholesterol is inserted between the long, saturated acyl chains of sphingomyelin and glycosphingolipids and stabilizes the microdomains by helping to alleviate the repulsive interactions between the large headgroups of sphingolipids in the water phase (Huang & Feigenson, 1999; Simons & Ikonen, 1997). At specific concentration, however, ceramide can exclude the cholesterol from the microdomains (Megha & London, 2004; Yu et al., 2005).

Ceramides with distinct fatty acyl chains exhibit specific physiological effects on microdomain formation. It has been shown that ceramides with long (C16- and C18-) compared to very long chain fatty acid (C24-) or saturated versus unsaturated chains (C18:1- and C24:1-) has differential impact on phosphatidylcholine model membranes. For example, ceramides with saturated chains have been shown to have greater impact on enhancing the order of fluid

membranes and inducing the phase separation compared to their unsaturated counterparts. Specifically, ceramides with unsaturated chains, such as C24:1, demonstrated a reduced ability to form gel domains, while ceramides with C18:1 unsaturated chains showed no effect on gel domain formation at physiological temperature. The effect of saturated ceramides on the properties of the membrane remained consistent and showed only minor changes with acyl chain length. However, each saturated ceramide led to formation of domains with difference sizes and morphologies. Moreover, it has been shown that the long chain unsaturated ceramides are incapable of increasing the order of the membrane and induce phase separation on the membrane (Pinto et al., 2011).

The length of the sphingoid bases of ceramides has been shown to have an impact on the properties of the bilayer. Maula et. al. conducted a study where they used ceramides with varying sphingoid bases (12, 14, 16, 18, or 20-carbon derived from sphingosine) coupled to palmitic acid (PCer). They investigated how the length of the sphingoid bases impacts the formation of gel phases in bilayers containing palmitoylphosphocholine (POPC) and/or palmitoylsphingomyelin (PSM), as well as in more complex bilayers comprising POPC, PSM, ceramide, and with or without cholesterol. They found that 16:1-, 18:1-, and 20:1-PCer facilitated the formation of the gel phase, while 12:1- and 14:1-PCer failed to do so (Maula et al., 2012). Another study performed by Al Sazzad et. al demonstrated that with increasing length of the sphingoid bases, less ceramide was required to induce lateral segregation and form ceramide-rich phases in POPC bilayers. A comparable yet less pronounced pattern was detected. when sphingosine (d18:1)-based ceramide containing N-linked acyl-chains of increasing length (ranging from 14:0 to 16:0–20:0 with increments of one carbon) were investigated. In the same study, they used two ceramides with equal carbon numbers (d16:1/17:0 or d20:1/13:0) and observed that those with longer sphingoid bases formed ceramide-rich phases at lower concentrations and had more stable gel phases compared to analogs with shorter sphingoid bases. These studies suggest that the sphingoid base length of ceramide has a greater impact on initiating lateral segregation and intermolecular interactions within the ceramide-rich gel phase compared to the length of the acyl chain (Al Sazzad et al., 2017).

1.5 Lipid Mediated EGFR Activation and Signaling

The epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptor tyrosine kinases and also called as HER-1 or ErBB-1. EGFR is composed of four parts including extracellular ligand binding and dimerization domain (I-IV), a single hydrophobic transmembrane domain (TMD), juxtamembrane domain (JMD), a cytoplasmic tyrosine kinase (KD) and C-terminal tail domain (Figure 4) (De Luca et al., 2008; Kovacs et al., 2022; Wee & Wang, 2017).

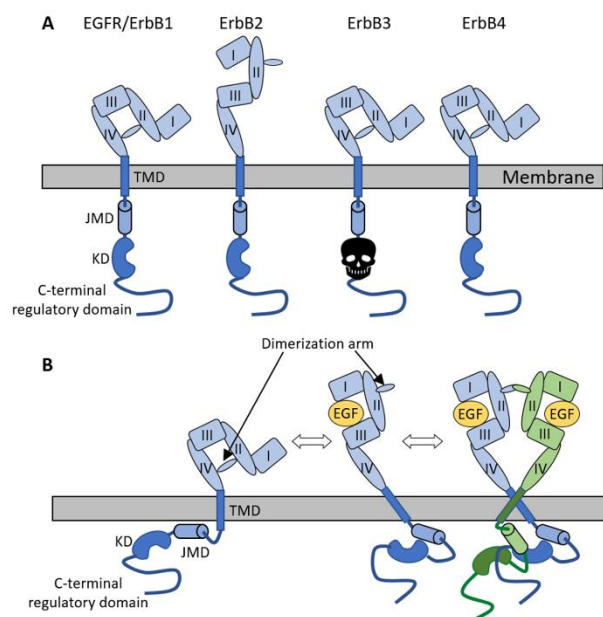


Figure 4. Organization of ErbB family receptors (A) in the absence of ligand and (B) their activation on upon ligand binding at the plasma membrane. (A) The ErbB family consists of four members (ErbB1- 4) and each has extracellular domain, transmembrane domain (TMD), the juxtamembrane domain (JMD), the kinase domain (KD), and the C-terminal regulatory domains on the intracellular side. When there is no available ligand, EGFR/ErbB1, ErbB3 and ErbB4 stays in closed conformation while ErbB2 remains in an extended conformation characterized by autoinhibitory interactions, which restrict access for growth factor binding. Mutations in ErbB3's KD can reduce or completely eliminate its activity (shown as skull). The components of the extracellular domains named I-IV. (B) The structure of the monomeric EGFR is in closed form as shown in the left side. In the absence of ligand, the dimerization arm (shown as oval shape) is hidden inside the protein which results in formation of intramolecular bridge between domains II and IV. Ligand binding to domain I and III causes a rearrangement, creates a binding pocket for the ligand and exposes the dimerization arm. Dimerization arm can now make an intermolecular bridge (shown as green oval shapes) with another EGFR having the extended conformation (shown as green on the right side) (Figure is from (Kovacs et al., 2022))

EGFR signaling plays a crucial role in coordinating fundamental cellular processes. It promotes cell proliferation, differentiation, migration and cell survival while inhibiting apoptosis. The dysregulation of EGFR signaling could lead to tumor development and progression. Therefore, its dysregulation is considered as a major driver of cancer (Sabbah et al., 2020).

There are seven EGFR ligands capable of initiating signaling event in the cells. These are epidermal growth factor (EGF), transforming growth factor- α (TGF- α), amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), and epigen (EPI). Interestingly, despite their diverse downstream effects, all these ligands activate EGFR in a similar manner (Wee & Wang, 2017). In the absence of a ligand, the extracellular domain of EGFR adopts a closed conformation. This configuration involves an intramolecular bridge formed between domain II and IV, which keeps the dimerization domain buried within the structure. EGFR activation initiates upon ligand binding, inducing a transition to an extended conformation. This transition involves rearrangement of domain I and III, resulting in the formation of a ligand-binding pocket and the release of the dimerization arm. Consequently, intermolecular interactions occur with another EGFR molecule in the extended conformation, leading to receptor dimerization. Within the active receptor dimer, the transmembrane domains come together to form a stable dimer which is promoted by the N-terminal dimerization motif. In the meantime, the juxtamembrane domain disengages from anionic phospholipids within the membrane. Subsequent events include transautophosphorylation of the receptor and the recruitment of signaling proteins and adaptors (Kovacs et al., 2022).

Lipids in the plasma membrane are recognized to interact with EGFR via its transmembrane and juxtamembrane domain. These interactions act to stabilize conformational state of the EGFR. They also modulate and fine-tune the proteins interactions happening at the boundary between the lipid bilayer and the surrounding environment (Kim et al., 2021). For example, the inactive monomeric form of EGFR (depicted on the left side in figure 2B) stays stable via the direct interactions between N-acetylglucosamine of EGFR and carbohydrate moiety of GM3 ganglioside (Coskun et al., 2011). This inactive state is further stabilized by facilitating the embedding of JMD into the plasma membrane. This occurs through electrostatic interactions between the negatively charged phosphatidylserine (PS) on the inner leaflet of the

plasma membrane and the positively charged amino acids within the JMD (Arkhipov et al., 2013).

The EGF binding to extracellular binding domain has been shown to induce twist in the JMD and consequently leading to rotation in the TMD. This rotation shifts the TMD conformation from the inactive C-terminal dimer to the N-terminal dimer, enhancing dimer stability and exposing the intrinsic catalytic kinase to tyrosine residues on the C-terminal domain and other substrates (Moriki et al., 2001). Phosphatidylcholine is suggested to function as a solvent, influencing the flexibility and dynamics of the TMD conformation (Kim et al., 2021).

Other studies have demonstrated the impact of lipids on EGFR signaling, yet they often lack an explanation of the underlying molecular mechanism. For example, in a study conducted by Mirkin et al., showed that gangliosides GT1b, GD1a, GM3 and GM1a have a negative impact on cell proliferation and phosphorylation of EGFR in the N-myc amplified human neuroblastoma cell line NBL-W. Among all, GT1b has been found to have the most inhibitory effect on the phosphorylation of EGFR (Mirkin et al., 2002). Similarly, Zhou and Guan, observed that overexpression of GM1a in MCF10A, MCF7, and MDA-MB-231 cells lead to suppression of EGFR activation and cell proliferation at high cell densities (Zhuo & Guan, 2020).

In the presence of cholesterol in the membrane and upon ligand binding, EGFR relocates from the microdomain for activation. However, when the microdomain is disrupted by depleting cholesterol with methyl- β -cyclodextrin treatment, EGFR has been shown to be abnormally activated, independent of ligand presence. In other words, the absence of cholesterol in the membrane triggers spontaneous ligand-independent autophosphorylation of EGFR. This phenomenon may be attributed to changes in the membrane's biophysical properties and receptor microenvironment, which could affect membrane fluidity, release of inhibitory constraints, consequently, receptor function. Although the precise mechanism remains elusive, effective EGFR activation necessitates initial presence within the microdomain followed by translocation out of it for full activation (Isik & Cizmecioglu, 2023; Takayama et al., 2024).

1.6 Aim of the Thesis

Sphingolipids are integral components of the cells, involved in the cellular organization and the dynamics of signaling processes. Among their diverse functions, sphingolipids play an essential role in the formation and maintenance of specialized membrane microdomains. These microdomains are known for their pivotal role in orchestrating localized signaling events, thereby regulating various cellular processes.

The length of the sphingoid bases, fundamental structural components of sphingolipids, is predicted to influence the tendency of ceramides to form ceramide rich microdomains and exerts more pronounced influence on intermolecular interactions to a higher degree as compared to the acyl chain length of ceramides. Recent studies suggest that longer sphingoid bases not only promote the formation of these microdomains but also enhance their stability, potentially influencing the duration and amplitude of associated signaling events. However, the function and biological significance of sphingoid bases with carbon chains exceeding 18 remains elusive.

The aim of this project is to generate cell lines producing longer sphingoid bases (>18 carbon) and examine the effect of the chain length of the sphingoid bases on cell signaling and overall cellular functionality. To achieve this goal, the strategy involved permanently deleting the small subunit A (SPTSSA) from the SPT complex in the neuroblastoma cell line SH-SY5Y. These engineered mutant cell lines were then planned to be used for comparison in terms of biochemical composition and cellular function.

2. METHODS AND MATERIALS

2.1 METHODS

2.1.1 Molecular Cloning

2.1.1.1 Designing and Annealing of gRNAs for Crispr/Cas9 Gene Editing

Guide RNAs targeting exon1 of SPTSSA gene were designed using CRISPOR tool (<http://www.crispor.tefor.net>) (Concordet & Haeussler, 2018). Tool ranked the predicted guide sequences for PAMs by default highest to lowest specificity score (Hsu et al., 2013). Among the top 10 guide RNAs with high MIT and CFD specificity, guide RNA93 (gRNA93) targeting the region that is closest to the sequence influencing the SPT activity and acyl-CoA selectivity was selected (Figure 5). The sense and antisense oligos contained a 5'G (for efficient transcription from U6 promoter) and additional sequence for *Bbs*I directed restriction cloning (Table 26).

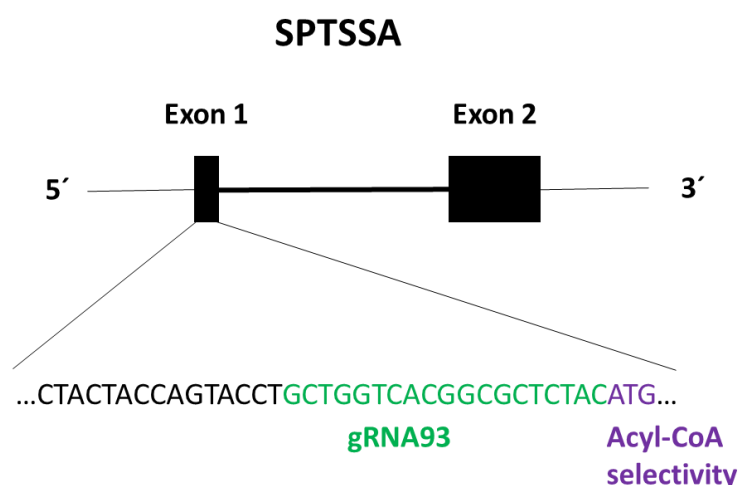


Figure 5. A partial sequence of exon 1 including the gRNA93 sequence in green and the first three base pairs of the sequence determining the acyl-CoA selectivity (purple) of SPTSSA is printed.

Complementary oligos were resuspended in nuclease free water to a final concentration of 100 μ M. They were annealed to themselves in an annealing reaction mix with a heating-cooling method using water bath (Table 1).

Table 1. Annealing reaction mix

Reagent	Volume (per reaction)
gRNA93 Sense	2 µl
gRNA93 Antisense	2 µl
NEBuffer 2	2 µl
Nuclease free ddH ₂ O	14 µl
Total	20 µl

Briefly, inside a closed 200 µL-sized polypropylene (PP) tube, they were placed in 500 ml beaker filled with 300 ml boiling water (around 95-100°C) and allowed to gradually cool to room temperature. The success of the annealing reaction was checked with 2% agarose gel. Annealed oligos were cut from the gel and purified with QIAGEN Gel Extraction kit following the manufacturer's instructions.

2.1.1.2 Restriction Digestion of pSpCas9(BB)-2A-GFP (PX458)

The CRISPR/Cas9 plasmid pSpCas9(BB)-2A-GFP (PX458) was digested with *Bbs*I enzyme in a mix described in Table 2 at 37°C for 2 hours. Digested plasmid was run on 1% agarose gel and purified with QIAGEN Gel Extraction kit following the manufacturer's instructions.

Table 2. Restriction digestion mix

Reagent	Volume (per reaction)
pSpCas9(BB)-2A-GFP	5 µg
BbsI (10 U/µl)	2 µl
10X Buffer G	3 µl
Nuclease free ddH ₂ O	20 µl
Total	30 µl

2.1.1.3 Ligation of gRNA93 and pSpCas9(BB)-2A-GFP

The annealed gRNA93 in (section 2.1.1.1) was inserted into a digested pSpCas9(BB)-2A-GFP with a ligation reaction described in Table 3. The molar ratio of 4 insert: 1 vector was utilized. A total of 100 µg of vector was used for ligation. The reaction was incubated for 2

hours at room temperature. An aliquot of ligation mixture was used for bacterial transformation.

Table 3. Ligation reaction mix

Reagent	Volume (per reaction)
pSpCas9(BB)-2A-GFP	x μ l
Insert	x μ l
T4 DNA Ligase Buffer	4 μ l
T4 DNA Ligase (1u/ μ l)	1 μ l
Nuclease free ddH ₂ O	Up to 20 μ l
Total	20 μ l

2.1.1.4 Competent Bacteria Preparation

E. coli DH5 α bacteria were spread over LB agar plate using sterile loop and incubated overnight at 37 °C. A single colony was picked and inoculated to 5 ml sterile LB media and the bacterial culture was incubated for 16 hour at 37°C with shaking at 200 rpm. 1 ml of the overnight culture was reinoculated to 200 ml fresh sterile LB media and incubated at 37 °C with shaking at 200 rpm until optical density at 550 nm (OD₅₅₀) reached 0.3-0.4. The bacterial culture was centrifuged for 5 minutes at 2500 rpm at 4°C. Supernatant was removed and the bacterial pellet was resuspended in 15 ml TFB1 buffer (Table 20). It was incubated on ice for 10 minutes and centrifuged 5 minutes at 2500 rpm at 4°C. Supernatant was removed and the pellet was resuspended in 1.2 ml TFB2 buffer (Table 20). Resuspended bacteria were incubated on ice for 45 minutes. The resultant competent bacteria were aliquoted and stored at -80°C

2.1.1.5 Transformation

Competent *E. coli* DH5 α were thawed while being kept on ice. Ligation product (4 μ l) was added to competent bacteria in a 1.5 ml PP tube and mixed by pipetting. Competent bacteria/DNA mixture was incubated on ice for 30 minutes and then it was subjected to the heat shock by placing the tube to a preheated blocks at 42°C for 90 seconds. Afterwards, tube was immediately placed back into ice for 2 minutes. A total of 500 μ L S.O.C medium was added to the tube and the bacteria were incubated in a shaker (at 800 rpm) at 37°C for 30 minutes. An appropriate volume of bacteria was plated onto a 10 cm LB Agar plates containing 100 μ g/ml ampicillin and incubated overnight in an incubator at 37°C.

2.1.1.6 Colony PCR

On the next day after transformation, single bacterial colonies were screened by PCR to verify whether the cloned plasmid was carrying the insert. The PCR reaction was set up by optimizing parameters for each reaction according to the primers used (Table 4). One colony was picked with a sterile pipette tip and briefly dipped into the PCR mix (Table 4) and the tip was placed in a well of a 96-well plate containing LB media for further use. After the PCR, the reaction mix was directly run on 2% agarose gel at 110V for 50 minutes. Pipette tips carrying positive clones dipped into 5 ml LB media (containing 100 µg/ml ampicillin) in a 15 ml tube and were cultured at 37°C overnight in shaking incubator (225 rpm).

Table 4. Colony PCR

Reagent	Volume (per reaction)
10X Reaction Buffer	1.5 µl
10 mM dNTPs	0.5 µl
5 µM F/R Primer Mix	1.5 µl
Taq Polymerase	0.25 µl
Nuclease free ddH ₂ O	11.25 µl
Total	15 µl

2.1.1.7 Plasmid Extraction and Purification (Mini-Prep)

Plasmids used in this study were initially extracted at a small scale and purified using the Qiaprep® Spin Miniprep Kit following the protocol provided by manufacturer for subsequent analysis. Briefly, bacterial plasmid DNAs were isolated from 4 ml of 5 ml bacterial culture (Aliquots of 1ml were stored for large scale culture of bacteria). Bacterial cells were lysed by alkaline lysis method and cleared from genomic DNA, proteins and cellular debris by centrifugation. The cleared lysates were transferred to columns where plasmid DNA binds to silica membrane. Here, plasmid DNAs were washed with specific buffers to eliminate any impurities and contaminants. Finally, pure plasmid DNAs were eluted in 30 µl of kit Buffer EB by centrifugation. All of the steps were explained in detail in manufacturer's protocol. The quantity and quality of plasmid DNAs were measured with a NanoDrop instrument.

2.1.1.8 Diagnostic Restriction Digestion by Restriction Endonucleases

Isolated pSpCas9(BB)-2A-GFP-gRNA93 was digested with *BbsI* and *EcoRV* enzymes in a mix described in Table 5 at 37°C for 2 hours.

Table 5. Restriction Digestion of pSpCas9(BB)-2A-GFP-gRNA93

Reagent	Volume (per reaction)
pSpCas9(BB)-2A-GFP-gRNA93	1 µg
<i>BbsI</i> (10 U/µl)	1 µl
<i>EcoRV</i> (10 U/µl)	2 µl
10X Buffer G	2 µl
Nuclease free ddH ₂ O	Up to 20 µl

Digested plasmid was run on 1% agarose gel. After confirming that the plasmid is ready for further use, it was amplified and purified in large scales as explained in section 2.1.1.9.

2.1.1.9 Plasmid Extraction and Purification (Maxi-Prep)

After the integrity of plasmid and correct insertion of DNA sequence is determined by sequencing and restriction digestion, 100 µl of bacteria from 1 ml stock (from section 2.1.1.7) was inoculated into 200 ml LB media (containing 100 µg/ml ampicillin) for large-scale production. Bacteria were cultured at 37°C overnight in shaking incubator (200 rpm). Plasmids were extracted and purified using the QIAGEN Maxi kit following the protocol provided by manufacturer instructions. All of the steps were explained in detail in manufacturer's protocol.

2.1.2 Cell Culture

All cell lines used in this study (Table 6) were cultured at 37°C and 5% CO₂ in a humidified incubator and handled in a sterile environment unless otherwise stated.

Table 6. Cell Lines used in this study

Cell Line	Origin	Source	Genetic Modification
SH-SY5Y	Human Neuroblastoma Cell Line	In House	WT (Parental)

(continued)

Table 6 (continued)

Cell Line	Origin	Source	Genetic Modification
SH-SY5Y 1F5	Human Neuroblastoma Cell Line	This Study	Monoclonal cell line- WT (see section 2.1.3.2)
SH-SY5Y 2G9	Human Neuroblastoma Cell Line	This Study	Monoclonal cell line- WT (see section 2.1.3.2)
SH-SY5Y 5C11	Human Neuroblastoma Cell Line	This Study	Monoclonal cell line- SPTSSA KO (see section 2.1.3.2)
SH-SY5Y 4D10	Human Neuroblastoma Cell Line	This Study	Monoclonal cell line- SPTSSA KO (see section 2.1.3.2)
SH-SY5Y 5D8	Human Neuroblastoma Cell Line	This Study	Monoclonal cell line- SPTSSA KO (see section 2.1.3.2)
SH-SY5Y Bulk Control	Human Neuroblastoma Cell Line	This Study	Polyclonal cell line-WT (see section 2.1.3.3)
SH-SY5Y Bulk SPTSSA KO	Human Neuroblastoma Cell Line	This Study	Polyclonal cell line- SPTSSA KO (see section 2.1.3.3)

2.1.2.1 Subculturing of Cell Lines

SH-SY5Y cells were cultured in complete media: DMEM/F12 with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). When the cells reached to 75-80% confluency in the flask, they were passaged to maintain growth. For subculturing, the medium of the cells was aspirated. The cells were washed with 1X PBS buffer and detached by adding 1-2 ml trypsin-EDTA solution (0.25% Trypsin, 0.5mM EDTA in 1X PBS) and incubating at 37°C for 5 minutes. Trypsin was inactivated by resuspending the detached cells with prewarmed complete medium. The cells were centrifuged at 1000 rpm for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 10 ml complete medium and diluted 1:10 for seeding into a new culture flask. Media of the cells were changed twice a week. The cells were maintained in incubator at 37°C and 5% CO₂ in a humidified incubator.

2.1.2.2 Thawing and Cryopreservation of Cells

The cells were frozen when they reached to 80-90% confluency. They were prepared for freezing following the same protocol during subculture. Shortly, cells were detached from the flask by adding 1-2 ml trypsin-EDTA solution (0.25% Trypsin, 0.5mM EDTA in PBS) and incubating at 37°C for 5 minutes. Trypsin was inactivated by resuspending the detached cells with prewarmed complete medium. The cells were centrifuged at 1000 rpm for 5 minutes. Supernatant was removed and the cell pellet was resuspended in 10 ml freezing media (90% FBS+10% DMSO) cooled in fridge. Aliquots of the cell suspension were dispensed into 2 ml cryovials. Then, cryovials were placed in quick-freeze container and stored at -80°C overnight. Next day, frozen cells were transferred to liquid nitrogen for long term storage.

The cells in cryovials were thawed rapidly by gentle agitation in a 37°C water bath. Afterwards, they were transferred to 9 ml warm complete media and centrifuged at 1000 rpm for 5 min. Supernatant was discarded and the cells were resuspended in 10 ml complete media to prepare them for seeding into the culture flask. Next day, the medium of the cells was changed to remove remaining DMSO and dead cells from the culture.

2.1.2.3 Cell Counting

Before an experiment, the cells were counted using a CASY cell counter and analyzer. They were prepared for counting following the same protocol during subculture (section 2.1.2.1). Shortly, the cells were washed, trypsinized and centrifuged. They were resuspended in fresh culture media and diluted 1:200 in isotonic CASYton buffer in CASYcups. The number of cells were measured according to the manufacturer's instructions.

2.1.3 Generation of SPTSSA KO and Control Monoclonal Cell Line

2.1.3.1 Transient Transfection of SH-SY5Y cells

CRISPR/Cas9-mediated genome editing was performed by transiently transfecting SH-SY5Y cells with a CRISPR/Cas9 targeting construct using FuGENE HD transfection reagent. Target cells were seeded a day in advance into 6-well plate at a density of 150,000 cells/well into a complete medium. According to the manufacturer's protocol, FuGENE® HD Transfection reagent was equilibrated to room temperature before using and mixed by vortexing briefly to dissolve possible precipitates. Components of the transfection solution were mix in a given order as described in Table 7 and Table 8. The final mix was vortexed and

incubated for 10 minutes at room temperature. A total of 200 μ L of the mix was added to the cells dropwise while gently swirling the plate. The cells were incubated for 24 hours before continuing with further experiments.

Table 7. FuGENE HD transfection mix

Reagent	Volume (per reaction)
Opti-MEM	Up to 200 μ l
Plasmid	2 μ g
FuGENE HD	12 μ l

Table 8. Plasmid list used for CRISPR/Cas9 genome editing

Plasmid	Features & Use
pSpCas9(BB)-2A-GFP	Cas9, GFP, no gRNA, control
pSpCas9(BB)-2A-GFP-gRNA93	Cas9, GFP, gRNA targeting SPTSSA

2.1.3.2 Single Cell Sorting by Flow Cytometry

After 24 hours of transfection, the cells were trypsinized and centrifuged following the same protocol, which had been used during subculture (section 2.1.2.1). The supernatant was removed and the cell pellet was resuspended in 500 μ l of 1X PBS/1.5% FBS/0.5 mM EDTA. The cell suspension was filtered through the 35 μ m strainer cap of the 5 ml round bottom polystyrene test tube. To distinguish GFP positive cells from background or nonspecific events during sorting, a negative control group (non-transfected fluorescence minus group) was prepared alongside the experimental groups. Fluorescence activated cell sorting (FACS) was performed by Flow Cytometry Core Facility at DKFZ. The facility prepared the FACS instrument (BD FACSAria Fusion, 100 μ M nozzle, 20psi) and set up the associated software (BD FACSDiva 8.0.2) for proper gating and event rate. GFP positive single cells were sorted into the wells of 96-well plate under sterile conditions. Sorted single cells were kept in special growth media (DMEM/F12+ 30% FBS+ 1% P/S+ 1X B27) to maintain cell viability and promote cell proliferation.

2.1.3.3 Bulk Cell Sorting by Flow Cytometry

After 24 hours of transfection, the cells were trypsinized and centrifuged following the same protocol, which had been used during subculture (section 2.1.2.1). The supernatant was removed and the cell pellet was resuspended in 500 μ l of 1X PBS containing 1.5% FBS and 0.5 mM EDTA. The cell suspension was filtered through a 35 μ m strainer cap placed on top of a 5 ml round bottom polystyrene test tube. To distinguish GFP positive cells from background or nonspecific events during sorting, a negative control group (non-transfected fluorescence minus group) was prepared alongside the experimental groups. FACS sorting was performed by the Flow Cytometry Core Facility at the DKFZ. The facility prepared the FACS instrument (BD FACSAria Fusion, 100 μ m nozzle, 20psi) and set up the associated software (BD FACSDiva 8.0.2) for proper gating and event rate. GFP-positive single cells were sorted into 6-well plates. A total of 30,000 cells were GFP-positive in control cells (named as SH-SY5Y bulk-control) transfected with pSpCas9(BB)-2A-GFP, while 20,000 cells (named as SH-SY5Y bulk-SPTSSA KO) were positive in SPTSSA KO cells transfected with pSpCas9(BB)-2A-GFP-gRNA93.

2.1.3.4 Extraction of Genomic DNA from Cells

SH-SY5Y SPTSSA KO and control cell clones were seeded into 6 cm dishes at a density of 500,000 cells. The following day, the media were aspirated, cells were washed with 1X PBS and then harvested in 1X PBS by scraping. The cell pellet was collected by centrifugation at 12,000 rpm for 5 minutes. Genomic DNAs were extracted using DNeasy Blood and Tissue kit following the provided protocol. Briefly, cells were lysed with Proteinase K and loaded onto the silica-based membrane contained within columns. Samples were centrifuged to allow genomic DNA to bind to the column in the presence of high concentration of chaotropic salt and to eliminate contaminants. Subsequent washing steps ensured the removal of any remaining impurities and enzyme inhibitors. Finally, genomic DNA was eluted in 100 μ l of Buffer AE of the kit.

2.1.3.5 PCR and Sanger Sequencing

Changes at the genomic level were monitored using Sanger sequencing. PCR was performed using 100 ng of genomic DNA from SH-SY5Y control and SPTSSA KO clones with primers spanning the targeting region of gRNA93. Primers (hSPTSSA 5UTRs/ hSPTSSA Int1as) used in PCR reaction was obtained from previous study by Yamaji et. al. (Yamaji et

al., 2019). The protocol outlined in the Table 9 and Table 10 was followed. After the reaction, the amplified fragments were run on a 3% agarose gel for 50 minutes at 120 V. DNA band corresponding to the correct size were excised from the gel and purified using the QIAquick Gel Extraction Kit according to the manufacturer's protocol. The eluted DNA was sent for Sanger sequencing using the forward primer. The Sanger sequencing was conducted by Eurofins Genomics GmbH. Sequence results of the clones were aligned to WT sequence of human SPTSSA gene (NCBI Gene ID: 171546) using Unipro UGENE program (Okonechnikov et al., 2012). Multiple Sequence Alignment is conducted by the software tool MUSCLE. The accuracy and reliability of the generated sequences were controlled by closely examining the trace file (chromatogram).

Table 9. PCR mix for SPTSSA KO Validation

Reagent	Volume (per reaction)
Q5 Reaction Buffer 5X	5 µl
10 mM dNTPs	0.5 µl
F/R Primers	2.5 µl
Genomic DNA	100 ng
Hot Start Q5 Polymerase	0.25 µl
Q5 High GC Enhancer	5 µl
Nuclease free dH ₂ O	Up to 50 µl

Table 10. Thermocycling program for PCR

Stages	Temperature	Time	
Initial Denaturation	98°C	2 minutes	1 cycle
Denaturation	98°C	20 seconds	45 cycles
Annealing	67°C	20 seconds	
Elongation	72°C	20 seconds	
Final Elongation	72°C	5 minutes	1 cycle
Hold	4°C	∞	Infinite

2.1.3.6 Raw Lipid Extraction and Total Acid Hydrolysis

SH-SY5Y SPTSSA KO and control cell clones were seeded in 10 cm dishes at a density of 1.5×10^6 cells. Next day, the media were aspirated, cells were washed with 1X PBS and then harvested in 1X PBS by scraping. The cell pellet was collected by centrifugation at 12,000 rpm for 5 minutes. The cell pellet was resuspended in 1 ml PBS and aliquoted into two reaction tubes: 850 μ l for raw lipid extraction and 150 μ l for total acid hydrolysis. Both samples were centrifuged at 13,000 rpm for 5 minutes at 4°C. The supernatant was discarded, and 50 μ l of internal standard lipid mix “IS-Mix-Neuro” (Table 11) was added to the lipid samples, while 50 μ l of internal standard lipid mix “IS-Mix-SB mix” was added to the hydrolysis samples (Table 12).

For lipid extraction, 400 μ l of chloroform/methanol/water (10:10:1) was added to each sample, followed by vortexing and incubation at 37°C for 15 minutes in an ultrasound bath, with 3 cycles of 3 minutes of sonication and 2 minutes of rest between cycles. Subsequently, samples were centrifuged at 13,000 rpm for 2 minutes at 25°C, and the supernatant (2x 200 μ l) was transferred to clean 1.8 ml flass tubes (GC-vials) with screw caps containing a pre-slit Silicon/PTFE-septum. The residual pellet was reextracted a second time as before, and the supernatant was collected in the same GC-vial. Finally, a third extraction step was performed similarly to the previous two, but this time, lipids were extracted with chloroform/methanol/water (30:60:8). All supernatants were collected in the same GC vial. The collective extracts were dried at 39°C around 30 minutes under nitrogen stream and dissolved in 200 μ l chloroform/methanol/water (10:95:5) for measurements with the liquid chromatography-coupled tandem mass spectrometer (LC-MS/MS) using a reverse phase column. Samples aliquoted for total acid hydrolysis were dried around 30 minutes under nitrogen stream and further processed following the total hydrolysis protocol. The residual pellet remaining in reaction tubes after lipid extraction and centrifugation was dried and used to determine the protein concentration. Briefly, 25 μ l of 1M NaOH was added to the sample pellet, which were then incubated in water bath at 50 °C for 2 hours. Subsequently, the 1M NaOH solution inside the reaction tubes was diluted to 0.1 M NaOH by adding 225 μ l of dH₂O. This was followed by continuing with the by bicinchoninic acid assays (BCA) protocol to determine the protein concentration.

Table 11. Internal standard (IS) mix - Neuro used for raw lipid extraction

Internal standard	pmol per sample and per 50 µl	Supplier
D7-C18- ketodihydrosphingosine (KDS)	10	Avanti Polar Lipids
D7-C18- sphinganine (Sa)	10	Avanti Polar Lipids
D5-C17-Deoxymethyl-Sa	10	Avanti Polar Lipids
D5-C18-Deoxy-Sa	9.6	Avanti Polar Lipids
D7- C18-sphingosine (So)	10	Avanti Polar Lipids
D7-C18-sphinganine 1-phosphate (Sa1P)	10	Avanti Polar Lipids
D7-C18-sphingosine 1-phosphate (So1P)	10	Avanti Polar Lipids
Ceramides (Cer): Cer(d18:1/14:0), Cer(d18:1/19:0), Cer(d18:1/25:0), and Cer(d18:1/31:0)	30 (each)	As published in (Jennemann et al., 2007)
C12-deoxy-dihydroceramide (doDHCer)	10	Avanti Polar Lipids
Sphingomyelins (SM): SM(d18:1/12:0), SM(d18:1/17:0), and SM(d18:1/31:0)	150 (each)	As published in (Jennemann et al., 2007)
Glucosyl(Glc)- and Galactosylceramides (GalCer): GlcCer(d18:1/14:0), GlcCer(d18:1/19:0), GlcCer(d18:1/25:0), and GalCer(d18:1/31:0)	30 (each)	As published in (Jennemann et al., 2007)
D5-GM1	60	Avanti Polar Lipids
D3-GM2	60	Cayman Chemical
D5-GM3	60	Avanti Polar Lipids
D3-GD3	60	Cayman Chemical
Internal standard	ng	Supplier
Int.PC(24/28/44/48) 1,2-Dilauroyl-sn-glycero-3-phosphocholine (PC24), 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (PC28), 1,2-dibehenoyl-sn-glycero-3-phosphocholine (PC44), and 1,2-dilignoceroyl-sn-glycero-3-phosphocholine (PC48)	200 (each)	Sigma-Aldrich-Chemie

Table 12. Internal standard (IS) mix - SB used for total lipid hydrolysis

Internal standard	pmol per sample	Supplier
D7-C18-KDS	60	Avanti Polar Lipids
D7-C18-Sa	60	Avanti Polar Lipids
D5-C17-Deoxymethyl-Sa	60	Avanti Polar Lipids
D3-C18-Deoxy-Sa	60	Avanti Polar Lipids
D7-C18-So	60	Avanti Polar Lipids
D7-C18-sphingadiene (Sde)	60	Avanti Polar Lipids

2.1.3.7 Total Acid Hydrolysis

Sample lipid raw extracts aliquoted for total acid hydrolysis were further processed. They were treated with a mixture composed of 43 μ l concentrated HCl, 47 μ l H₂O and 410 μ l methanol. The samples were incubated at 80°C for 20 hours in a heating block and by insulating with aluminum foil. Following this incubation, the samples were dried under a nitrogen stream at 40°C to remove the solvent. To ensure complete drying, the samples underwent an additional drying step in the SpeedVac for approximately 1 hour. Subsequently, 500 μ L of methanol and 500 μ L of 25% NH₄OH_{aq} were added, and the samples were once again incubated at 80°C to hydrolyze previously formed methyl esters. Samples were dried first under nitrogen stream at 40°C and then in SpeedVac about 1 hour. Finally, the samples were dissolved in 200 μ l chloroform/methanol/water (10:95:5) for measurement by LC-MS/MS.

2.1.3.8 SPT Activity Assay

SH-SY5Y SPTSSA KO and control cell clones were seeded in T175 flask and cultured until they reached 60% confluency. The cells were washed with 10 ml PBS and dissociated with PBS containing 0.25% Trypsin and 0.5mM EDTA by incubating 5 minutes. Trypsin was inactivated by resuspending the detached cells with prewarmed complete medium. The cells were centrifuged at 1000 rpm for 5 min and the supernatant was discarded. The cell pellet was resuspended in 1X PBS cooled in fridge for washing and were centrifuged again at 1000 rpm for 5 min. The supernatant was discarded and 1ml SPT SPT activity assay (AA) buffer (50 mM HEPES, 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 1X protease inhibitor cocktail, 1X phosphatase inhibitor) was added. The cells were resuspended and transferred to 2 ml PP-reaction tube. The cells were homogenized by passing the suspension through a 23-gauge

needle for five times. This step is repeated by increasing the needle size first to 27-gauge and then 30-gauge. The cell lysate was vortexed and sonicated in an ice bath five times, with each cycle consisting of 1 minute of sonication followed by 1 minute of rest. Two microliter of the sample was mixed with two microliter trypan blue (1:1 ratio), placed on a glass slide with coverslip and check under microscope for proper homogenization. The homogenate was incubated on ice for 20 minutes and centrifuged at 2,000 g for 10 minutes at 4°C to eliminate the debris, nuclei and unbroken cells. The supernatant containing cytoplasm, and membranes including mitochondria was transferred into a new tube. Five microliter (2 x 2.5 µl) aliquot was saved for protein determination and protein concentration for each sample was determined by Bradford assay. The rest of the sample was centrifuged at 21,000 g for 2 hours at 4 °C. The pellet containing ER membranes and mitochondria was dissolved in 130 µl SPT AA buffer. Protein concentration was determined. The 200 µl reaction mixture was prepared by combining 0.025 mg protein dissolved in SPT reaction buffer, with 5mM L-serine ($^{13}\text{C}_3^{15}\text{N}$) (2 µl), 50 µM FA-CoA (5 µl) and 12.5 µM PLP (2 µl). The SPT activity assay was performed by incubating the samples at 37°C for 7.5 minutes (n=3) and for 15 minutes (n=3) in two separate reactions. The reaction was stopped by adding 200 µl reaction solution into 4 ml chloroform/methanol (1:1) in a 15 ml Falcon. Samples were stored at -20°C for lipid extraction.

2.1.3.9 Raw Lipid Extraction for SPT Activity Assay

After the reaction for the SPT activity is completed, lipids were extracted as described in the following. Internal standard (IS) mix in 50 µL was added to each sample (Table 13). Lipids were extracted with 2 ml chloroform/methanol/water (10:10:1) (1st time). Samples were incubated at 37°C for 15 minutes with 3x 3 minutes of ultrasonication. Then, they were centrifuged at 5,000 rpm for 5 min at room temperature. Supernatant was transferred to a 12 ml glass vial. Lipids were re-extracted (2nd time) with 2 ml chloroform/methanol/water (10:10:1) + 2% formic acid. The same steps as in the case for first extraction was repeated. Supernatant was transferred to the same glass vial and pooled together with the first extract. Last and third extraction was performed the same way as in the previous steps by adding 2 ml chloroform/methanol/water (30:60:8)+ 2% formic acid and supernatant again was pool with the first two extracts. The pooled lipid extracts were dried under nitrogen flow at 37°C. The extracts were dissolved in 2 ml chloroform/methanol/water (10:10:1) and concentrated. It was dissolved in 1 ml chloroform/methanol/water (10:10:1) in transferred into a GC-vial and concentrated. Afterwards, they were dried under a gentle nitrogen flow at 37°C and re-

dissolved with 200 μ l chloroform/methanol/water (10:95:5). Dissolved lipids were transferred into glass inserts, which were placed back into GC-vials. They were centrifuged for 5 minutes at 2,000 rpm. The amount of labeled KDS- $^{13}\text{C}^{15}\text{N}$, which had been synthesized by SPT complex, was measured in MRM-mode, as described previously (Pilz et al., 2022).

Table 13. Internal standard (IS) mix used in SPT Activity Assay for LC-MS/MS analysis

Internal standard	pmol per sample	Supplier
D7-KDS (m18:0)	50	Avanti Polar Lipids Inc., Alabaster, AL, USA
D7-Sa (d18:0)	30	Avanti Polar Lipids Inc., Alabaster, AL, USA
D7-So (d18:1)	30	Avanti Polar Lipids Inc., Alabaster, AL, USA
Cer(d18:1;14:0), Cer (d18:1;19:0), Cer (d18:1;25:0), and Cer (d18:1;31:0)	20	As published in (Jennemann et al., 2007)
3-Keto-dihydroceramide Cer (m18:0;14:0)	50	As published in (Pilz et al., 2022)

2.1.4 Functional Analysis of SPTSSA KO and Control Monoclonal Cell Line

2.1.4.1 Crystal Violet Assay for Determining Viability of Cells

Cells were seeded into 96 well plate at a density of 1×10^4 cells/well as eight replicates. They were cultured in complete growth media for 24, 48 or 72 hours (for each time point different culture plates were used). Afterwards, their media were discarded and the cells were fixed with 4% Formaldehyde (FA) for 10 min. FA was removed and the fixed cells were washed with 1X PBS. Plates were dried under the fume hood overnight. Next day, 100 μ l 0.01% crystal violet solution was added to each well and they were incubated for 30 minutes at room temperature. Crystal violet solution was discarded and the plates were washed with water two times. Plates were dried overnight. When the wells were completely dried, 100 μ l 10% acetic acid was added to the wells and plates were incubated around 2 hours in a shaking plate (200-300 rpm) until crystal violet dye completely dissolved. The absorbance at 570 nm was measured with a Synergy H1 microplate reader.

2.1.4.2 Cell Cycle Analysis

SH-SY5Y SPTSSA KO and control clones were seeded in 6 well plates (8×10^5 cells/well as three replicates) in complete growth media. The following day, the cells were washed once with warm 1X PBS, trypsinized, and then collected in 2 ml tubes with complete growth media. The cells were counted and aliquot of 5×10^5 cells were transferred into new tubes. Cell pellet were obtained by centrifugation at 1,000 rpm for 5 minutes and the supernatants were discarded. The cell pellets were dissolved in 125 μ l 1X PBS by gently pipetting up and down for approximately five times. Ice-cold methanol (350 μ l) was added dropwise while slowly vortexing the tubes. The cells were incubated at 4°C for at least one hour and 4 ml 1X PBS containing 1% FCS were added to the FACS tubes. The fixed cells were passed through 30 μ M cell strainer cap into the FACS tubes and centrifuged at 1200 rpm for 5 minutes. They were stained with 200 μ l 0.01mg/ml Propidium iodide (PI) containing 0.25mg/ml RNase A solution. Cells were incubated at room temperature in the dark for 30 minutes and measured by FACS at 585nm in linear mode in the FL2 channel (FACS Canto, with DIVA software). Quantification was performed using FlowJo software.

2.1.5 Proteomic and Phosphoproteomic Analyses

2.1.5.1 Cell Seeding and Protein Isolation

SH-SY5Y SPTSSA KO and control clones were seeded in 10 cm dishes at a density of 5×10^5 cells as four replicates. Next day, cells were washed three times with ice-cold PBS. They were scraped in ice-cold PBS and centrifuged at 14,000 rpm for 5 minutes to obtain the cell pellet. The supernatant was discarded and 50 μ l lysis buffer was added (Table 20). Samples were kept on ice for 1 hour and centrifuged at 20,000g for 60 minutes. Supernatant was transferred to 1.5 ml reaction tube. Protein concentration was determined by bicinchoninic acid assays (BCA) following the protocol from manufacturer. Protein samples were submitted to Proteomics Core Facility at DKFZ Heidelberg for proteomic analysis. The experiment was named as AK0735. The Proteomics Core Facility team further processed samples for measurements and performed the bioinformatic analysis following the protocol below.

The proteomic analysis was repeated for SH-SY5Y SPTSSA KO and control cells after over 30 passages following the previously described protocol and experiment named as AK0800-1. The initial cell density was increased to 3×10^6 cells/10 cm dish. Furthermore, phosphoproteomic analysis was conducted alongside the proteomic analysis in experiment

AK0800-2. The difference between experiments AK0800-1 and AK0800-2 lies in the acquisition method used. Specifically, data were collected using data independent analysis (DIA) in experiment AK0800-1 and using data dependent analysis (DDA) in experiment AK0800-2.

2.1.5.2 Sample Preparation and LC-MS/MS Analysis

2.1.5.2.1 AK0735

Extracted proteins (10 µg) were digested with trypsin using an AssayMAP Bravo liquid handling system (Agilent technologies) running the autoSP3 protocol according to Muller et al. (Muller et al., 2020).

2.1.5.2.2 AK0800-1 & 2

Proteins (55 µg) were digested (trypsin) using an AssayMAP Bravo liquid handling system (Agilent technologies) running the autoSP3 protocol according to Muller et al. (Muller et al., 2020). After digestion 3 µg of peptides were separated for full proteome analysis. The residual amount was used for phosphopeptide enrichment workflow using Fe(III)-NTA Cartridges (Agilent technologies) according to the manufacturers protocol (PhosphoPeptide Enrichment v2.0).

2.1.5.3 MS Method Orbitrap Exploris 480

2.1.5.3.1 AK0735 and AK0800-2 (Full Proteome)

Analysis was performed following the protocol from Bonadonna et.al. (Bonadonna et al., 2022). Briefly, LC-MS/MS analysis was carried out on an Ultimate 3000 UPLC system (Thermo Fisher Scientific) directly connected to an Orbitrap Exploris 480 mass spectrometer for a total of 120 minutes. Peptides were online desalted on a trapping cartridge (Acclaim PepMap300 C18, 5µm, 300Å wide pore; Thermo Fisher Scientific) for 3 min using 30 ul/min flow of 0.05% TFA in water. The analytical multistep gradient (300 nl/min) was performed using a nanoEase MZ Peptide analytical column (300Å, 1.7 µm, 75 µm x 200 mm, Waters) using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). For 102 min the concentration of B was linearly ramped from 4% to 30%, followed by a quick ramp to 78%, after two minutes the concentration of B was lowered to 2% and a 10 min equilibration step appended. Eluting peptides were analyzed in the mass spectrometer using data independent acquisition (DIA) mode. A full scan at 120k resolution (380-1400 m/z, 300%

AGC target, 45 ms maxIT) was followed by 47 fragment mass windows covering a mass range of 400-1200 m/z. Fragment mass spectra were recorded at 30k resolution (100% AGC target, 54 ms maxIT).

2.1.5.3.2 AK0800 -1 (Phosphoproteome)

Analysis was performed following the protocol from Bonadonna et.al. (Bonadonna et al., 2022). A LC-MS/MS analysis was carried out on an Ultimate 3000 UPLC system (Thermo Fisher Scientific) directly connected to an Orbitrap Exploris 480 mass spectrometer for a total of 90 minutes. Peptides were online desalted on a trapping cartridge (Acclaim PepMap300 C18, 5µm, 300Å wide pore; Thermo Fisher Scientific) for 3 min using 30 µl/min flow of 0.05% TFA in water. The analytical multistep gradient (300 nl/min) was performed using a nanoEase MZ Peptide analytical column (300Å, 1.7 µm, 75 µm x 200 mm, Waters) using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile). For 73 min the concentration of B was linearly ramped from 2% to 28%, followed by a quick ramp to 78%, after two minutes the concentration of B was lowered to 2% and a 10 min equilibration step appended. Eluting peptides were analyzed in the mass spectrometer using data dependent acquisition (DDA) mode. A full scan at 120k resolution (380-1400 m/z, 300% AGC target, 45 ms maxIT) was followed by up to 1.5 seconds of MS/MS scans. Peptide features were isolated with a window of 1.0 m/z, fragmented using 30% NCE. Fragment spectra were recorded at 30k resolution (200% AGC target, 110 ms maxIT). Unassigned and singly charged eluting features were excluded from fragmentation and dynamic exclusion was set to 25 s.

2.1.5.4 Data Analysis

2.1.5.4.1 AK0735

The protocol from Erwied et. al used in this analysis (Erwied et al., 2024). Briefly, analysis of DIA RAW files was performed with Spectronaut (Biognosys, version 17.1.221229.55965) in directDIA+ (deep) library-free mode. Default settings were applied with the following adaptations. Within the *Pulsar Search in Peptides* the *Max Peptide Length* was set to 35, in *Result Filters* the *Peptide Charge* was enabled and the *Max Charge* set to 6 and the *Min Charge* set to 2. Within *DIA Analysis* under *Identification* the *Precursor PEP Cutoff* was set to 0.01, the *Protein Qvalue Cutoff (Run)* set to 0.01 and the *Protein PEP Cutoff* set to 0.05. In *Quantification* the *Proteotypicity Filter* was set to *Only Protein Group Specific*, the *Corss-Run Normalization* was disabled, the *Quantification window* was set to *Not*

Synchronized and the *Major Group Quantity* was set to *Sum peptide quantity*. The data was searched against the human proteome from Uniprot (human reference database, containing 81,837 unique entries from 26.10.2022) and the contaminants FASTA from MaxQuant (246 unique entries from 22.12.2022).

2.1.5.4.2 AK0800-1

Briefly, data analysis was carried out by MaxQuant (version 2.1.4.0) (Tyanova et al., 2016) using an organism specific database extracted from Uniprot.org (human reference database with one protein sequence per gene, containing 20,591 unique entries from 01.03.2023). Settings were set to default with the following adaptations. Match between runs (MBR) was enabled to transfer peptide identifications across Raw files based on accurate retention time and m/z. Fractions were set in a way that MBR was only performed within replicates of the full proteome or the phospho fraction respectively. Separate parameter groups were assigned for full proteome and phospho fractions. For the phospho fractions PTM was set to *True* and in the parameter group settings *Phospho (STY)* was added to the variable modifications. For the full proteome fraction label free quantification (LFQ) was enabled with default settings. Quantification on the full proteome fraction was carried out using the LFQ approach based on the MaxLFQ algorithm (Cox et al., 2014). A minimum of 2 quantified peptides per protein was required for protein quantification. Additionally, iBAQ-values were generated (Schwanhaussner et al., 2011).

2.1.5.4.3 AK0800-2

The protocol from Erwied et. al used in this analysis (Erwied et al., 2024). Briefly, analysis of DIA RAW files was performed with Spectronaut (Biognosys, version 17.1.221229.55965) in directDIA+ (deep) library-free mode. Default settings were applied with the following adaptations. Within the *Pulsar Search* in *Peptides* the *Max Peptide Length* was set to 35, in *Result Filters* the *Peptide Charge* was enabled and the *Max Charge* set to 6 and the *Min Charge* set to 2. Within *DIA Analysis* under *Identification* the *Precursor PEP Cutoff* was set to 0.01, the *Protein Qvalue Cutoff (Run)* set to 0.01 and the *Protein PEP Cutoff* set to 0.05. In *Quantification* the *Proteotypicity Filter* was set to *Only Protein Group Specific*, the *Corss-Run Normalization* was disabled, the *Quantification window* was set to *Not Synchronized* and the *Major Group Quantity* was set to *Sum peptide quantity*. The data was searched against the human proteome from Uniprot (human reference database with one

protein sequence per gene, containing 20,591 unique entries from 01.03.2023) and the contaminants FASTA from MaxQuant (246 unique entries from 22.12.2022).

2.1.5.5 Statistical Analysis

2.1.5.5.1 AK0735

For the statistical analysis log2 transformed, MaxLFQ normalized, quantity values were used. Contrasts were performed for the individual clones and comparing control vs knockout across all clones. Filtering and imputation were performed individually for each contrast, not across the whole data matrix. Adapted from the Perseus recommendations (Tyanova & Cox, 2018) valid intensity values in 70% of the samples of at least one condition were used for statistics. Adapted from the Perseus recommendations missing values being completely absent in one condition, were imputed with random values drawn from a downshifted (2.2 standard deviation) and narrowed (0.3 standard deviation) intensity distribution of the individual samples. For missing values with no complete absence in one condition the R package missForest was used for imputation (Stekhoven & Buhlmann, 2012). The statistical analysis was performed according the chapter 9.2 *Two Groups* of the R-package *limma* (Ritchie et al., 2015). Alongside the limma first author recommendations, the setup was adapted for proteomics data via setting the options trend and robust to *TRUE* in the eBayes function. The p-values were adjusted with the Benjamini-Hochberg method for the multiple testing (Benjamini & Hochberg, 1995).

2.1.5.5.2 AK0800-1

For the full proteome statistics log2 transformed LFQ and iBAQ values were used, for the phospho fraction the log2 transformed multiplicity specific site intensity values. Contrasts were performed for the individual clones and comparing control vs knockout across all clones. Filtering, normalization and imputation was done individually for each contrast, not across the whole data matrix. Adapted from the Perseus recommendations valid intensity values in 70% of the samples of at least one condition were used for statistics. The iBAQ values and phospho site intensities were normalized via variance stabilization normalization (vsd)(Huber et al., 2002). For the LFQ values no further normalization was performed. Adapted from the Perseus recommendations (Tyanova & Cox, 2018) missing values being completely absent in one condition, were imputed with random values drawn from a downshifted (2.2 standard deviation) and narrowed (0.3 standard deviation) intensity distribution of the individual

samples. For missing values with no complete absence in one condition the R package *missForest* was used for imputation (Stekhoven & Buhlmann, 2012).

The statistical analysis was performed according the chapter 9.2 *Two Groups* of the R-package *limma* (Ritchie et al., 2015). Alongside the *limma* first author recommendations, the setup was adapted for proteomics data via setting the options *trend* and *robust* to *TRUE* in the *eBayes* function. Some samples of the full proteome were technically underperforming their weight was therefore adjusted in the statistical contrasts. For sample 2, 17 and 18 to 30% and for sample 1 to 15%. For the phospho fraction the weight of samples 11, 12, 19, 21 was set to 30%. The p-values were adjusted with the Benjamini–Hochberg method for the multiple testing (Benjamini & Hochberg, 1995).

2.1.5.5.3 AK0800-2

For the statistical analysis log2 transformed, MaxLFQ normalized, quantity values were used. Contrasts were performed for the individual clones and comparing control vs knockout across all clones. Filtering and imputation were performed individually for each contrast, not across the whole data matrix. Adapted from the Perseus recommendations (Tyanova & Cox, 2018) valid intensity values in 70% of the samples of at least one condition were used for statistics. Adapted from the Perseus recommendations missing values being completely absent in one condition, were imputed with random values drawn from a downshifted (2.2 standard deviation) and narrowed (0.3 standard deviation) intensity distribution of the individual samples. For missing values with no complete absence in one condition the R package *missForest* was used for imputation (Stekhoven & Buhlmann, 2012).

The statistical analysis was performed according the chapter 9.2 *Two Groups* of the R-package *limma* (Ritchie et al., 2015). Alongside the *limma* first author recommendations, the setup was adapted for proteomics data via setting the options *trend* and *robust* to *TRUE* in the *eBayes* function. The p-values were adjusted with the Benjamini-Hochberg method for the multiple testing (Benjamini & Hochberg, 1995).

2.1.6 EGF Stimulation

EGF receptor phosphorylation at Tyr992 in WT SH-SY5Y cells was optimized to achieve maximum phosphorylation. This was achieved by determining the ideal dosage of EGF and the duration of stimulation. Briefly, 1×10^6 cells were seeded into 6 cm dish, cultured overnight and treated with 100 ng/ml of EGF for 0, 5, 10, 15, 20, or 30 minutes. Immediately

after stimulation, the cells were washed with cold 1X PBS, harvested by scraping, and subsequently centrifuged at 12,000 rpm for 5 minutes. The supernatant was discarded, and the cell pellet was stored at -80°C for later protein extraction and Western blot analysis. For the subsequent experiment, cells were stimulated with increasing concentration of EGF (0, 12.5, 25, 50, 100 ng/ml) for 10 minutes, which was determined as the optimal time point from the initial experiment, to determine the ideal EGF dosage. Building upon the results from the dose and time-dependent EGF stimulation, SH-SY5Y SPTSSA KO and control cells were stimulated with 12.5 ng/ml EGF for 10 minutes for following functional analyses. The cell harvest, protein extraction, and Western blot procedures were conducted in accordance with the protocol used in the initial experiments.

2.1.6.1 Protein Extraction and Western Blotting

Depending on the experiment, cells were cultured in corresponding vessel with or without treatment. To harvest the cells, media were removed and cells were washed with and scraped in cold 1X PBS. Cell pellet was obtained by centrifugation at 12,000 rpm for 5 min at 4°C. The cells were lysed with cold RIPA lysis buffer supplemented with 1X Complete EDTA free protease inhibitor and 1X PhosStop phosphatase inhibitors. The pellet was sonicated (15 seconds on/ 1 min 45 sec off) in ice-water bath and then incubate on ice for 20 min. Supernatant containing protein was collected by centrifugation at 14,000 rpm at 4°C for 10 min. Protein concentration was determined by bicinchoninic acid assays (BCA) following the protocol from manufacturer. Total of 20-30 µg protein from each sample was diluted in 4X Laemmli buffer and boiled for 5 min at 95°. Proteins were separated on 4-10% SDS-PAGE gels (the smaller size proteins, ≤ 30 kD, were separated on 4-15% gel) at constant volt. Proteins were run in stacking gel at 90V until all protein samples lined in running gel and run at 120V around 75 min. They were transferred onto nitrocellulose membranes at constant 250 mAmp for 2 hours at 4°C in a wet blot chamber. After transfer, membranes were blocked with 5% skim milk in 1X Tris-Buffered Saline (TBS) + 0.1% Tween-20 (1X TBS-T) for 1 hour at room temperature and washed three times with 1X TBS-T for 10 min. Membranes were incubated with primary antibodies overnight at 4°C by following the manufacturer's protocol and washed again three times with 1X TBS-T for 10 minutes. They were incubated with corresponding secondary antibodies at room temperature for 1 hour by following the manufacturer's protocol. Proteins were visualized using BioRad imaging system. Quantification was performed using the Image Lab software.

2.1.7 RNA-Related Methods

2.1.7.1 Total RNA Isolation

Total RNA was isolated from the cells using Peqlab peqGOLD TriFast™, DNA/RNA/protein purification reagent. For 5×10^6 - 1×10^6 cells, 500 μ l Trifast reagent was used. The cells were homogenized by pipetting quickly several times and they were incubated at RT for 5 min to separate nucleoprotein complexes. For phase separation, 100 μ l chloroform was added and vortexed to homogenize samples. The homogenate was incubated at RT for 5 minutes and centrifuged at 12,000 g for 15 minutes at 4°C. Aqueous phase was transferred to new RNA-free tube and 250 μ l 100% isopropanol was added. The samples were stored at -20°C overnight to precipitate RNA and centrifuged at 12,000 g for 15 minutes at 4°C. Supernatant was removed and pellet was washed with 900 μ l RNase-free 75% ethanol. Sample was centrifuged at 12,000 g for 15 minutes at 4°C. Ethanol was discarded and RNA pellet was air-dried for 5 minutes. RNA was resuspended in 10-20 μ l of RNase-free water. The quantity and quality of RNA was measured with a NanoDrop instrument.

2.1.7.2 cDNA Synthesis

Total RNA extraction was followed by cDNA synthesis for further downstream applications. A total of 1 μ g RNA is added to the RNase-free reaction tubes and the volume was filled with nuclease free water up to 7 μ l. Mix 1 in Table 14 was prepared, and 5.5 μ l was aliquoted to each reaction tubes. Tubes were incubated in thermal cycler following the protocol for Mix1 (37°C for 15 minutes, 70 °C for 15 minutes, 4 °C for 2 minutes) for digestion of any contaminating DNA and priming single-stranded RNA for cDNA synthesis.

Table 14. Mix 1 for cDNA synthesis

Reagent	Volume (per reaction)
HexaN 0.5 μ g/ μ L	2.25 μ l
dNTP 10 mM	1.00 μ l
DNase I (1000u/ml)	1.00 μ l
DNase Buffer	1.25 μ l
Total	5.5 μ l

Mix 2 in Table 15 was prepared, 7.5 μ l was aliquoted into tubes of the first reaction and vortexed. Tubes were incubated in thermal cycler following the protocol for Mix 2 (25°C for 10 minutes, 42 °C for 50 minutes, 70 °C for 15 minutes) for the synthesis of cDNA. The cDNAs were diluted 1:10 in nuclease-free water and stored in -20°C.

Table 15. Mix 2 for cDNA synthesis

Reagent	Volume (per reaction)
M-MLV RT Buffer 5X	4 μ l
RNAse Free Water	2.4 μ l
Ribolock RNAse Inhibitor (40 U/ μ L)	0.1 μ l
M-MLV Reverse Transcriptase (200u/ μ l)	1 μ l
Total	7.5 μ l

2.1.7.2.1 Quantitative Real-Time PCR (qPCR)

Reaction mix for qPCR was prepared according to Table 16 and 6 μ l of the mix was aliquoted into wells of 96-well reaction plates containing 4 μ l cDNAs. For each sample, three biological and three technical replicates were used. For each gene, the corresponding no template control (NTC) samples were included. The qPCRs were run on a StepOnePlus Real-Time PCR System (Applied Biosciences) with the setting in Table 17. Gene expression values were normalized to the expression of RPL13 gene and relative fold changes were calculated using $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak, 2008). Primers used in this study were designed to span exon-exon junction to prevent amplification of genomic DNA sequences.

Table 16. Reaction mix for RT-qPCR

Reagent	Volume (per reaction)
F/R primer (5 μ M)	1 μ l
SYBER Green Master Mix 2X	5 μ l
cDNA (1:10)	4 μ l
Total	10 μ l

Table 17. Thermocycling program for the qPCR

Step	Temperature	Time	Cycle
Holding Stage	95°C	20 sec	1 cycle
Cycling Stage	95°C	3 min	40 cycles
	60°C	30 sec	
Melt Curve Stage	95°C	15 sec	1 cycle
	60°C	1 min	+ 0.3°C/cycle
	95°C	15 sec	

2.1.8 Transformation of SH-SY5Y Cell Line into Neuron-Like Cells

On day 0, 5×10^3 cells were seeded into 96-well plates and cultured in 200 μ l of normal growth media (DMEM/F12+10%FCS+1%P/S) overnight. On day 1, media was changed with either control (DMEM/F12+10%FCS+1%P/S) or differentiation media. Two different differentiation media were prepared and treatment groups were named as either B27 only or B27+ATRA depending on the media they received. B27 only group was cultured in Neurobasal Plus media+ 1X B27-supplement + 1X Glutamax. B27+ATRA group was cultured in Neurobasal Plus media+ 10 μ M all-trans retinoic acid +1X B27-supplement + 1X Glutamax. The medium of the cells was refreshed every 3 days. The cells were treated for 8 days. To determine the cell viability the cells were fixed with 4% formaldehyde (FA) for 10 min on Day 1, Day 4, Day 6 and Day 8 and crystal violet protocol was followed .

2.1.9 Live Cell Imaging

SH-SY5Y SPTSSA KO and control clones were seeded into 96-well at a cell density of 5×10^3 for performing differentiation experiment as explained in section 2.1.8. Each clone was seeded as four replicates per treatment. Plates were transferred to the Incucyte S3 Live Cell Analysis System, where they were incubated at 37°C for approximately 20-30 minutes to allow them to warm up before scanning. Plates were scanned using adherent Cell-by-Cell mode every 6 hours for 5 days and phase- contrast images of cells (4 images/well) were taken at 20x magnification. Images were analyzed using IncuCyte® Neurotrack Analysis Software Module.

2.1.10 Assays for Mitochondria Labelling

2.1.10.1 Analysis of Mitochondrial Membrane Potential

On day 0, SH-SY5Y SPTSSA KO and control clones were seeded in 6 well plates at a density of 2×10^4 cells as duplicates and cultured in normal growth media overnight. The following day, the media were replaced with either control or differentiation media (see section 2.1.8). The cells were incubated for 21 hours and subsequently treated with 100 nM MitoTracker® Red CMXRos for 20 minutes at 37°C in the incubator. After the incubation period, cells were washed with 1X PBS, trypsinized and collected with normal growth media. They were centrifuged at 1000 rpm for 5 minutes, and the cell pellet was resuspended in 1X PBS supplemented with 1% FBS. The cells were analyzed by BD FACSCanto II and data was evaluated using FlowJo v10 software.

2.1.10.2 Quantification of Mitochondrial Mass

On day 0, SH-SY5Y SPTSSA KO and control clones were seeded in 6 well plates at a density of 5×10^4 cells as duplicates and cultured in normal growth media overnight. The following day, the media were replaced with either control or differentiation media (see section 2.1.8). The cells were incubated for 21 hours and subsequently treated with 100 nM MitoView Green for 20 minutes at 37°C in the incubator. After the incubation period, cells were washed with 1X PBS, trypsinized and collected with normal growth media. They were centrifuged at 1000 rpm for 5 minutes, and the cell pellet was resuspended in 1X PBS supplemented with 1% FBS. The cells were analyzed by BD FACSCanto™ II and data was evaluated using FlowJo v10 software.

2.1.11 Quantification of Total ATP level

On day 0, SH-SY5Y WT cells were seeded in 96-well plates at a density of 1×10^4 cells with six replicates and cultured in normal growth media overnight. Another plate was prepared for crystal violet staining for normalization purposes later on. The following day, the media were replaced with either 200 µl of control or differentiation media (refer to section 2.1.8). The cells were then incubated for 21 hours in the incubator. To calculate the total ATP level in the cells post-treatment, the CellTiter-Glo® Luminescent Cell Viability Assay was used following the manufacturer's protocol. Briefly, a serial 10-fold dilution of ATP was prepared in the cultured media and aliquoted into empty wells on the plate to generate an ATP standard curve. The cell culture medium was then replaced with a reduced volume (50 µl) of the same medium,

and an equal volume of CellTiter-Glo® Reagent was added to each well. Both the ATP standards and the cells were treated in the same manner. The 96-well plate was placed in a plate reader, incubated for two minutes with shaking for cell lysis followed by a 10-minute stabilization period for the luminescent signal and measured. Total ATP levels were normalized to the cell number obtained from crystal violet staining.

2.1.12 Analysis of Polar Metabolites and Lipids

2.1.12.1 Extraction of Water Soluble Metabolites and Lipids from Cells

SH-SY5Y SPTSSA KO and control clones were seeded in 6 well plates at a density of 4×10^5 cells as five replicates. The cells were cultured for 48 hours and afterwards, media were aspirated, cells were washed with PBS and then frozen in liquid nitrogen. Cells were scrapped in a mixture containing 700 μ L methanol/water (4:1) and internal standards (Tables 18 and 19). Then, they were transferred to a reaction tube. Tert-butyl methyl ether (1 ml) was added to samples, which were vortexed thoroughly for 30 seconds and stirred in thermomixer at 1000 rpm for 1 hour at 20 °C. A total of 300 μ L of ultrapure water were added to samples and they were kept in ice-cold bath for 10 min. After centrifugation at $10,000 \times g$ for 10 min at 4°C, the organic phase containing the total lipid extract was transferred to a glass vial, dried under nitrogen stream, and dissolved in 80 μ L of isopropanol. The aqueous phase was collected, dried using SpeedVac and dissolved into 80 μ L of H₂O/ACN (25/75). The pellet obtained after extraction was used for protein quantification using BCA method. LC-MS/MS conditions for lipidomic water-soluble metabolite analyses were set as described below.

Table 18. Internal standard (IS) mix used in the analysis of lipids

Internal standard	nmol/sample	Supplier
PC (15:0/18:1-d7)	2.125	Avanti Polar Lipids Inc., Alabaster, AL, USA
PE (15:0/18:1-d7)	0.070	Avanti Polar Lipids Inc., Alabaster, AL, USA
PS (15:0/18:1-d7)	0.066	Avanti Polar Lipids Inc., Alabaster, AL, USA
PG (15:0/18:1-d7)	0.405	Avanti Polar Lipids Inc., Alabaster, AL, USA
PI (15:0/18:1-d7)	0.118	Avanti Polar Lipids Inc., Alabaster, AL, USA
PA (15:0/18:1-d7)	0.105	Avanti Polar Lipids Inc., Alabaster, AL, USA
LPC (18:1-d7)	0.473	Avanti Polar Lipids Inc., Alabaster, AL, USA

(continued)

Table 18 (continued)

Internal standard	nmol/sample	Supplier
LPE (18:1-d7)	0.103	Avanti Polar Lipids Inc., Alabaster, AL, USA
SM (d18:1/18:1-d9)	0.406	Avanti Polar Lipids Inc., Alabaster, AL, USA
MG (18:1-d7)	0.055	Avanti Polar Lipids Inc., Alabaster, AL, USA
DG (15:0/18:1-d7)	0.170	Avanti Polar Lipids Inc., Alabaster, AL, USA
TG (15:0/18:1-d7)/15:0)	0.677	Avanti Polar Lipids Inc., Alabaster, AL, USA
CE (18:1-d7)	5.318	Avanti Polar Lipids Inc., Alabaster, AL, USA
Cholesterol-d7	2.540	Avanti Polar Lipids Inc., Alabaster, AL, USA
CoQ6	0.085	Avanti Polar Lipids Inc., Alabaster, AL, USA
CL (14:0x4)	0.980	Avanti Polar Lipids Inc., Alabaster, AL, USA
Cer (d18:1/17:0)	0.453	Avanti Polar Lipids Inc., Alabaster, AL, USA
HexCer (d18:1/17:0)	0.350	Avanti Polar Lipids Inc., Alabaster, AL, USA
CAR (18:1)-d3	0.107	Avanti Polar Lipids Inc., Alabaster, AL, USA

Table 19. Internal standard (IS) mix used in the analysis of water soluble metabolites

Internal standard	nmol/ sample	Supplier
PC (15:0/18:1-d7)	1.25	Eurisotop, Cambridge Isotope Laboratories, Inc
Amino acid mix (list in Supplementary Figure 20)	5.00	Eurisotop, Cambridge Isotope Laboratories, Inc
Sodium pyruvate (D3)	10.00	Eurisotop, Cambridge Isotope Laboratories, Inc
Sodium lactate (3,3,3-D3)	5.00	Eurisotop, Cambridge Isotope Laboratories, Inc
Citric acid (2,2,4,4-D4)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
alpha-Ketoglutaric acid (D6)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
2-Hydroxyglutaric acid (2,3,3,-D3)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
Succinic acid (D6)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
Fumaric acid (D4)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
Malic acid (2,3,3-D3)	5.00	Eurisotop, Cambridge Isotope Laboratories, Inc

(continued)

Table 19 (continued)

Internal standard	nmol/ sample	Supplier
Cystine (3,3,3',3', -D4)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
Ornithine:HCl (3,3,4,4,5,5-D6)	2.50	Eurisotop, Cambridge Isotope Laboratories, Inc
Creatine (Methyl-D3)		Eurisotop, Cambridge Isotope Laboratories, Inc

2.1.12.2 LC-MS/MS Analysis of Water Soluble Metabolites

The protocol from Wang et. al was followed for the analysis (Wang et al., 2024). Briefly, water soluble metabolites were analysed by LC-MS/MS using a Q-Exactive Plus quadrupole-Orbitrap mass spectrometer interfaced with an ultra high-performance liquid chromatography (Ultimate 3000, Thermo Fisher Scientific). The analytical gradient was carried out using an SeQuant ZIC-pHILIC column (5 μ m, 2.1 mm x 150 mm, Millipore Sigma) with solvent A (20 mM ammonium carbonate + 0.1% ammonium hydroxide in water) and solvent B (acetonitrile). The samples (3 μ L) were injected into the column at 25 °C, and the analytical gradient lasted 30 minutes, as follows: linear gradient from 80% to 20% within 20 minutes before returning to 80% in 0.5 min. and appended with a 7.5-minute equilibration step. The flow rate was maintained at 150 μ L/min. The eluents were analysed with MS in ESI positive/negative mode with ddMS2. The full scan at 70k resolution (69-1000 m/z scan range, 1e6 AGC-Target, 50 ms maximum Injection Time (maxIT)) was followed by a ddMS2 at 17.5k resolution (1 x 105 AGC target, 20 ms maxIT, 1 loop count, 2 s to 10 s apex trigger, 2 x 103 minimum AGC target, 20 s dynamic exclusion). The HESI source parameters were set as 40 sheath gas flow rate, 15 auxiliary gas flow rate, 1 sweep gas flow rate, spray voltage: 3.0 kV in positive mode, 3.1 kV in negative mode, 320 °C capillary temperature, and the heater temperature of auxiliary gas was 120 °C. The annotation of the metabolites was performed using the EI-Maven software (Elucidata, <https://www.elucidata.io/el-maven>) with an offset of $\pm$ 15 ppm. The intensity of each target was normalized to the internal standards (positive/negative standards for metabolites) and protein concentration.

2.1.12.3 LC-MS/MS Analysis of Lipids

The protocol from Chaves et. al was followed for the analysis (Chaves et al., 2019). Briefly, total lipid extract and total fatty acids were analysed by LC-MS/MS using a Q-Exactive

Plus quadrupole-Orbitrap mass spectrometer (Thermo) interfaced with an ultra high-performance liquid chromatography (Vanquish, Thermo). The samples were loaded into a BEH® (UPLC® C18 column, 1.7 μ m, 2.1 mm i.d. x 100 mm) with a flow rate of 0.2 mL min⁻¹ and the oven temperature maintained at 40 °C. For reverse-phase LC, mobile phase A consisted of water/acetonitrile (60:40), while mobile phase B composed of isopropanol/acetonitrile/water (88:10:2). Mobile phases A and B contained 10 mM ammonium formate and 0.1% formic acid for experiments performed in negative or positive ionization modes. Lipids were separated by a 20 min linear gradient as follows: from 40 to 100% B over the first 10 min., hold at 100% B from 10–12 min., decreased from 100 to 40% B during 12–13 min., and hold at 40% B from 13–20 min.

For lipidomic analysis, the MS was operated in both positive and negative ionization modes, and the scan range was set at a mass-to-charge ratio of 350-1600 Da and 350-1300 Da, respectively. The mass spectrometry was operated using sheath gas flow rate: 30; auxiliary gas flow rate: 10; sweep gas flow rate: 0; spray voltage: 3.6 kV (positive)/2.5 kV (negative); capillary temperature: 320 °C; S-lens RF level: 55.0; auxiliary gas heater temperature: 120 °C. Data acquisition on MS was performed using the following settings: mass resolution = 70,000 at m/z 200, Automatic Gain Control (AGC) = 1×10^6 ; and Injection Time (IT) = 50 ms. Data for lipid molecular species identification and quantification was obtained by data dependent acquisition (DDA) using the following settings: mass resolution = 17,500 at m/z 200, AGC = 1×10^5 ; IT = 50 ms; isolation window = 1.2 m/z ; collision energy (CE) = 20/40/60; dynamic exclusion = 10s; and Minimum AGC target to trigger MS/MS = 2×10^3 . For total fatty acid analysis, the MS was operated in negative ionization mode, and the scan range was set at a mass-to-charge ratio of 180-500 Da. Data acquisition on MS was performed using the same settings described for lipidomics.

Lipid molecular species were manually identified based on their specific fragments and/or neutral losses, retention time and exact m/z . Data was analysed with the help of SeeMS® and an in-house Excel-based macro developed by our group. After identification, the area of lipid species was obtained by MS data using EI-Maven. Each peak integration was carefully inspected for correct peak detection and accurate area determination. The area ratio obtained for each lipid was normalized by the peak area of its corresponding internal standard. The concentration of lipid molecular species and fatty acids was estimated by multiplying the area

ratio by the concentration of the corresponding internal standard. The concentration of lipids was expressed in nmol/mg of protein.

2.2 MATERIALS

2.2.1 Buffers and Solutions

Buffers and solutions used in this study were prepared using either autoclaved MilliQ water or Aqua ad injectabilia Braun unless otherwise specified.

Table 20. Buffers/Solutions and their compositions used in this study

BUFFERS & SOLUTIONS	
Protein Studies Related	
Name	Composition
Cell lysis buffer (for proteomic analysis)	10 mM NaF, 1mM Na ₃ VO ₄ , 250 U/ml Benzonase (Merck Millipore, Cat. No: 70664-3), 1 tablet Complete EDTA free protease inhibitor (Merck, Cat.4693159001), tablet PhosSTOP (Merck, Cat. 4906845001), 10 U/ml DNase I (Qiagen, Cat. No: 14325) in 10 ml RIPA buffer (Thermo Scientific, Cat. No: 89900),
10x PBS pH 7.4	1.5M NaCl, 30mM KCl, 80mM Na ₂ HPO ₄ x 2H ₂ O, 20mM KH ₂ PO ₄
10x TBS pH 7.5	0.2M Tris-Base, 1.5M NaCl
10x TBS-T pH 7.5	20mM Tris-Base, 150 mM NaCl, 1% Tween-20
4X Laemmli buffer pH 6.8	40% Glycerol, 250mM Tris-HCl, 8% SDS, 400mM DTT, 0.02% Bromophenol Blue
4x Resolving buffer pH 8.8	1.5 M Tris-Base, 0.4% SDS
4x Stacking buffer pH 6.8	0.5 M Tris-Base, 0.4% SDS
Running buffer (10x)	0.25M Tris-Base, 1.92M Glycine, 1% SDS
Transfer buffer (10x)	0.25M Tris-Base, 1.92 M Glycine

Transfer Buffer (1X)	10% Transfer Buffer (10X), 70% MilliQ Water, 20% Methanol (added just before using)
Blocking Buffer (1X)	5% skimmed milk (w/v) in 1X TBS-T
DNA & RNA Related	
<i>Name</i>	<i>Composition</i>
50x TAE buffer pH 8.0	50mM EDTA, 2M Tris-Base, adjust pH using 100% acetic acid
Gel Loading Buffer	0.25% Xylene cyanol in 1:1 Glycerol/ Tris-HCl
Buffer EB	10 mM Tris/HCl, pH 8.5
Buffer AE	10 mM Tris/HCl, 0.5 mM EDTA, pH 9.0
1X Buffer G	10 mM Tris-HCl (pH 7.5), 10 mM MgCl ₂ , 50 mM NaCl, 0.1 mg/mL BSA
Molecular Cloning Related	
<i>Name</i>	<i>Composition</i>
Transformation buffer 1 pH 5.8	30mM Kac, 10mM CaCl ₂ x 2H ₂ O, 50mM MnCl ₂ x 4H ₂ O, 100mM KCl, 15% glycerol (v/v), adjust pH with 0.2M acetic acid
Transformation buffer 2 pH 7.0	10mM MOPS, 10mM KCl, 75mM CaCl ₂ , 15% glycerol (v/v), adjust pH with 1M NaOH
LB Media pH 7.0	0.5% NaCl, 0.5% Yeast Extract, 1.0% Tryptone
LB Agar Plates pH 7.0	0.5% NaCl, 0.5% Yeast Extract, 1.0% Tryptone, add 4.5g agar per 300mL
Others	
<i>Name</i>	<i>Composition</i>
Crystal Violet Solution	0.1% (w/v) crystal violet in 20% ethanol
FACS Buffer	1% FBS (v/v) in 1X PBS

(continued)

Table 20 (continued)

<i>Name</i>	<i>Composition</i>
FACS Cell Sorting Buffer	1X PBS, 1.5% FBS, 0.5 mM EDTA
Cell freezing Media	90% FBS, 10% DMSO (v/v)
SPT AA buffer	50 mM HEPES, 1 mM DTT, 150 mM NaCl, 1 mM EDTA, 1× protease inhibitor cocktail, 1x phosphatase inhibitor

2.2.2 Media and Supplements for Cell Culture

Table 21. Media and supplements used to culture, differentiate and stimulate the cells

Growth Media	Company	Reference Number
DMEM/F12 (1:1) (1X) media ^{1, 2}	Gibco™, Thermo Fisher Scientific	11320074
Neurobasal™ Plus Medium ²	Gibco™, Thermo Fisher Scientific	A3582901
Pen/Strep (10,000 U/mL) ^{1, 2}	Gibco™, Thermo Fisher Scientific	15140122
Value HI FBS ¹	Gibco™, Thermo Fisher Scientific	A5256801
GlutaMAX™ Supplement ²	Gibco™, Thermo Fisher Scientific	35050038
B-27™ Supplement (50X), minus vitamin A ²	Gibco™, Thermo Fisher Scientific	12587010
All-Trans-Retinoic acid, ≥98% (HPLC), powder ²	Sigma Aldrich	R2625
Opti-MEM I Reduced Serum Medium ⁴	LIFE Technologies	31985-062
Human EGF Recombinant Protein ³	Thermo Fisher Scientific	PHG0311

¹ For culture of SH-SY5Y cells

² For culture of differentiation of SH-SY5Y cells into neuron-like cells

³ EGF Stimulation

⁴ Transfection of the cells

Table 22. Additional solutions used to culture cells

Solution	Company	Reference Number
Trypsin (2.5%), no phenol red	Gibco™, Thermo Fisher Scientific	15090046
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537
CASYton	OLS	5651808

2.2.3 Kits, Reagents and Solutions

Table 23. List of kits, reagents and solutions used in this study

Name	Company	Reference Number
<i>Bbs</i> I	Invitrogen, Thermo Fisher Scientific	ER1011
Benzonase, Purity > 99%	Merck Millipore	70664-3
Buffer G (10X)	Invitrogen, Thermo Fisher Scientific	BG5
cOmplete Mini, EDTA-free Protease I	Sigma-Aldrich	4693159001
DNeasy Blood & Tissue Kit	QIAGEN	69504
dNTP Set (100 mM)	Invitrogen, Thermo Fisher Scientific	10297018
<i>Eco</i> RV	Invitrogen, Thermo Fisher Scientific	15425
FuGENE® HD Transfection Reagent	Promega	E2311
GeneRuler DNA Ladder Mix	Thermo Fisher Scientific	SM0331
Hexanucleotide Mix (HexaN)	Sigma-Aldrich	11277081001
Midori Green Advance	Biozym	617004
MitoTracker® Red CMXRos	Cell Signaling Technology	9082
MitoView Green	Hözel Biotech	B-70054-T
M-MLV Reverse Transcriptase, 10,000u	Promega	M1701
NEBuffer 2	New England Biolabs	B7002S

(continued)

Table 23 (continued)

Name	Company	Reference Number
PageRuler Plus Prestained Protein Ladder, 10 to 180 kDa	LIFE Technologies	26616
PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa	LIFE Technologies	26619
PEQGOLD TRIFAST	VWR International	30-2010
PhosSTOP (phosphatase inhibitor)	Sigma-Aldrich	4906837001
Pierce BCA Protein Assay Reagent A	Thermo Fisher Scientific	23223
Pierce BCA Protein Assay Reagent B	Thermo Fisher Scientific	23224
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific	A25780
Q5 High Fidelity DNA Polymerase	New England Biolabs	M0491S
Q5 High GC Enhancer	New England Biolabs	B9028A
Q5 Hot Start High-Fidelity DNA Polymerase	New England Biolabs	M0493S
QIAGEN Plasmid Maxi Kit	QIAGEN	12163
QIAprep Spin Miniprep kit	QIAGEN	27104
QIAquick Gel Extraction Kit	QIAGEN	28704
QIAquick PCR purification kit	QIAGEN	28104
RiboLock RNase Inhibitor (40 U/uL)	LIFE Technologies	EO0381
RIPA Lysis and Extraction Buffer	LIFE Technologies	89900
RNase-Free DNase Set	QIAGEN	79254
RQ1 RNase-Free DNase	Promega	M6101
S.O.C. Medium	Invitrogen, Thermo Fisher Scientific	15544034
T4 DNA Ligase Buffer	Invitrogen, Thermo Fisher Scientific	46300018
T4 Ligase	Invitrogen, Thermo Fisher Scientific	15224017

2.2.4 Chemicals

Table 24. List of chemicals used in this study

Chemicals	Company
2-Propanol LC-MS CHROMASOLV®	Thermo Fisher Scientific, Waltham, MA, USA
Agarose	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ampicillin	Sigma Aldrich Inc., St. Louis, MI, USA
Bromophenol Blue	Sigma Aldrich Inc., St. Louis, MI, USA
Chloroform HPLC-grade	Honeywell International Inc., Morristown, NJ, USA
DMSO	Sigma Aldrich Inc., St. Louis, MI, USA
EDTA	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Ethanol	Sigma Aldrich Inc., St. Louis, MI, USA
Glycerol	Honeywell International Inc., Morristown, NJ, USA
HEPES	Thermo Fisher Scientific, Waltham, MA, USA
KCl	AppliChem GmbH, Darmstadt, Germany
KH ₂ PO ₄	Merck KGaA, Darmstadt, Germany
Methanol	Thermo Fisher Scientific, Waltham, MA, USA
Methanol LC-MS CHROMASOLV®	Thermo Fisher Scientific, Waltham, MA, USA
Na ₃ VO ₄	Sigma Aldrich Inc., St. Louis, MI, USA
NaCl	Fisher Scientific GmbH, Hampton, New Hampshire, USA
NAF	Thermo Fisher Scientific, Waltham, MA, USA
NaOH	Thermo Fisher Scientific, Waltham, MA, USA

(continued)

Table 24 (continued)

Chemicals	Company
SDS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
TEMED	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Tris Base	Sigma Aldrich Inc., St. Louis, MI, USA
Tween-20	Sigma Aldrich Inc., St. Louis, MI, USA
Water LC-MS CHROMASOLV®	Thermo Fisher Scientific, Waltham, MA, USA

2.2.5 Primers and gRNA Target Sequences

Table 25. List of primers for PCR or qPCR

Primer Name	Sequence 5' -> 3'	Product Length	T _m	Application
SPTLC1- F	AGCGGCTAACTATGGCGACC	77 bp	62 °C	qPCR
SPTLC1-R	AGCAGGAGCCTCGTAAAGCG			
SPTLC2-F	GAAGTGCATCATGGGGCAGGA	164 bp	62 °C	qPCR
SPTLC2- R	TGGCAGGCATGTAGAGCATCAA			
SPTLC3- F	CCATGAGCCCACCGATAGCA	84 bp	62 °C	qPCR
SPTLC3- R	TCTGCAGCCCTTGAGTGGTC			
SPTSSA-F	AGACTGACGTGTGAGCTGCAT	158 bp	62 °C	qPCR
SPTSSA-R	ACCAGCATGGAATTGAACACCG			
SPTSSB-F	CACCTAAGCCGCAGGGAGAT	144 bp	62 °C	qPCR
SPTSSB- R	GGCCCAGGAATGAGCACTAAGA			
hSPTSSA 5UTRs	GACAGACTGACGTGTGAGCTG	361 bp	67 °C	PCR
hSPTSSA Int1as	GGATCTCAAGAGTTCTCGTCTCC			
GAP43- F	TTGACTTTCTGGATTTC AAGGGTTG	122 bp	60 °C	qPCR
GAP43- R	CCACGGAAGCTAGCCTGAAT			

(continued)

Table 25 (continued)

Primer Name	Sequence 5' -> 3'	Product Length	Tm	Application
MAP2- F	CAGCCTGTGGGGATAATGCT	100 bp	60 °C	qPCR
MAP2- R	ACAAGCTGAAGAATCAGCGCA			

Table 26. Guide RNA sequences targeting the SPTSSA locus. The BbsI shown in italic and additional G nucleotide was shown underlined.

Guide	Sequence 5' -> 3'
exon1_gRNA93rvSenseSPTSSA	<i>CACCGTGTAGAGCGCCGTGACCAGC</i>
exon1_gRNA93rvAntisenseSPTSSA	<i>AAACGCTGGTCACGGCGCTCTACAC</i>

2.2.6 Antibodies

Table 27. List of primary and secondary antibodies used in Western blot

1° Antibody	Species	Dilution	Dilution Buffer	Supplier
Akt	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 9272S
c-cbl	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Cell Signaling Cat No. 2747
CRABP1	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Proteintech Cat No.
DCLK1	Rabbit, Polyclonal	1:2000	5% milk in 1X TBS-T	Proteintech Cat No. 21699-1-AP
EGFR	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 2232
GAPDH (FL-335)	Rabbit, Polyclonal	1:5000	5% milk in 1X TBS-T	Santa Cruz Biotechnology Cat No. F0316

(continued)

Table 27 (continued)

1° Antibody	Species	Dilution	Dilution Buffer	Supplier
ITGA2	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 88228
MT-CO2	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Proteintech Cat No. 55070-1-AP
MVP/LRP	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Proteintech Cat No. 16478-1-AP
P-Akt (Ser473)	Rabbit, Monoclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 4058S
P-Akt (Thr308)	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 9275S
P-EGFR (Tyr1069)	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Proteintech Cat No. 30277-1-AP
P-EGFR (Tyr845)	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 2231S
P-EGFR (Tyr992)	Rabbit, Polyclonal	1:1000	5% BSA in 1X TBS-T	Cell Signaling Cat No. 2235
PLEKHA5	Rabbit, Polyclonal	1:1000	5% milk in 1X TBS-T	Proteintech Cat No. 13045-1-AP
UQCRC2	Mouse, Monoclonal	1:2000	5% milk in 1X TBS-T	Abcam, Cat No. ab14745
2° Antibody	Species	Dilution	Dilution Buffer	Supplier
StarBright Blue 520	Goat Anti- Rabbit IgG	1:2500- 1:5000	5% milk in 1X TBS-T	Bio-Rad Laboratories Cat No. 12004159
StarBright Blue 700	Goat Anti- Mouse IgG	1:2500- 1:5000	5% milk in 1X TBS-T	Bio-Rad Laboratories Cat No. 12004159

2.2.7 Instruments

Table 28. List of instruments used in this study

Instruments	Supplier
BD FACSCanto™ II Clinical Flow Cytometry System	BD Biosciences, New Jersey, USA
Casy cell counter and analyzer	Roche Life Science, Basel, Switzerland
Centrifuge 5417R and 5804R	Eppendorf AG, Hamburg, Germany
ChemiDoc™ MP Imaging System	Bio-Rad Laboratories, Hercules, CA, USA
Incubator B6030 and B6060	Heraeus Holding GmbH, Hanau, Germany
Incubator BD115	BINDER GmbH, Tuttlingen, Germany
Incucyte S3 Live Cell-Analysis System	Sartorius AG, Goettingen, Germany
Julabo SW20 water bath	ULABO GmbH, Seelbach, Germany
Magnetic Stirrer MR3002	Heidolph Instruments GmbH, Schwabach, German
Mini-PROTEAN® tetra	Bio-Rad Laboratories, Hercules, CA, USA
Nanodrop spectrophotometer ND-1000	Peqlab Biotechnology GmbH, Erlangen, Germany
Nikon Eclipse Ts2-FL inverted microscope	Nikon Corporation, Tokyo, Japan
Safety cabinet MAXISAFE 2030i	Thermo Fisher Scientific, Waltham, MA, USA
Scale Abt I20-4M	Kern & Sohn GmbH, Balingen-Frommern, Germany
StepOnePlus™ Real-Time PCR System	ThermoFisher Scientific, , Waltham, MA, USA
Synergy H1 microplate reader	BioTek Instruments GmbH, Bad Friedrichshall, Germany
Thermomixer comfort	Eppendorf AG, Hamburg, Germany
Ultrasonic water bath Sonorex Super	Bandelin electronic GmbH & Co. KG, Berlin, Germany

(continued)

Table 28 (continued)

Instruments	Supplier
Ultrasonic water bath Sonorex Super	Bandelin electronic GmbH & Co. KG, Berlin, Germany
Vortex mixer K 7-2020	NeoLab Migge GmbH, Heidelberg, Germany
Waters I-class UPLC system	Waters GmbH, Eschborn, Germany
WTW pocket pH meter pH 3110	Xylem Analytics Germany Sales GmbH & Co.KG, Weilheim, Germany
Xevo TQS mass spectrometer	Waters GmbH, Eschborn, Germany

2.2.8 Software

Table 29. List of software used for data evaluation and statistical analysis

Software	Version	Supplier
BioRender	2023	BioRender
EndNote	21	Clarivate
FlowJo	10.8.1	BD Life Sciences
GraphPad Prism	9.3.1	GraphPad Software
Image Lab	6.0.1	Bio-Rad Laboratories
MassLynx	-	Waters Corporation
Microsoft Office	2016	Microsoft Corporation
SnapGene Viewer	5.0.7	SnapGene
Unipro UGENE	50	Unipro

3. RESULTS

3.1 Analysis of SPT complex Expression and Ceramide Profile of SH-SY5Y WT (parental) Cells

To elucidate the expression profile of SPT complex subunits in SH-SY5Y wt (parental) cells, I extracted total RNA from these cells and performed cDNA synthesis. Afterwards, I made qPCR with primers designed for each subunit of SPT complex and run qPCR product on agarose gel. MCF7 cells were used as positive control which expresses three large (SPTLC1, SPTLC2 and SPTLC3) and two small subunits (SPTSSA and SPTSSB) (Figure 6).

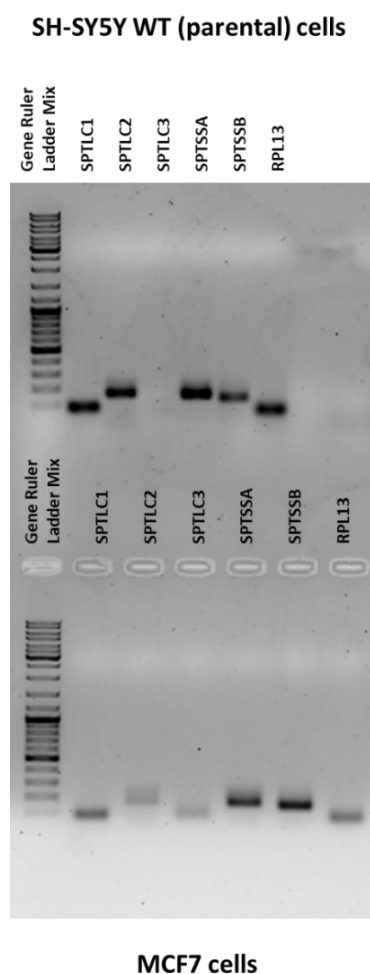


Figure 6. The agarose gel of qPCR products reveals that SH-SY5Y wt (parental) cells expresses the SPT complex large subunits SPTLC1 and SPTLC2 as well as the two SPT small subunits SPTSSA and SPTSSB. MCF7 cells expresses all subunits of SPT complex. RPL13 was loaded as positive control. Expression of SPT complex subunits in SH-SY5Y wt (parental) (upper part) and in MCF7 cells (lower part)

Parental SH-SY5Y (wt) cells were shown to express two of the three large subunits: SPTLC1 and SPTLC2 but they were lack of SPTLC3 expression. On the other hand, they expressed both of the small subunits. SH-SY5Y wt (parental) cells appeared to have stronger expression of SPTSSA than that of SPTSSB with a Ct values 30.8 and 35.7, respectively.

Lipid profile of SH-SY5Y wt (parental) were analyzed by measuring ceramide content of the cells. More specifically, we measured NS- and NDS-ceramides with a fatty acid of variable chain length (C16–C26) and containing either C18- or C20- sphingoid bases as backbone. Our main focus was to understand whether SH-SY5Y cells produce ceramide with C20-sphingoid bases. Subsequently, we aimed to quantify the percentage of ceramide composed of C20-sphingoid bases relative to the total ceramide content within the cell.

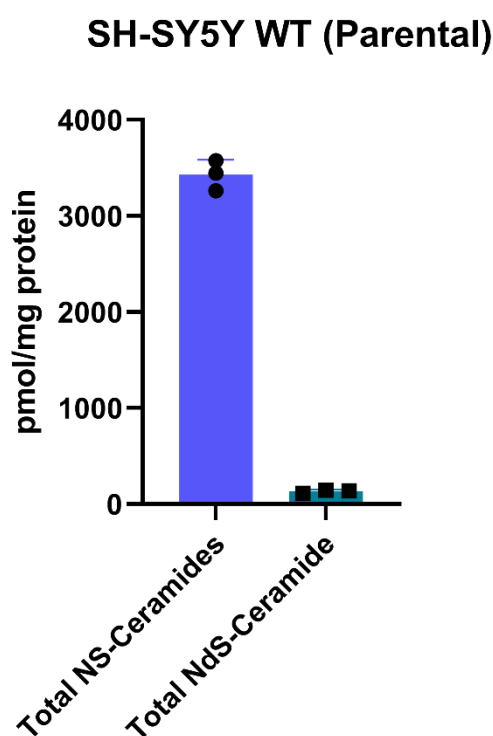


Figure 7. Total amount of NS- or NdS- ceramides in SH-SY5Y wt (Parental) cells as determined by LC-MS/MS in MRM mode. MRM mode targeted those compounds with either a C18-, C20-, C22- or a C24-base containing C16:0, C18:0, C20:0, C22:0, C24:0, C26:0, or C24:1 acyl chains. N refers to non-hydroxy acyl chain and S to sphingosine in NS-Ceramides and dS to dihydrosphingosine (sphinganine) in NdS-Ceramides.

The mean total amounts of NS-ceramides and NdS-ceramides measured in the cells were 3427.7 and 132.6 pmol/mg protein, respectively. This indicates that SH-SY5Y cells contain approximately 25.8 times more NS-ceramides than NdS-ceramides (Figure 7).

I calculated the distribution of NS-ceramides containing 18-, 20-, 22-, or 24-carbon sphingoid bases with different fatty acyl chain lengths by dividing the total amount of NS-ceramides with specific sphingoid base chain lengths by the overall number of NS-ceramides. Then, I determined the percentage of the total as shown in Figure 8. The results indicated that C18-sphingoid bases were the predominant species, constituting approximately 95% of NS-ceramides. The cells also contained around five percent NS-ceramides with C20-sphingoid bases. NS-ceramides with C22- or C24- sphingoid bases were not detected in SH-SY5Y wt (parental) cells by targeted LC-MS/MS.

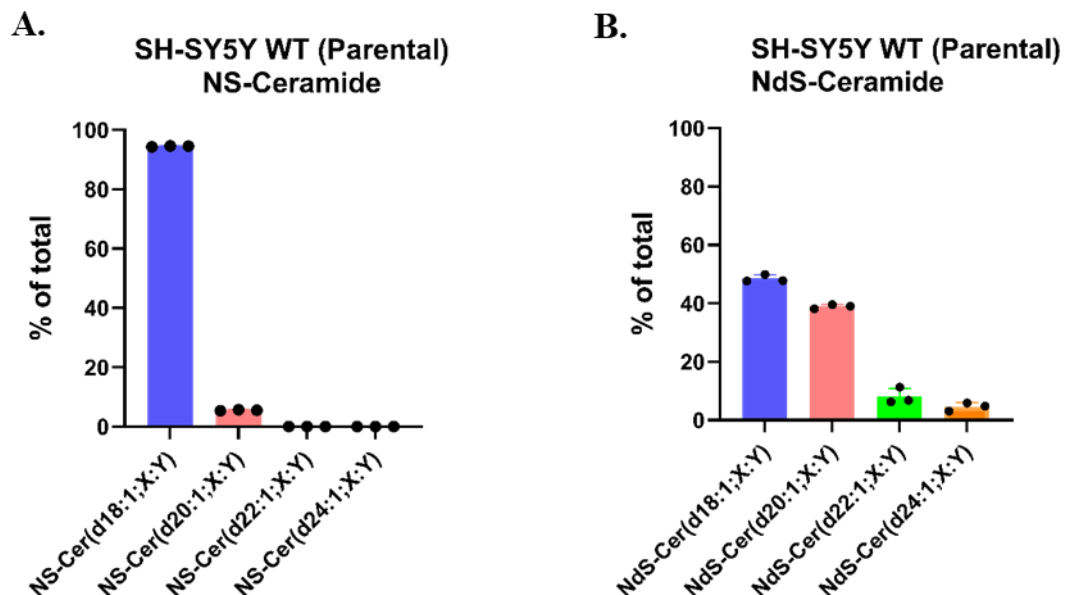


Figure 8. Distribution of NS-ceramides or NdS- ceramides containing 18-, 20-, 22- or 24- carbon sphingoid bases with various fatty acyl chain length in SH-SY5Y wt (Parental) cells. Calculations were performed in LC-MS/MS data of Figure 7.

I further investigated the sphingoid profile of NDS-ceramides by making the same calculation that I did for NdS-ceramides. The results were surprisingly different as the cells contained almost equal amount of C18- (48.5%) and C20- (38.9%) sphingoid bases in NdS-ceramides. Here, we observed that the cells produced NDS-ceramides with even longer sphingoid bases. SH-SY5Y wt (parental) cells contained 8.1% NDS-ceramide with C22-sphingoid bases and 5.9% NdS-ceramide with C24-sphingoid bases. Since the contribution of

NdS-ceramides to the total ceramide pool in SH-SY5Y wt (parental) cells is minor, we decided to concentrate on exploring the distribution of individual species of ND-ceramides with various sphingoid bases and fatty acid chains.

I determined the relative abundance of each NS-Cer(d18:1; X:Y) and NS-Cer(d20:1; X:Y) species percent of the total amount of NS-ceramides recorded (Figure 9). The NS-ceramide profiles indicate that nearly half of the total NS-ceramides comprised NS-Cer(18:1; 24:1) (45.1%), making it the most abundant species. It was followed by NS-Cer(18:1; 16:0) with 20.8%, the second most abundant species identified. Among NS-Cer(d20:1; X:Y) species, NS-Cer(20:1; 24:1) was determined to be the predominant species with 3.5% followed by NS-Cer(20:1; 22:0) and NS-Cer(20:1; 24:0) with 0.8% and 0.7%, respectively. NS-Cer(d18:1; 16:0), NS-Cer(20:1; 20:0) and NS-Cer(20:1; 26:0) species were not detected in SH-SY5Y wt (parental) cells by mass spectrometry.

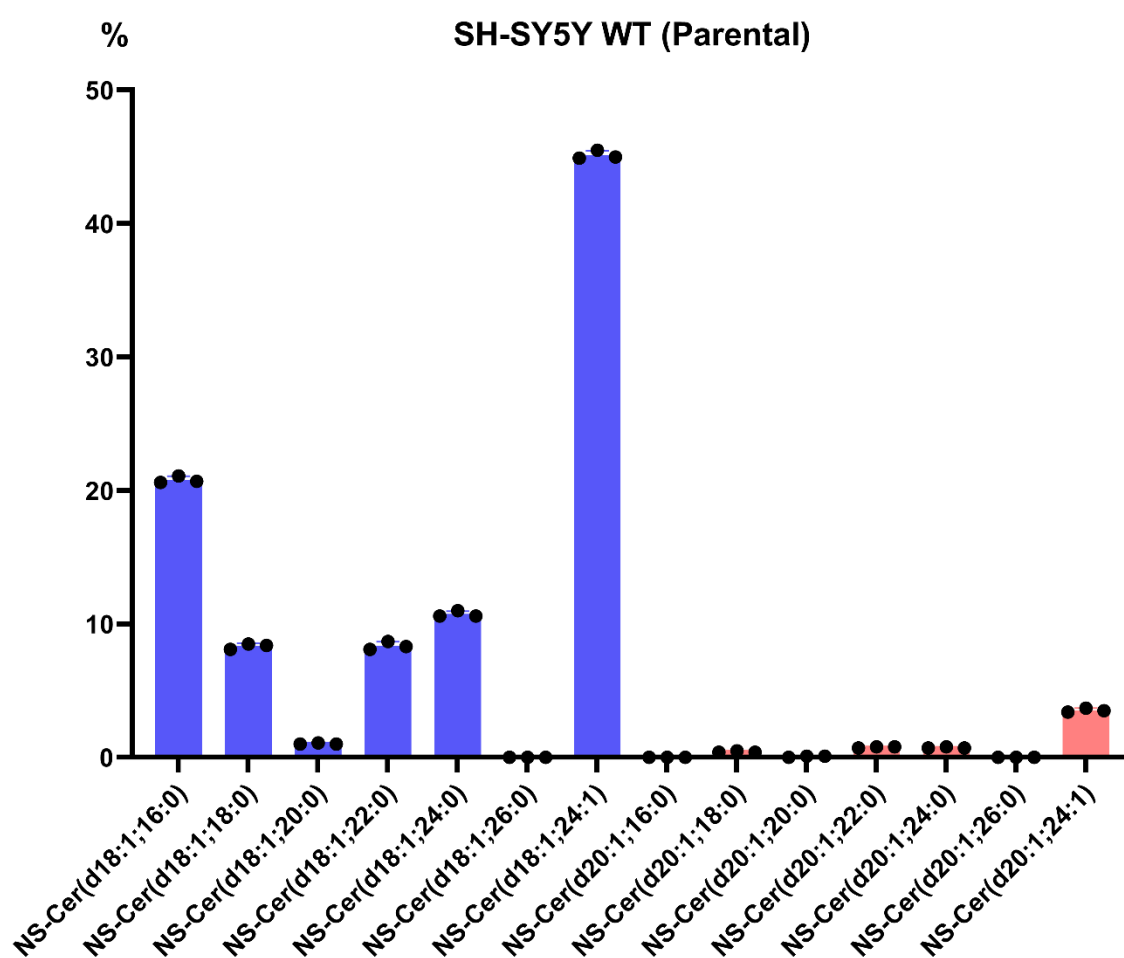


Figure 9. Relative abundance of NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) in SH-SY5Y wt (Parental) cells as calculated from the targeted LC-MS/MS data.

3.2 Generation of SPTSSA KO SH-SY5Y cells

The presence of SPTSSA in SPT complex favors the production of d18-sphingoid bases. In order to increase the concentration of sphingoid bases having more than 18 carbons, SH-SY5Y wt (parental) cells were edited by Crispr-Cas9 technology to knock out SPTSSA. I transiently transfected SH-SY5Y cells with px458-GFP vector (with or without a targeting gRNA). Transfected cells were sorted by FACS with the help of Flow Cytometry Core Facility to obtain single cell colonies (Figure 10). After two weeks of sorting four colonies for SPTSSA KO and two colonies for the empty vector control were obtained. However, one of the colonies from SPTSSA KO stopped dividing and died.

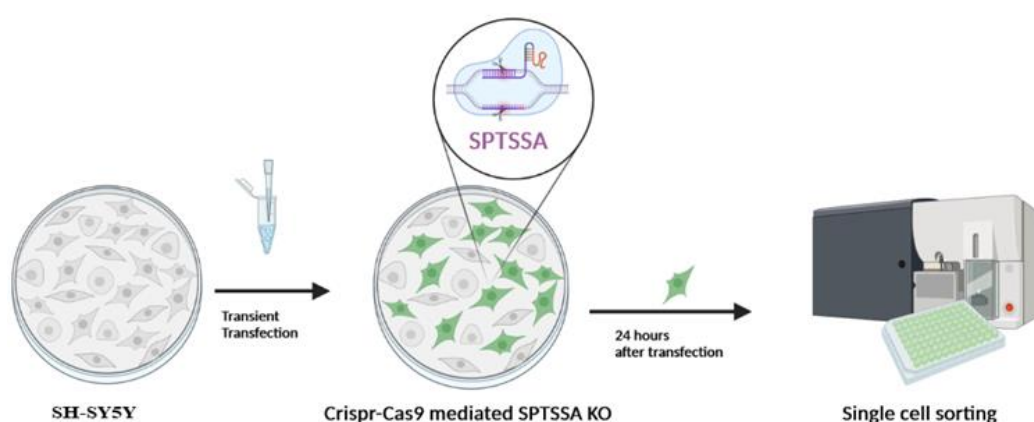


Figure 10. Scheme illustrating the work flow to generate SPTSSA KO SH-SY5Y cells using Crispr-Cas9 technology and single cell sorting by FACS to obtain mutant monoclonal cell lines (Image generated by Biorender).

The remaining cell populations derived from single cells were expanded to obtain monoclonal cell lines. Control clones were named as 1F5 and 2G9. The SPTSSA KO clones were named as 5C11, 4D10 and 5D8.

3.3 Validation of SPTSSA KO in SH-SY5Y Clones

3.3.1 Sanger DNA Sequencing

I extracted genomic DNA from colonies obtained after FACS sorting, performed a PCR to amplify the region targeted by the gRNA93, and sent sample for sequencing. The samples were sequenced by Eurofin Genomics GmbH by Sanger sequencing. The results showed that Cas9-mediated DNA cleavage at the predicted cutting site (G nucleotide in region 111 of the SPTSSA original sequence- Figure 11) and subsequent DNA repair gave rise to small indels in the target DNA of the three knock-out clones. However, no mutation was detected in both of

the control clones. The number of generated indels were not a multiple of three and this resulted in frameshift mutations in all three KO clones. Therefore, I further analyzed the sequencing results to understand whether the genotype of SPTSSA KO clones was homozygous or heterozygous for the mutation of SPTSSA KO. I searched the DepMap database (<https://depmap.org/portal/>) and found that the copy number of SPTSSA gene is two in SH-SY5Y cells. Then, I examined the trace file (chromatogram) of sequencing results using Unipro UGENE. Two out of three clones (4D10 and 5D8) had the homozygous mutation whereas 5C11 had the heterozygous mutation (Supplementary Figure 3, 4 and 5). Even though both alleles of SPTSSA were edited in 5C11 clone, each allele contained different mutations.

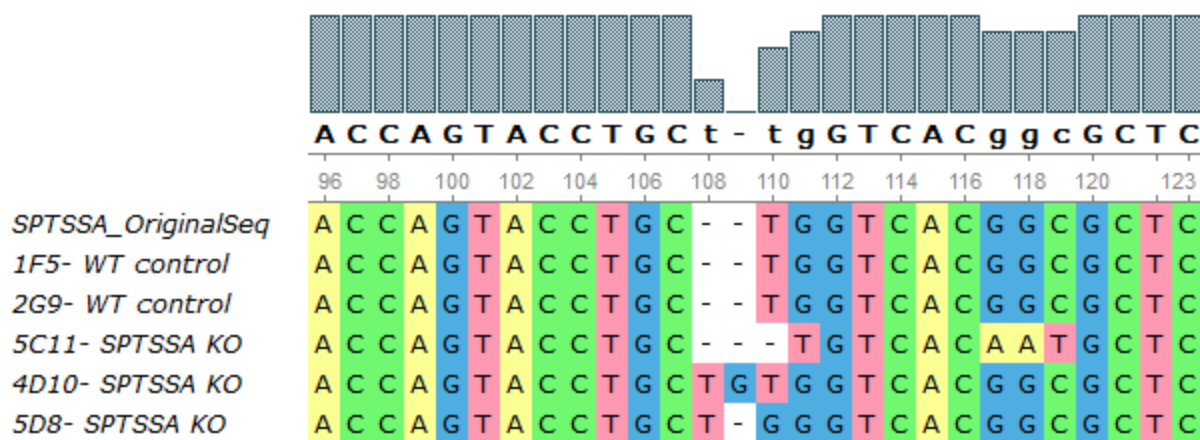


Figure 11. Sequence alignment of amplified PCR product of SPTSSA gene from wilt-type, control and knock out cells.

3.3.2 Analysis of Ceramide Profile

3.3.2.1 Monoclonal SPTSSA KO and Control Cells

After LC-MS/MS analysis of NS-ceramides from these clones had been performed, I calculated the percent distribution of NS-ceramides containing 18- and 20- carbon sphingoid bases taking all acyl chains of the targeted MRM-analysis into account (Figure 12). On average, around 98% of NS-ceramide contained C18-sphingoid bases and around 2% NS-ceramide had C20-sphingoid bases in WT clones (1F5 and 2G9). These results were similar to the SH-SY5Y wt (parental) cells from which the clones were derived. Crispr-Cas9 editing of SPTSSA in clone 4D10, 5C11 and 5D8 led to a decrease of NS-ceramides containing C18-sphingosine down to around 62.7% on average. Accordingly, the percentage of NS-Ceramides with C20-sphingosine were increased around more than 7-fold in SPTSSA KO clones (around 37.3% of total on average) compared to SH-SY5Y wt (parental). NS-ceramides with C22- or C24-

sphingoid bases were not detected in both WT control and SPTSSA KO clones as in the case for SH-SY5Y wt (parental) cells.

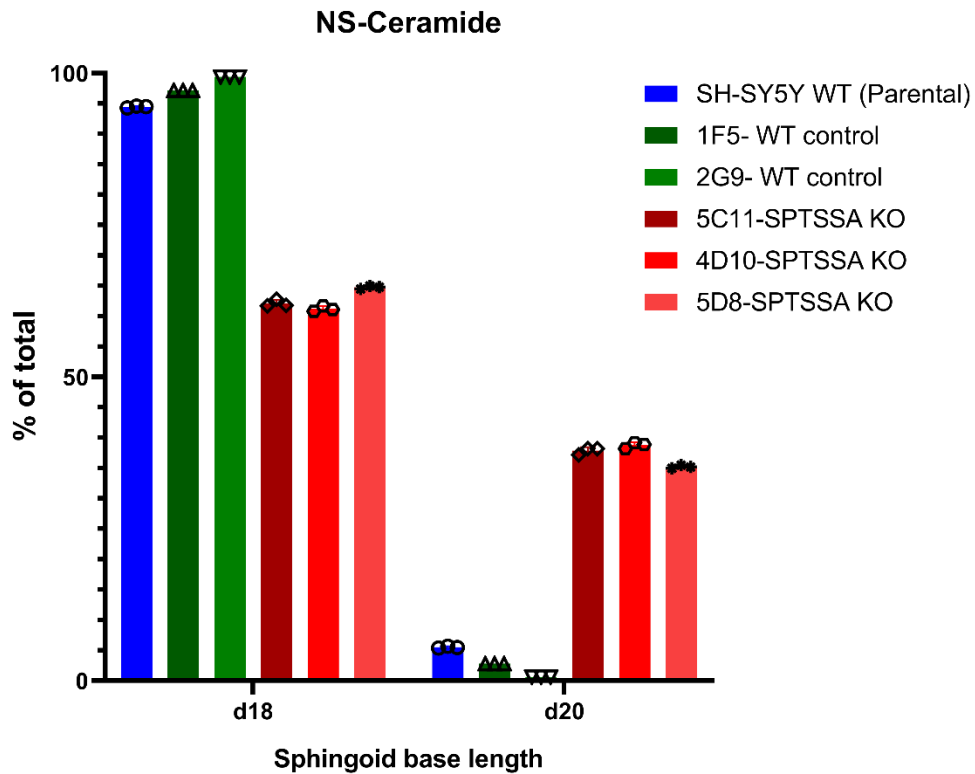


Figure 12. Targeted LC-MS/MS analysis in MRM mode revealed the distribution of NS-ceramides containing 18- and 20-carbon sphingosine in SH-SY5Y wt (parental), WT-clones and SPTSSA KO clones. More than 7 fold increase in the proportion of NS-ceramides with C20-sphingosine was observed in the SPTSSA KO clones compared to wt (parental) cells (red bars)

I evaluated the sphingoid profile of NdS-ceramides the same way that I did for NS-ceramides. Here, there was a slight increase in the average percentage of NdS-ceramide with C20-sphinganine from 38.9% to 47.9% as compared to the SH-SY5Y wt (parental). We did not observe any increase in NdS-ceramides with C22- or C24-sphinganine in SPTSSA KO as compared to parental and wt controls. Since one control clone showed a similar increase in NdS-ceramides with C20-sphinganine, there was no significant difference between control and parental clones on one side as compared to SPTSSA KO clones on the other side with respect to the percent of C18- and C20- sphinganine in NdS-ceramides (Figure 13).

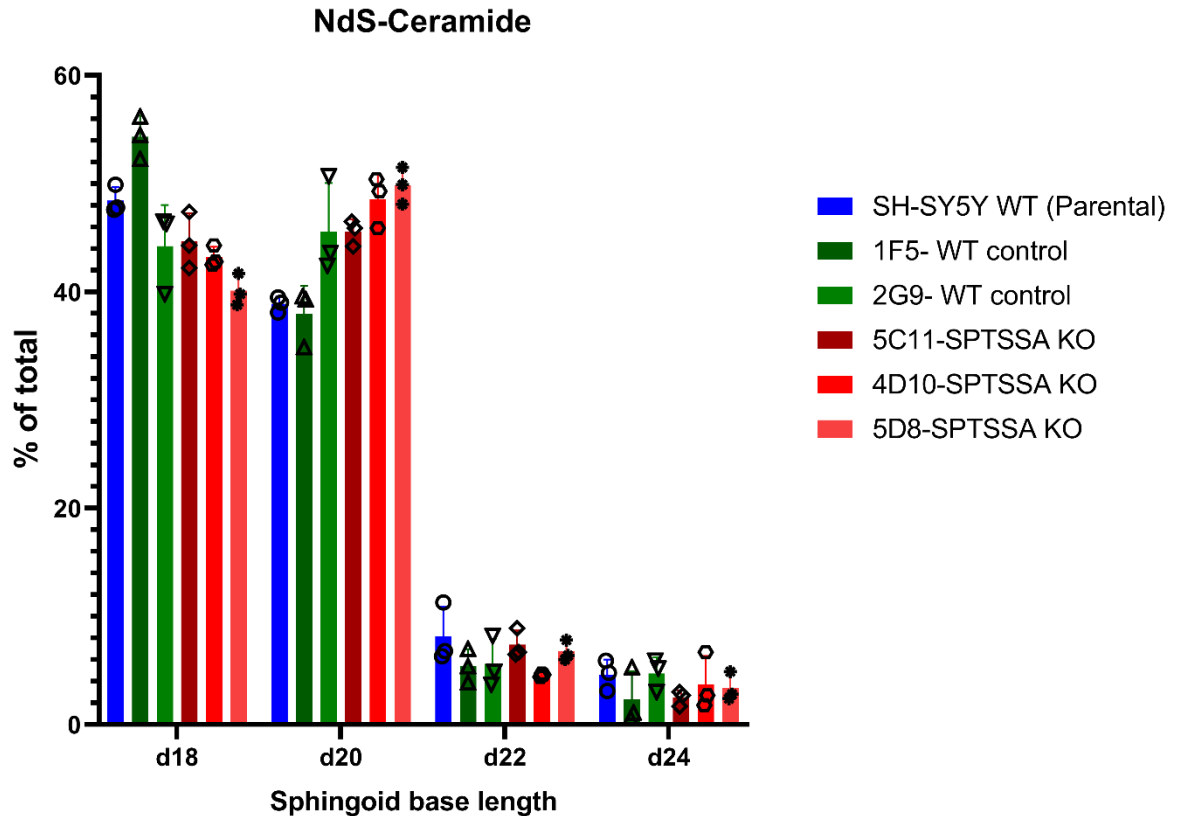


Figure 13. Distribution of NdS-ceramides containing 18-, 20-, 22- or 24- carbon sphinganine in SH-SY5Y wt (Parental, in blue), WT-clones (in green) and SPTSSA KO clones (red bars).

I analyzed the NS-ceramides to find out the relative contribution of each NS-Cer(d18:1; X:Y) and NS-Cer(d20:1; X:Y) to the total of NS-ceramides (Figure 14). This pattern was quite similar in parental cells and control clones with the dominant species being NS-Cer (18:1; 24:1), with 45.8% of the total on average. However, knocking out SPTSSA in KO clones altered this pattern. Whereas resulting in NS-Cer with C22- and C24-acylchain decreased strongly, no significant relative decrease was observed for those with C16- and C18-acyl chains. While NS-Cer with C16-acyl chain made up 20 and more percent within the group of C18-sphingosine containing ceramides, it was almost absent in the group of C20-sphingosine containing ceramides in the KO clones.

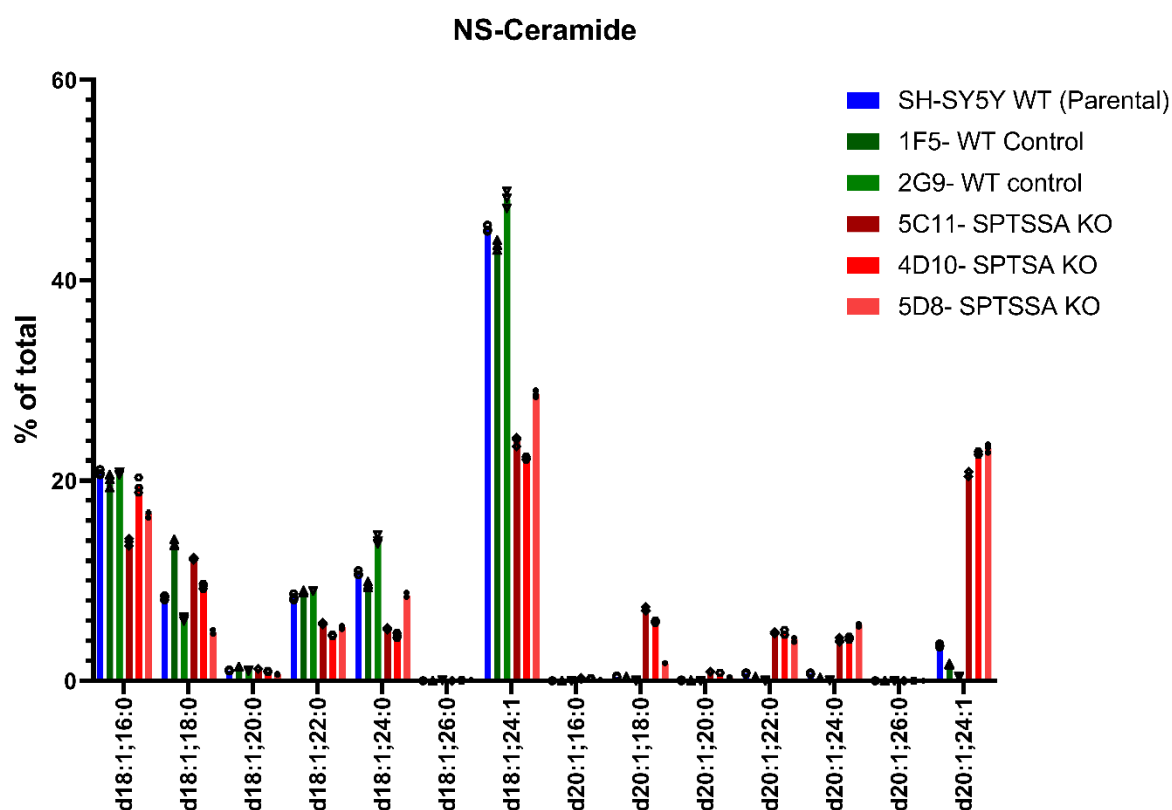


Figure 14. Relative abundance of NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) among all NS-ceramides in SH-SY5Y wt (Parental), control clones and SPTSSA KO clones

3.3.2.2 Polyclonal SPTSSA KO and Control Cells

LC-MS/MS analysis of NS-ceramides were performed for the evaluation of changes in the sphingoid base chain length after the cells were bulk sorted. For evaluation, I performed the same analysis that I did for Figure 12. I calculated the percent distribution of NS-ceramides containing 18- and 20- carbon sphingoid bases taking all acyl chains of the targeted MRM-analysis into account (Figure 15). On average, around 95% of NS-ceramide contained C18-sphingoid bases and around 5% NS-ceramide had C20-sphingoid bases in Bulk-WT control cells. These results were almost the same as SH-SY5Y wt (Parental) cells from which the polyclonal population were derived. Crispr-Cas9 editing of SPTSSA in bulk sorted polyclonal cells led to a decrease of NS-ceramides containing C18-sphingosine down to 84.6% on average. Accordingly, the percentage of NS-Ceramides with C20-sphingosine were increased around more than 3-fold in Bulk- SPTSSA KO cells (around 16.4% of total on average) compared to SH-SY5Y wt (parental). NS-ceramides with C22- or C24- sphingoid bases were not detected

in both Bulk- WT control and bulk- SPTSSA KO cells as in the case for SH-SY5Y wt (Parental) cells.

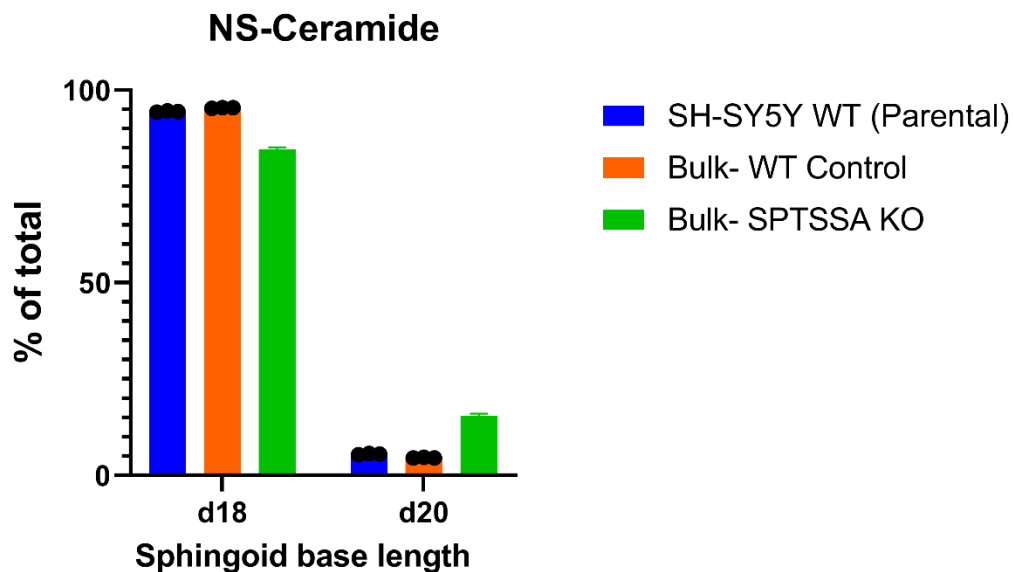


Figure 15. Targeted LC-MS/MS analysis in MRM mode revealed the distribution of NS-ceramides containing 18- and 20-carbon sphingosine in SH-SY5Y wt (parental), Bulk WT-control and Bulk-SPTSSA KO clones. Around 3 fold increase in the proportion of NS-ceramides with C20-sphingosine was observed in the SPTSSA KO clones compared to wt (parental) cells (green bars).

Again, I analyzed the NS-ceramides to find out the relative contribution of each NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) to the total of NS-ceramides in Bulk- WT control and Bulk- SPTSSA KO cells (Figure 16). This pattern was quite similar in parental cells and Bulk- WT control cells with the dominant species being NS-Cer (18:1; 24:1), with 41.7% of the total on average. Knocking out SPTSSA in cells did not cause a strong decrease in NS-Cer with C22- and C24-acyl chain as in the case for SPTSSA KO clones in Figure 14. However, the general relative pattern of how NS-ceramides changed over SPTSSA KO remained consistent both in monoclonal and polyclonal SPTSSA KO cells.

The relative increase in NS-Ceramides with C20-sphingosine in SPTSSA KO cells to control cells was higher in monoclonal compared to polyclonal cells. Therefore, we decided to continue our project using the monoclonal SPTSSA KO and control cells.

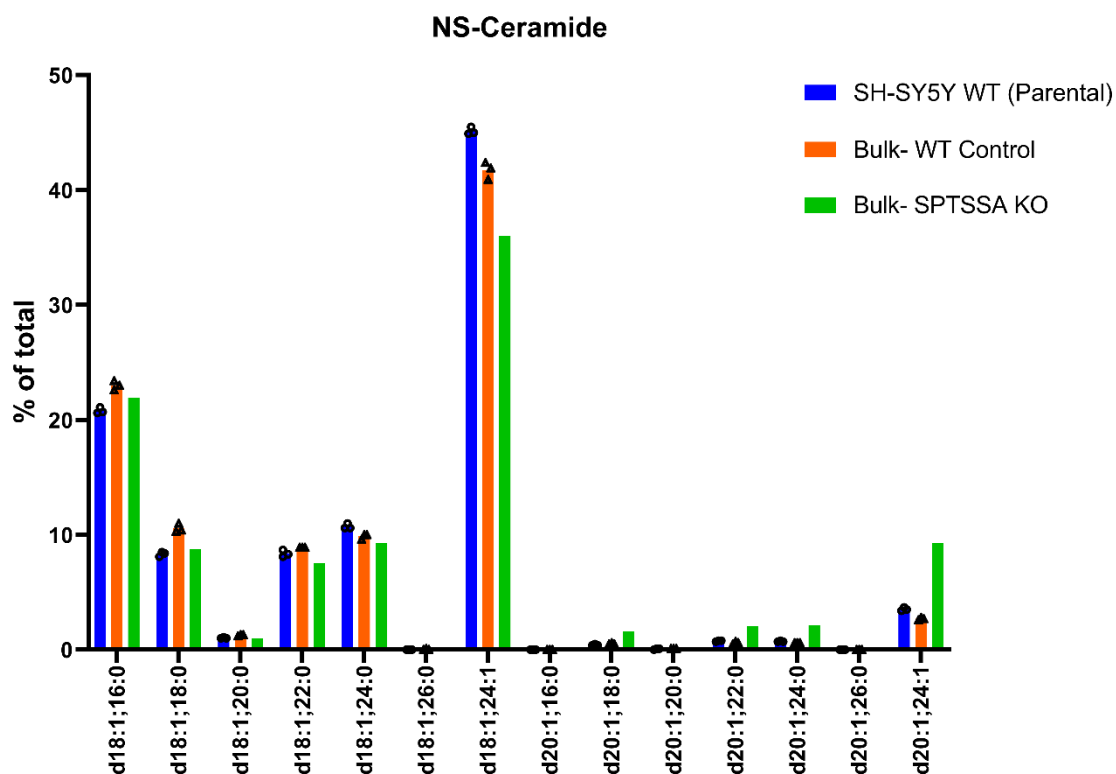


Figure 16. Relative abundance of NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) among all NS-ceramides in SH-SY5Y wt (Parental), Bulk- WT control and Bulk-SPTSSA KO cells

3.3.3 SPT Activity Assay

The SPT complex catalyzes the condensation of L-serine and acyl-CoA to produce 3-ketophingosine (3-KDS). To track changes in SPT activity following SPTSSA KO, we used the $^{13}\text{C}_3$, ^{15}N -isotopically labelled L-Serine and monitored the incorporation of labelled L-serine into 3-KDS. The content of 3-KDS in the cells was measured at 7.5 and 15 minutes after the reactions was induced by adding isolated cell membranes to the reaction mixture. The linear increase of product over time (slope of the regression curve in the graph of Figure 17) was used to calculate the SPT activity. The results revealed a great difference in enzymatic activity among the control clones (1F5 and 2G9). Interestingly, one of the SPTSSA KO clone (4D10) showed enzymatic activity similar to that of the control clone 1F5. However, the enzymatic activity decreased over 3 fold in 5C11 and 5D8 compared to control clones.

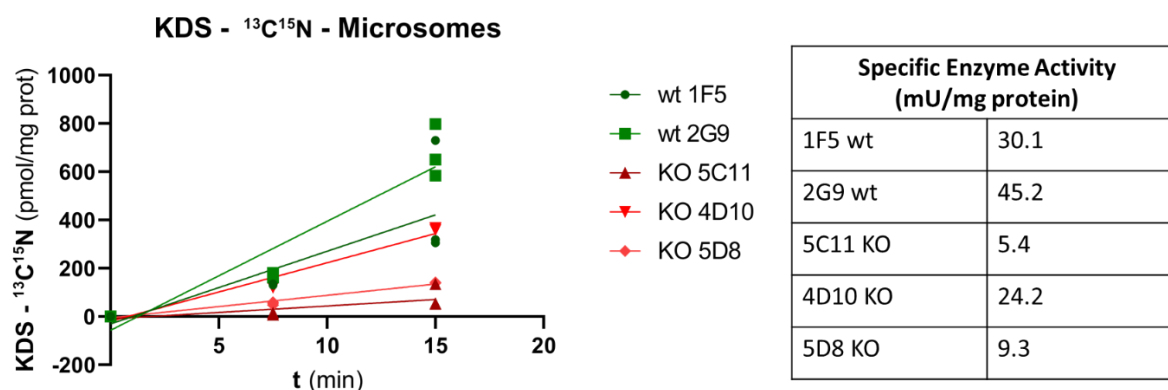


Figure 17. Examining the functional impact of SPTSSA knockout on SPT activity in both control (1F5 and 2G9) and SPTSSA knockout (5C11, 4D10, and 5D8) clones of SH-SY5Y. In an initial experiment with the wild type control cells it was established that under these conditions the substrate turnover is linear within the first 15 min.

3.4 Investigating the Effect of the LCB Chain Length on Cell Signaling and Cell Functionality

3.4.1 Proteome Analysis of SH-SY5Y SPTSSA KO and Control Clones

3.4.1.1 General Overview

Two separate analyses were performed to evaluate the impact of SPTSSA absence on the proteome of SH-SY5Y cells. The experimental setting for the analyses was same except for the passage number of the cells. The initial experiment (AK0735) was conducted 2-3 passages after the generation of SPTSSA KO cells and only the data-independent acquisition (DIA) technique was employed. The second experiment was performed after the cells were maintained in culture for over 30 passages and data were acquired by DIA (Data-Independent Acquisition- AK0800-2) and DDA (Data-Dependent Analysis- AK0800-1). In the first analysis, an average of 8629 and 8693 proteins were detected in control and SPTSSA KO cells, respectively. The average number of quantified proteins in control cells was 7958, whereas in SPTSSA KO cells, it was 8030 (Figure 18A). In the second analysis, an average of 8056 and 8125 proteins were detected in control and SPTSSA KO cells, respectively. The average number of quantified proteins in control cells was 7528, whereas in SPTSSA KO cells, it was 7624 (Figure 18C). Even though, the number of quantified proteins in general were less in the second measurement, we observed that there was slightly more protein on average in the SPTSSA KO cells in both of the measurement. This information is important to understand the

impact of SPTSSA KO on the proteome of the cells and suggests a specific role of SPTSSA in protein homeostasis

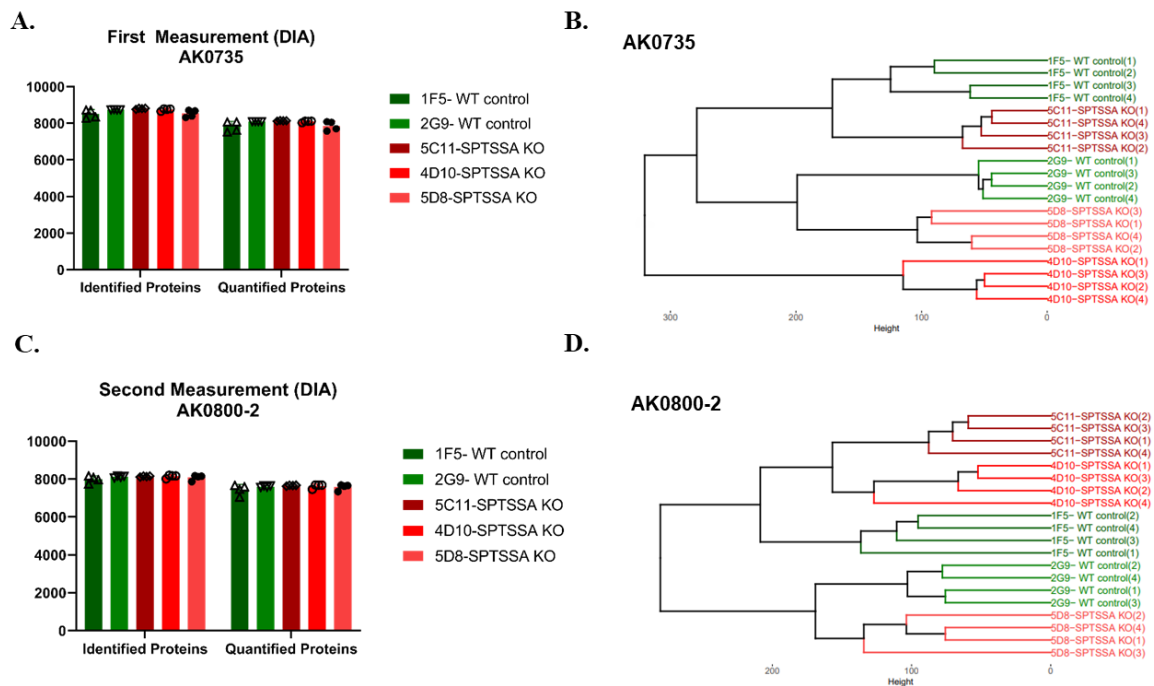


Figure 18. The first (A and B) and second (C and D) total proteome analysis of control and SPTSSA KO clones. (A and C) number of proteins identified and quantified, (B and D) DIA based hierarchical clustering

Hierarchical clustering based on DIA revealed distinct clonal clusters in the initial measurement, indicating heterogeneity within cell populations. SPTSSA KO clone 5C11 and wt control clone 1F5 clustered together, as did SPTSSA KO clone 5D8 and wt control clone 2G9. SPTSSA KO clone 4D10 formed a distinct branch in the clustering (Figure 18B). The clustering pattern shifted in the second measurement, with two main branches emerging. In the first branch, SPTSSA KO clones 5C11 and 4D10 clustered together with WT control 1F5, while in the second branch, SPTSSA KO clone 5D8 clustered with WT control 2G9 (Figure 18D). In both measurements, technical replicates behaved the same way as they clustered in the same branch. However, distinct heterogeneity was observed within the control clones.

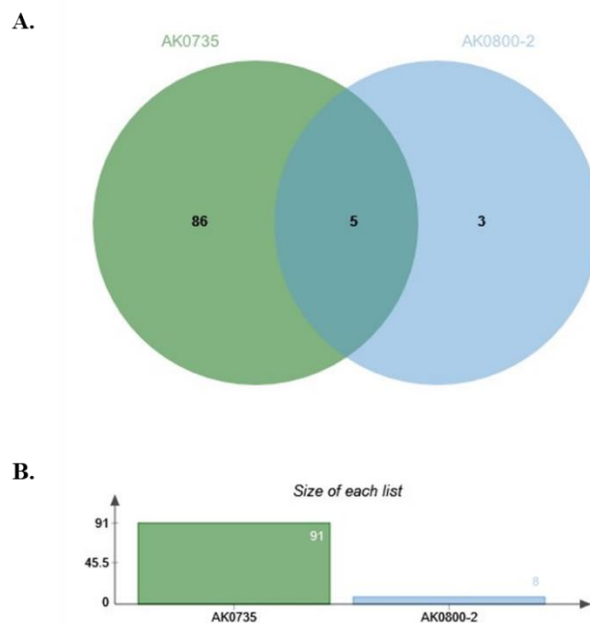


Figure 19. Venn diagram illustrating (A) the number of common and exclusive proteins of AK0735 and AK0800-2, (B) total number of proteins in each measurement passing predefined filters

Proteomics Core Facility conducted statistical analysis by comparing KO clones collectively as one group and control clones collectively as another group for both measurement (AK0735 and AK0800-2). From the data generated, I identified the proteins that were differentially expressed between these two groups using predefined filters ($\text{Log2FC} \geq 1$ or $\text{Log2FC} \leq -1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$). In the first measurement (AK0735), 91 proteins met these criteria and in the second measurement (AK0800-2), only 8 proteins passed the filters. I realized that there were changes in the expression of proteins that passed the filters between two measurements. Most of the proteins passing the predefined filters in the initial analysis failed to pass the filters in the second measurement despite meeting them initially. To determine which proteins were consistently differentially expressed across both measurements, I compared the lists of differentially expressed proteins from the two measurements using the online tool jvenn (<https://jvenn.toulouse.inrae.fr/app/index.html>) (Bardou et al., 2014). Venn diagram provided a visual representation and revealed the common and unique proteins identified in each measurement (Figure 19). It also provided the list of the proteins with consistent differential expression between KO and control group across two measurement. Only five proteins were identified to have persistent differential expression between the KO and control groups across both measurements, and they consistently exhibited

upregulation compared to the control group, indicating that their behavior did not change between the two measurements (Table 30).

Table 30. The list of common proteins detected both in AK0735 and AK0800-2 proteome analyses, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$ Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7 . Log fold change and adjusted p-values were calculated based on imputed data

<i>Common proteins detected in AK0735 and AK0800-2 proteome analyses</i>			
Gene names	Protein names	AK0735 Log_2FC	AK0800-2 Log_2FC
AHNAK	Neuroblast differentiation-associated protein AHNAK	2.17	1.31
SLC27A6	Long-chain fatty acid transport protein 6	1.25	1.22
MVP	Major vault protein	2.43	1.13
CNTNAP1	Contactin-associated protein 1	1.35	1.08
SH3PXD2A	SH3 and PX domain-containing protein 2A	1.03	1.05

3.4.1.2 First Measurement- AK0735

I conducted proteomic analyses comparing SPTSSA KO clones to control clones using two approaches with the help of Proteomics Core Facility. In the first approach, we compared each SPTSSA KO clones individually to both control clones. We identified the set of proteins exhibiting significant differences in expression levels.

Then, I filtered those proteins using predefined filters ($\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$, Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7). Using Venn diagrams, I compared these individual comparisons and identified a core set of four genes consistently upregulated in the same manner across all comparisons (Figure 20). The proteins that were regulated are DCLK1, ITGA2, MVP/LRP, and PLEKHA5. Interestingly, no commonly downregulated proteins were detected.

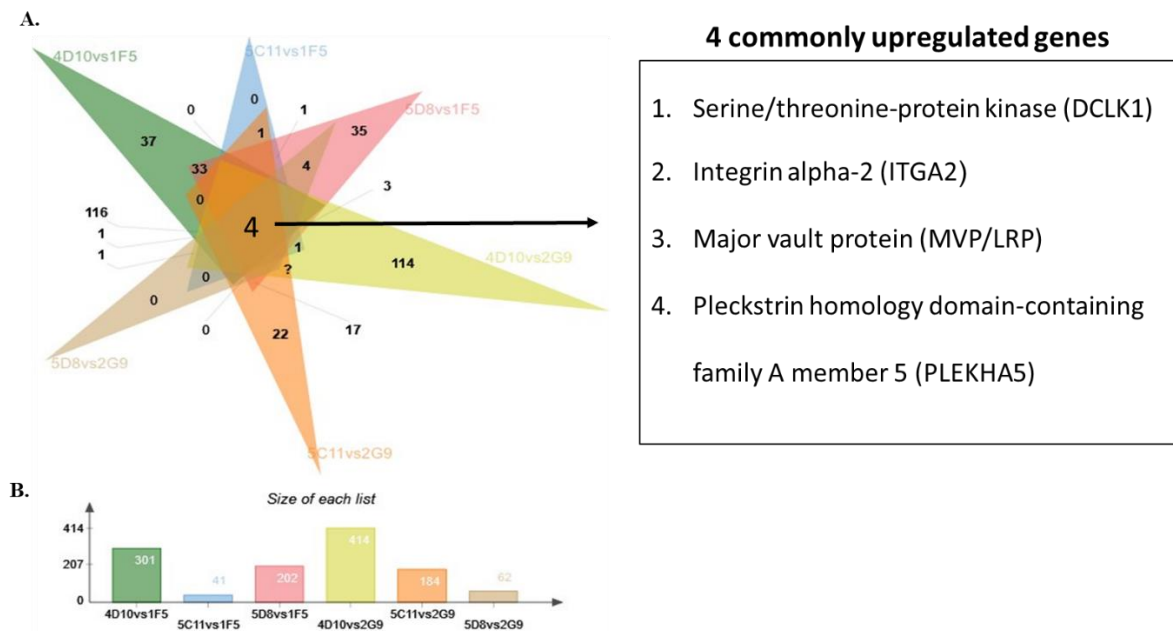


Figure 20. Venn diagram illustrating (A) the number of common and exclusive proteins of pairwise comparison, (B) total number of differentially expressed proteins in each pairwise comparison passing predefined filters in AK0735 analysis

In the second approach, I made a collective comparison where I treated KO clones as one group and compared against the collective control groups (Figure 21). Strikingly, the same set of proteins that I detected in the pairwise comparison appeared in the list of differentially expressed proteins (Supplementary table 1). They ranked among the top five differentially expressed proteins in the collective comparison. This finding reinforced the robustness and reproducibility of our findings.

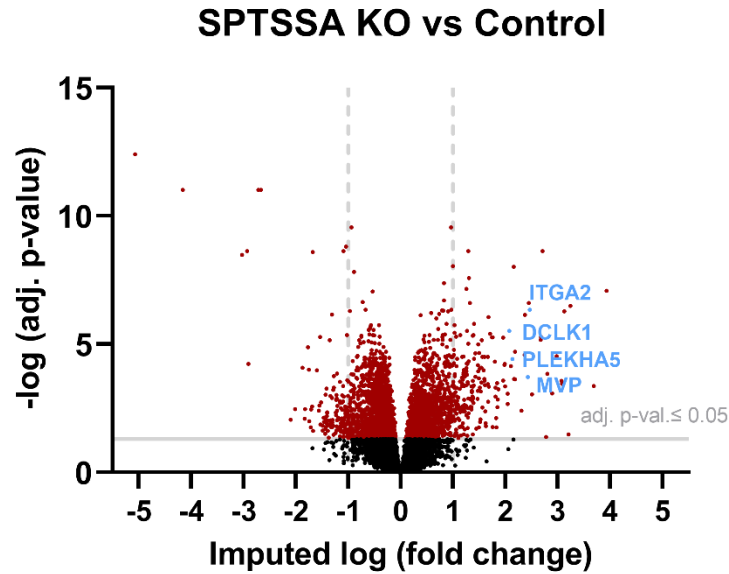


Figure 21. Volcano plot representing differentially expressed proteins in the collective analysis of SPTSSA KO and control clones. Red dots represents the proteins with adj. p val ≤ 0.05 and blue dots shows the core set of four genes consistently upregulated

3.4.1.2.1 Validation of Differentially Expressed Protein from AK0735 via Western Blot

I tried to validate the findings obtained from proteomic analysis by conducting Western blot experiments on a set of four proteins identified as differentially expressed in both pairwise and collective comparisons of SPTSSA knockout (KO) and control proteomes (Figure 22). However, despite my efforts, I encountered challenges during the validation process and could not confirm the observed differential expression.

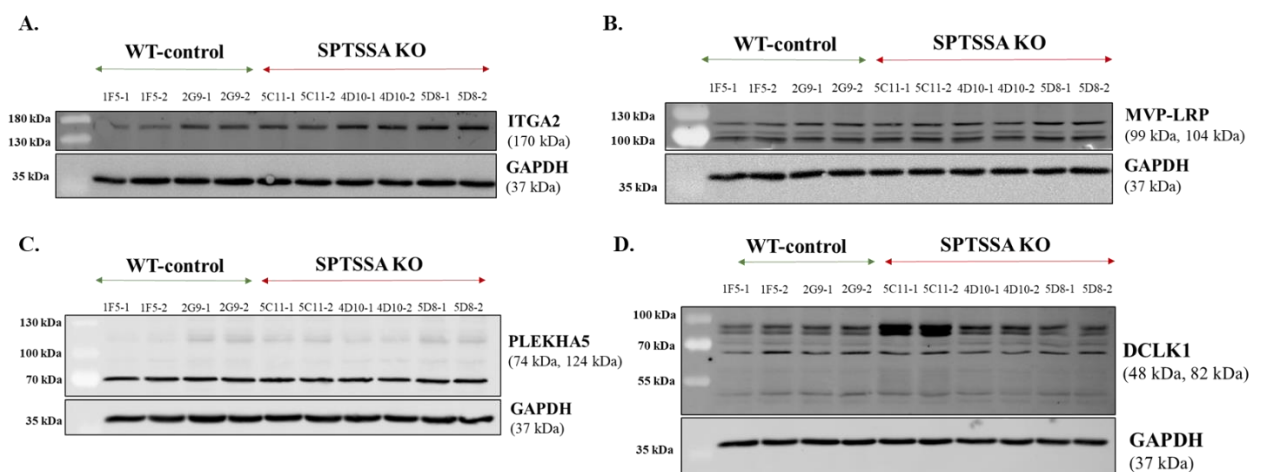


Figure 22. Western blot analysis of four candidate proteins using protein samples from SPTSSA KO and control cells

The protein expressions of MVL-LRP and PLEKHA5 were visually similar in both SPTSSA KO and control groups. The quantification of the blots for these proteins were parallel with the observation (Supplementary Figure 6). Variations in band thickness were observed for both isoforms of DCLK1 within SPTSSA KO groups. The SPTSSA KO clones 5C11 and 4D10 appeared to express long isoform of DCLK1 (≈ 82 kDa) more than others while its expression was similar both in 5D8 clones and control clones. Clone 5C11 exhibited the strongest expression among all SPTSSA KO clones. The expression of short isoform (≈ 48 kDa) also showed variation among all SPTSSA KO clones, with clone 5C11 displaying the strongest expression, followed by 5D8 clones, and with 4D10 showing the weakest expression. Lastly, I analyzed the expression profile of ITGA2 in SPTSSA KO and control clones. Even though, expression pattern on Western blot showed promising results visually (especially for clone 4D10 and 5D8), variations in band thickness led to statistical analyses yielding p-value of 0.1 which was above the conventional significance threshold of 0.05 (Supplementary Figure 6). Nevertheless, average expression of ITGA2 in SPTSSA KO was around two fold more compared to that of control clones. While the Western blot result did not reach statistical significance, a trend consistent with the proteomic data was noticeable for the selected proteins. A potential source of discrepancy between proteomic and Western blot results could have been caused by the protein samples used for Western blot validation as proteins used for Western blot were extracted using a different protocol than those utilized for proteomic analysis. Although the same cellular sources were employed for both experiments, the differing extraction methods may have introduced variability in protein composition and abundance, thereby hindering the validation process. To eliminate possible effect of methodological variations and ensure robust and reproducible results, the proteomic analysis was repeated as discussed in section 3.4.1.5.

3.4.1.2.2 STRING Interaction Network Analysis

I performed interaction network analysis using online tool STRING (<https://string-db.org/>). My aim was to explore the data for more insight and gain a deeper understanding from it. I used the list of 91 proteins which were differentially expressed and passed the predefined filters ($\text{Log2FC} \geq 1$ or $\text{Log2FC} \leq -1$ Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7) (Supplementary Table 1 and Supplementary Table 2). STRING performed gene set enrichment analysis using default settings and returned associated Gene Ontology (GO) terms for three main ontologies: Molecular Function, Biological Process and Cellular Components. Each ontology ranked using strength in ascendant order. The connection within the proteins based

on the enriched terms and the highlight of the potential functional roles were summarized in Supplementary Figure 7 and Supplementary Figure 8. Strikingly, the top five GO-terms for biological process were related to mitotic cell cycle. Another noteworthy finding from STRING analysis is that the GO terms for molecular function were directly or indirectly related to the lipid signaling and metabolism, with a particular emphasis on phospholipids and glycosphingolipids.

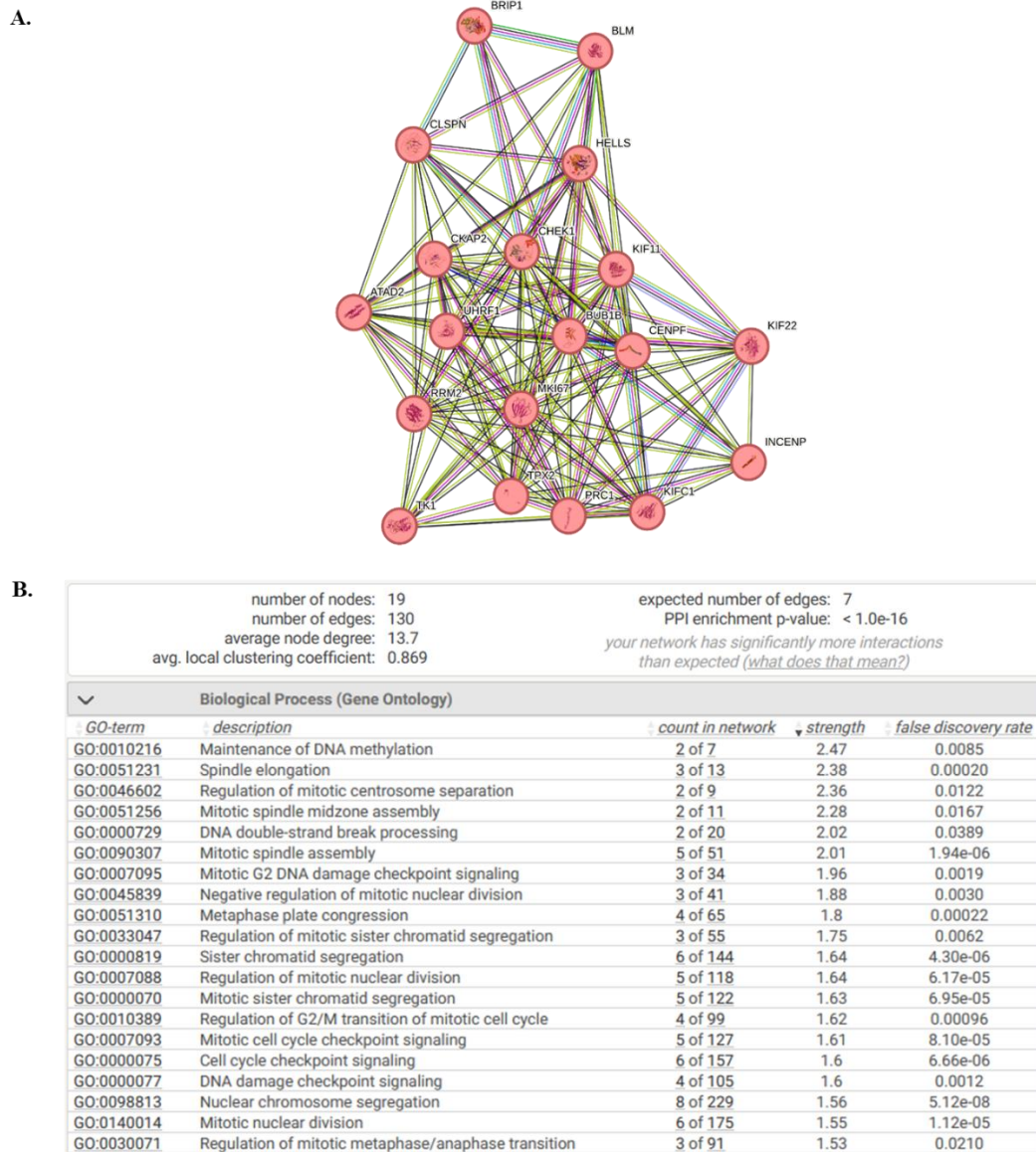


Figure 23. The largest cluster (Cluster 1) out of 19 clusters, which contains the proteins involved in cell cycle, generated by STRING interaction network analysis. (A) Proteins were clustered using MCL algorithm using the data from supplementary table 1 and supplementary table 2. (B) Gene ontology (GO) terms for biological processes were ranked based on strength in descendent order.

Proteins were further clustered using the MCL (Markov Cluster) algorithm to maximize the interconnection between them. Total of 19 clusters with varying number of proteins were identified. The largest cluster, Cluster 1 being the biggest cluster had 19 proteins and was associated with mitotic cell division (Figure 23A, Table 31 and Supplementary Figure 7). The identification of two GO terms, "Mitotic G2 DNA damage checkpoint signaling" and "Regulation of G2/M transition of mitotic cell cycle," provides insights into potential alterations occurring during the mitotic cell cycle, or the specific processes affected by these changes (Figure 23B).

Table 31. The list of protein present in Cluster 1 (in Figure 23) which were associated with cell cycle processes. Expression changes in SPTSSA KO compared to control for all proteins were listed in descending order.

<i>Proteins involved in cell cycle process</i>			
Gene names	Protein names	Log₂FC	Adj p-val
BUB1B	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	-1.01	3.78E-03
ATAD2	ATPase family AAA domain-containing protein 2	-1.01	1.20E-02
BRIP1	Fanconi anemia group J protein	-1.02	1.26E-02
KIF11	Kinesin-like protein KIF11	-1.05	1.45E-02
BLM	RecQ-like DNA helicase BLM	-1.07	7.15E-03
CHEK1	Serine/threonine-protein kinase Chk1	-1.08	1.16E-02
TK1	Thymidine kinase, cytosolic	-1.09	4.21E-02
MKI67	Proliferation marker protein Ki-67	-1.09	4.00E-02
UHRF1	RING-type E3 ubiquitin transferase	-1.11	1.30E-02
KIFC1	Kinesin-like protein KIFC1	-1.12	1.28E-02
CENPF	Centromere protein F	-1.14	1.69E-02
HELLS	Lymphoid-specific helicase	-1.21	9.18E-03
RRM2	Ribonucleoside-diphosphate reductase subunit M2	-1.28	6.07E-04
PRC1	Protein regulator of cytokinesis 1	-1.34	9.37E-03
KIF22	Kinesin-like protein KIF22	-1.34	1.44E-02
INCENP	Inner centromere protein	-1.39	1.45E-02
TPX2	Targeting protein for Xklp2	-1.43	1.68E-02
CLSPN	Claspin	-1.43	7.96E-03
CKAP2	Cytoskeleton-associated protein 2	-1.46	1.09E-02

The proteomics data associated with the proteins in Cluster 1 showed similar changes between SPTSSA KO and control groups, with proteins downregulated 2-3 fold in SPTSSA KO compared to control (Table 31).

3.4.1.3 Cell Cycle Analysis

The proteomics data suggested changes in mitotic cell division. To confirm these changes and detect cell populations at different stages of the cell cycle, we prepared and stained with PI. Richard Jennemann performed the flow cytometry and analyzed the data. The results showed that there are less cells in the G2/M phase and S phase in SPTSSA KO cells compared to the control (Figure 24). Presence of fewer cells in these phases indicate that the cells were in the G0 or G1 phase and therefore, they were not actively preparing to divide. It also points out that the more cells might have been arrested in either G0/G1 or G2/M phases.

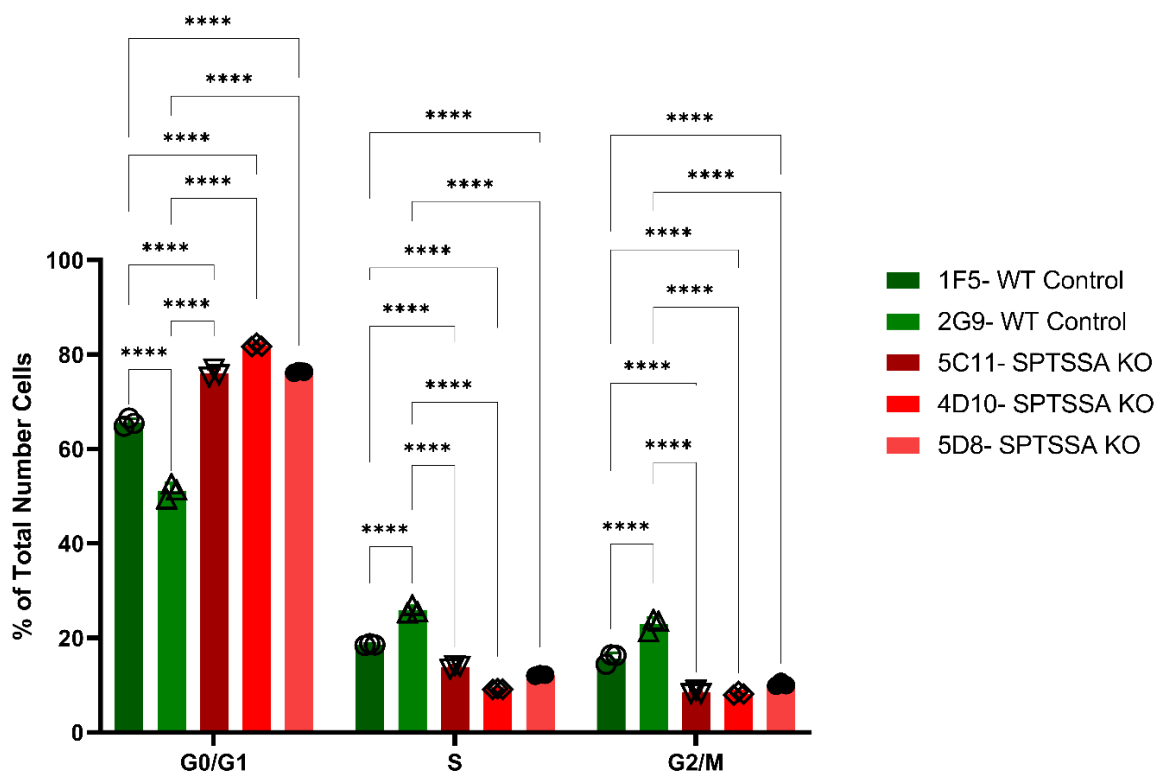
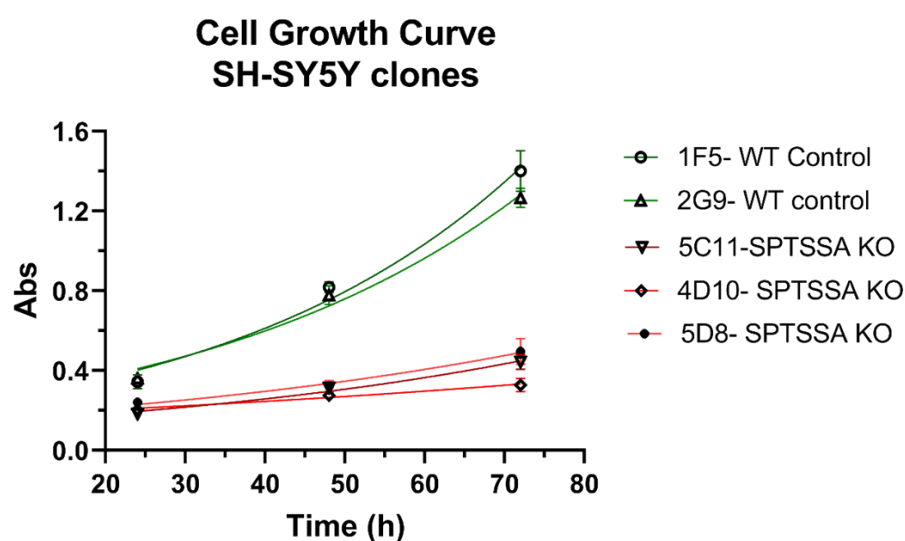


Figure 24. Cell cycle analysis by PI staining in SH-SY5Y control (1F5 and 2G9) and SPTSSA KO (5C11, 4D10 and 5D8) clones. Note that each KO clone differs significantly from each control clone.

3.4.1.4 Cell Proliferation

In parallel with cell cycle analysis, proliferation rate of each cell clones were evaluated by crystal violet staining. The division rate of SPTSSA KO SH-SY5Y clones decreased between 1.5-fold to 2.6-fold compared to the control clones. On average control cells divided every 27.8 hours whereas, SPTSSA KO cells exhibited significant variation in division time. Clone 4D10 showed the most dramatic change, dividing every 72.9 hours (Figure 25). The reduction in the percentage of the cells in the S phase supported the data from crystal violet staining indicating a decrease rate of cell proliferation in SPTSSA KO cells.



	1F5	2G9	5C11	4D10	5D8
Doubling Time (h)	26.4	29.1	39.9	72.9	43.8

Figure 25. Cell viability of SH-SY5Y control (1F5 and 2G9) and SPTSSA KO (5C11, 4D10 and 5D8) clones as determined by Crystal Violet assay. Assay was performed 2-3 passages after clonal selection.

3.4.1.5 Second Proteome Measurement- AK0800-2

Attempts for validation of the first proteomic analysis (AK0735, Figure 22) was not successful. Therefore, we performed a subsequent proteomic analysis to eliminate potential methodological or batch-related issues, or both. In this follow up study (after the cells were maintained in culture for over 30 passages), I compared the SPTSSA KO clones to the control clones using the same approach as in the initial measurement (AK0735). Initially, I performed

a pairwise comparison for each knock out and control clones. Then, I filtered those proteins using predefined filters ($\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$). Using Venn diagrams, I compared these individual comparisons and tried to identify common differentially expressed proteins. Interestingly, no common proteins were detected (Figure 26).

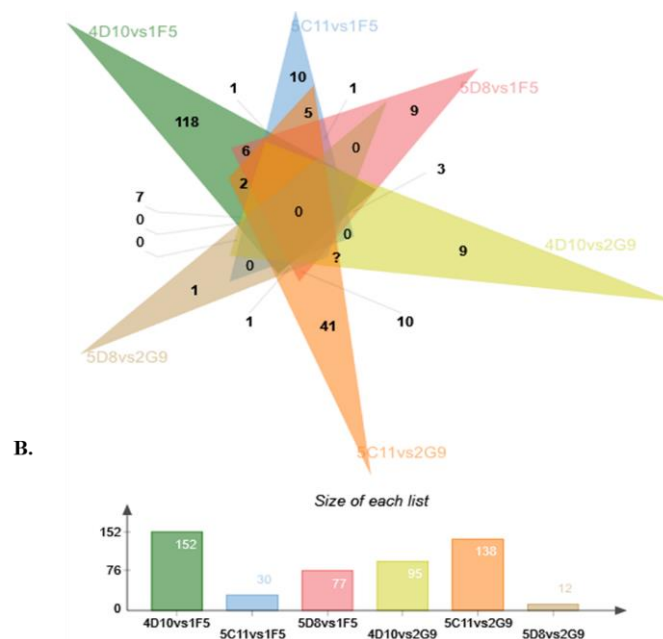


Figure 26. Venn diagram illustrating (A) the number of common and exclusive proteins of pairwise comparison, (B) total number of differentially expressed proteins in each pairwise comparison passing predefined filters in AK0800-2 analysis

In the second approach, I made a collective comparison where I treated KO clones as one group and compared against the collective control groups by setting the same filters as in pairwise comparison. Only eight proteins passed all the filters (Figure 27). These proteins upregulated in SPTSSA KO clones around $\log_2$ fold change of 1.05 to 1.43, which corresponds to around 2-3 fold higher expression as compared to control (Table 32). Due to the limited number of proteins passing the filter criteria, enrichment analysis could not be performed.

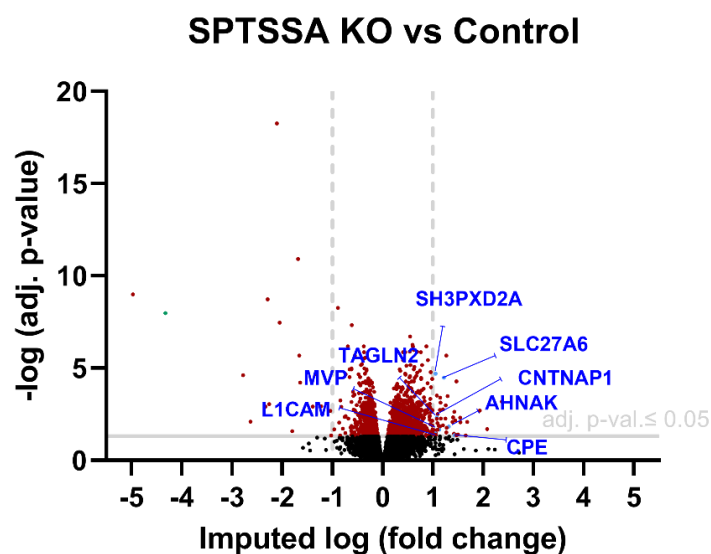


Figure 27. Volcano plot representing differentially expressed proteins in the collective analysis of SPTSSA KO and control clones in AK0800-2. Red dots represents the proteins with adj. p val ≤ 0.05 and blue dots shows the genes passing predefined filters

Table 32. The list of differentially regulated proteins from the AK0800-2 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$ Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7 . Log fold change and adjusted p-values were calculated based on imputed data

<i>Differentially regulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
CPE	Carboxypeptidase E	1.43	4.40E-02
AHNAK	Neuroblast differentiation-associated protein AHNAK	1.31	1.51E-02
SLC27A6	Long-chain fatty acid transport protein 6	1.22	3.34E-05
MVP	Major vault protein	1.13	2.17E-02
TAGLN2	Transgelin-2	1.08	5.14E-03
CNTNAP1	Contactin-associated protein 1	1.08	4.76E-03
SH3PXD2A	SH3 and PX domain-containing protein 2A	1.05	2.05E-05
L1CAM	Neural cell adhesion molecule L1	1.05	3.54E-02

3.4.1.5.1 Comparison of Two Proteomic Analyses via Western Blot

In the first set of proteomics data (AK0735), the ITGA2 gene was identified as upregulated. However, although initial Western blot results showed increased ITGA2 protein expression in KO compared to control, this difference was not statistically significant due to variations in expression among different clones in both SPTSSA KO and control groups. In the second set of proteomic data (AK0800-2), ITGA2 gene expression also appeared to be upregulated, but it did not meet the threshold of $\text{Log}_2\text{fold} \geq 1$, showing a log_2 fold change of only 0.95 (1.93 fold increase). To compare the two proteomic measurements and eliminate the possibility of technical issues, I made another Western blot for the ITGA2 gene using an aliquot from the same sample that was sent for proteomic analysis (Figure 28).

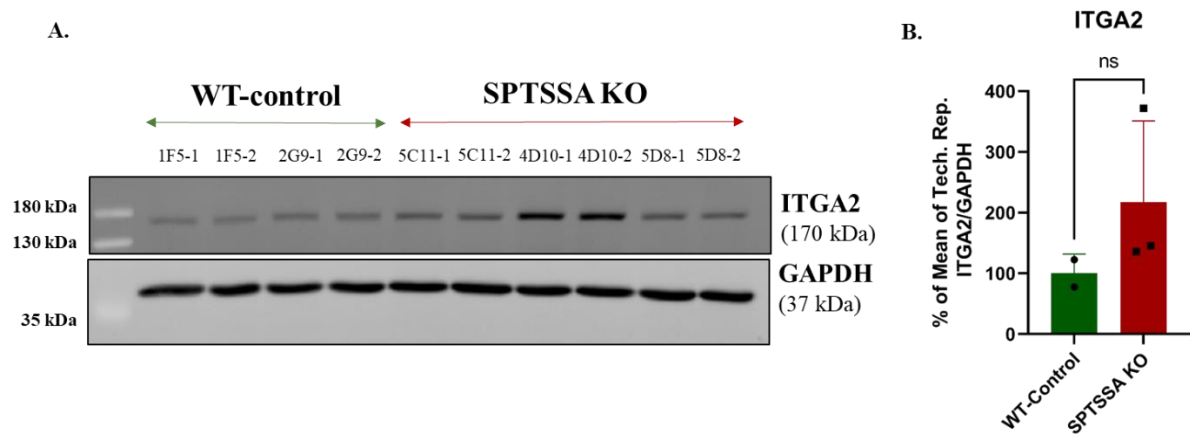


Figure 28. (A) Expression analysis of ITGA2 proteins via Western blot (B) Quantification of the Western blot. Expression of ITGA2 was normalized to GAPDH. Samples are aliquots from AK0800-2 proteome analysis.

In the previous experiment done for project AK0735, the 4D10 clone exhibited higher expression compared to 5C11, although it was lower than 5D8 (Figure 22A and Supplementary Figure 6). However, in the second experiment using aliquots of the samples analyzed for proteome project AK0800-2 (Figure 28A and B), 4D10 took the top ranking with higher expression, while the expression of 5D8 appeared to decrease in the Western blot analysis. Once again, clonal variation was observed. However, this time, both 5D8 and 5C11 showed similar expression levels to the control groups. This overall change in the expression pattern of ITGA2 in SPTSSA KO clones compared to the first measurement provided eventually also an initial indication that the cells may have undergone changes over time in culture and developed an adaptive mechanism to compensate for the loss of the SPTSSA gene.

3.4.1.5.2 Cell Adhesion

To extract the maximum information from the data, I adjusted the filter settings. While I kept the settings for the maximum contrast value and p-value unchanged, I reduced the threshold for $\log_2$ fold change to ± 0.5 , compared to the previous setting of ± 1 . I performed interaction network analysis using online tool STRING (<https://string-db.org/>) as in the case for AK0800-2. I used the list of 89 proteins which were differentially expressed and passed the new predefined filters ($\log_2\text{FC} \geq 0.5$ or $\log_2\text{FC} \leq -0.5$ Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7) (Supplementary Table 3 and Supplementary Table 4). STRING performed gene set enrichment analysis using default settings and returned associated Gene Ontology (GO) terms. Each ontology ranked using strength in ascendant order. The connection within the proteins based on the enriched terms and the highlight of the potential functional roles were summarized in Supplementary Figure 9 and Supplementary Figure 10. The identification of two GO terms, "Cadherin binding" and "Cell adhesion molecule binding," provides insights into potential alterations occurring during the cell adhesion, or the specific processes affected by these changes. Proteomic data indicates that the proteins associated with these GO terms were upregulated in knockout clones compared to control.

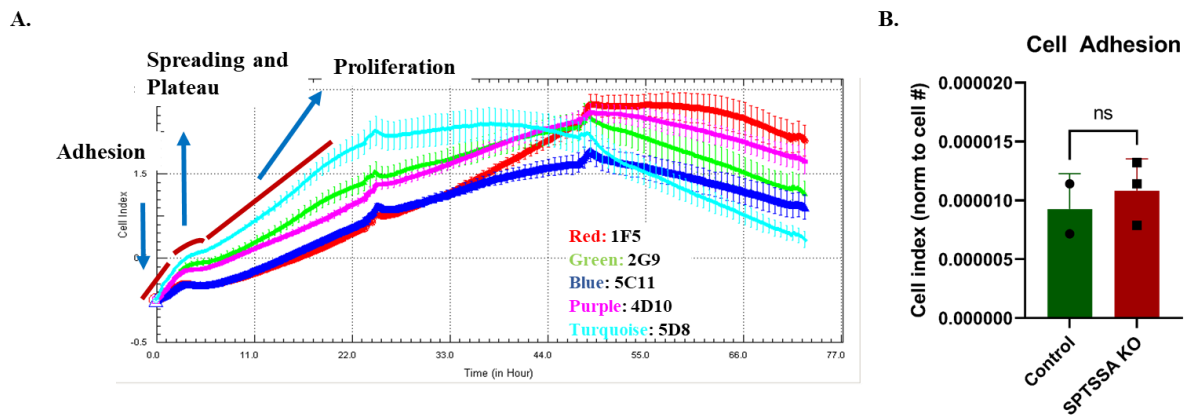


Figure 29. (A) Cell index adhesion curve of control clones (1F5 and 2G9) and SPTSSA KO (5C11, 4D10 and 5D8). The time period (0-4h) shows the initial phase of cell adhesion, followed by spreading (4-7 h). Subsequently, it reached to the plateau phase and followed by proliferation. The assay was performed by xCELLigence RTCA instrument (Agilent, USA). The curve shows the mean index value of 4 replicates with standard deviation. (B) Quantification of cell index was done by normalizing to cell number. The average expression values of proteins in the SPTSSA KO group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant).

To further validate this hypothesis, I conducted a cell adhesion assay using the xCELLigence RTCA instrument (Agilent, USA). The results revealed that SPTSSA KO clones 4D10 and 5D8 exhibited similar behavior to control clone 2G9, showing higher cell adhesion. Conversely, SPTSSA KO clone 5C11 displayed a behavior similar to that of control clone 1F5. Therefore, the results remained inconclusive (Figure 29A and B).

3.4.1.6 Third Measurement-AK0800-1

In project AK0800-1, we conducted phosphoproteomic analysis, which included total protein analysis using DDA as a component of this study. Since a different acquisition method was employed in this analysis compared to the first two, direct comparison with AK0735 and AK0800-2 is not feasible. However, it contributed valuable insights to the project. I performed a collective comparison where I treated KO clones as one group and compared against the collective control groups by setting the following filters: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$, $\text{Adj. p-value} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Only four proteins passed all the filters (Figure 30 and Table 32). Due to the limited number of proteins passing the filter criteria, enrichment analysis could not be performed.

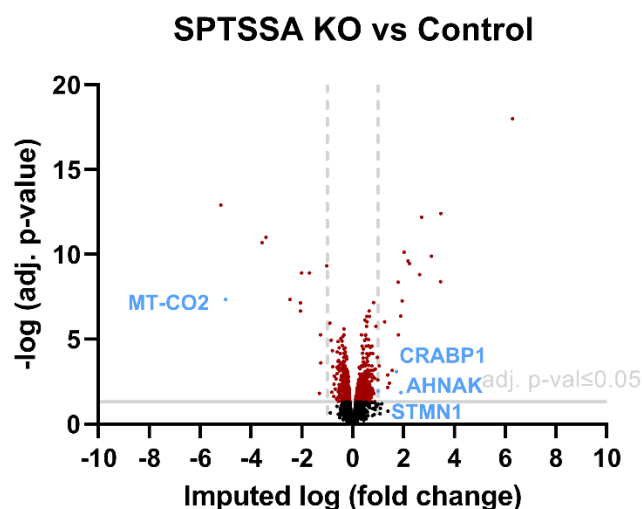


Figure 30. Volcano plot representing differentially expressed proteins in the collective analysis of SPTSSA KO and control clones in project AK0800-1. Red dots represents the proteins with $\text{adj. p-value} \leq 0.05$ and blue dots shows the genes passing predefined filters

Among the four differentially regulated proteins, three were upregulated while one was downregulated. The downregulated protein MT-CO2 exhibited a significant change, with a $\log_2$ fold-change of 5, corresponding to a 32-fold decrease (Table 33).

Table 33. The list of differentially regulated proteins from the AK0800-1 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$ Adj.p-val ≤ 0.05 , and ContrastMaxScore ≥ 0.7 . Log fold change and adjusted p-values were calculated based on imputed data

<i>Differentially regulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
AHNAK	Neuroblast differentiation-associated protein	1.91	1.39E-02
CRABP1	Cellular retinoic acid-binding protein 1	1.72	8.13E-04
STMN1	Stathmin	1.01	1.13E-02
MT-CO2	Cytochrome c oxidase subunit 2	-5.00	4.62E-08

I adjusted the filter settings to be able to perform enrichment analysis. I kept the settings for the maximum contrast value and p-value unchanged, I reduced the threshold for log₂ fold change to ± 0.35 which corresponds to a 1.5 fold change. I performed interaction network analysis using online tool STRING (<https://string-db.org/>). The list of the proteins that were used in the analysis is provided in Supplementary Table 5 and Supplementary Table 6. STRING performed gene set enrichment analysis using default settings and returned associated Gene Ontology (GO) terms. The relationships among the protein, indicated by the enriched GO terms, are summarized in Supplementary Figure 11 and Supplementary Figure 12. A notable discovery from STRING analysis is that the GO terms for biological processes were directly or indirectly related to the mitochondrial function, with a particularly highlighting mitochondrial electron transport chain.

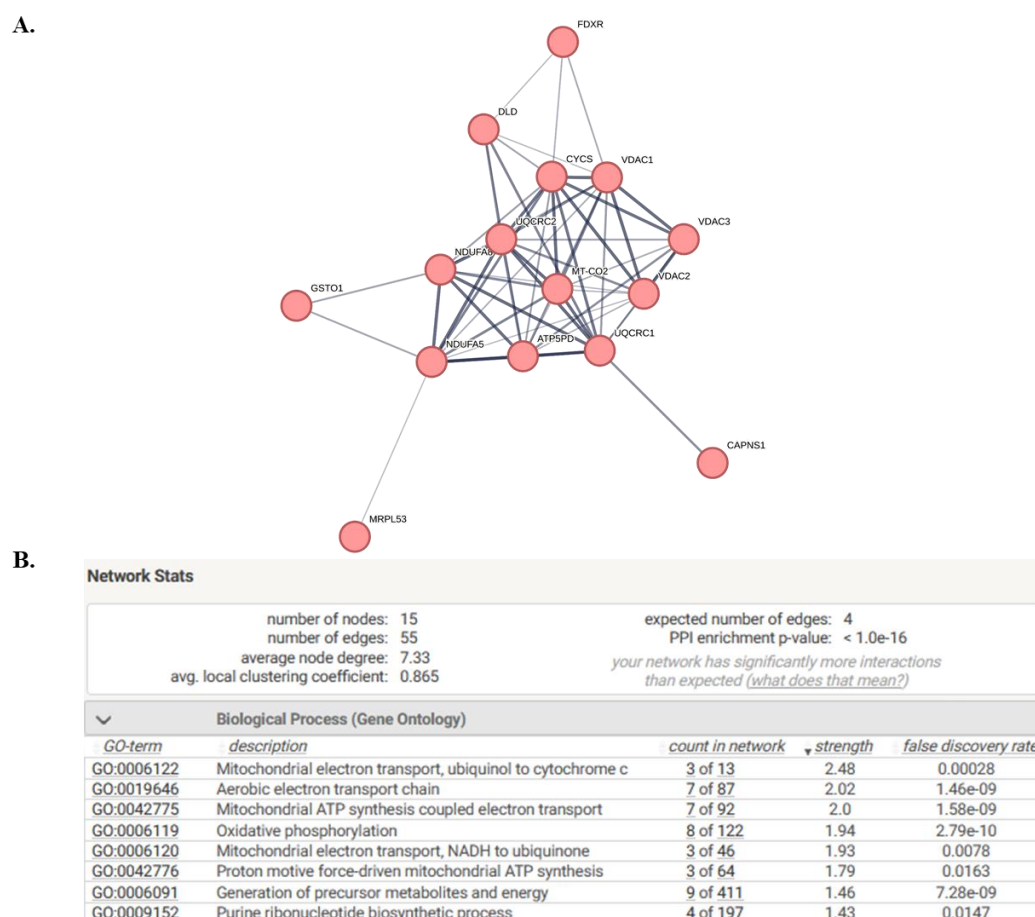


Figure 31. The largest cluster (Cluster 1) out of 34 clusters, which contains the proteins involved in mitochondrial function, was generated by STRING interaction network analysis. (A) Proteins were clustered using MCL algorithm using the data from Supplementary Table 5 and Supplementary Table 6. (B) Gene ontology (GO) terms for biological processes were ranked based on strength in descendent order.

Proteins were further clustered using the MCL (Markov Cluster) algorithm to maximize the interconnection between them. Total of 34 clusters with varying number of proteins were identified. The largest cluster, Cluster 1 being the biggest cluster had 15 proteins and was associated with mitochondrial electron transport chain (Table 34 and Figure 31A and B). Based on the change in the expression pattern of proteins related to mitochondrial function mitochondrial respiratory chain complexes (I, III and IV) appeared to be affected in different direction. Specifically, proteins within complexes I and III exhibited upregulation, while complex IV related proteins were downregulation.

Table 34. The list of protein present in Cluster 1 (in Figure 31) which were associated with mitochondrial function. Expression changes in SPTSSA KO compared to control for all proteins were listed in descending order.

<i>Proteins associated with mitochondrial function</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
ATP5H (or ATP5PD)	ATP synthase subunit d, mitochondrial	0.65	9.02E-03
MRPL53	39S ribosomal protein L53, mitochondrial	0.61	7.63E-03
CYCS	Cytochrome c	0.60	6.48E-04
NDUFA8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	0.56	1.15E-02
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	0.52	5.44E-06
CAPNS1	Calpain small subunit 1	0.50	3.74E-04
GSTO1	Glutathione S-transferase omega-1	0.50	5.85E-06
UQCRC2	Cytochrome b-c1 complex subunit 2	0.48	7.39E-07
NDUFA5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	0.46	2.16E-02
FDXR	NADPH:adrenodoxin oxidoreductase, mitochondrial	0.40	4.14E-04
DLD	Dihydrolipoyl dehydrogenase, mitochondrial	-0.35	1.99E-02
VDAC1	Voltage-dependent anion-selective channel protein 1	-0.39	3.35E-02
VDAC2	Voltage-dependent anion-selective channel protein 2	-0.40	1.98E-02
VDAC3	Voltage-dependent anion-selective channel protein 3	-0.45	3.32E-02
MT-CO2	Cytochrome c oxidase subunit 2	-5.00	4.62E-08

3.4.1.6.1 Validation of Differentially Expressed Proteins Detected in AK0800-1 via Western Blot

Initially, I conducted Western blot experiments on proteins identified as differentially expressed in collective comparisons of SPTSSA knockout (KO) and control proteomes (Figure 32). I used an aliquot from the same sample that was sent for proteomic analysis. I chose two proteins for validation. The first candidate was cellular retinoic acid-binding protein 1 (CRABP1), which successfully passed all the applied filters (Table 33) and is assumed to be involved in retinoic acid-mediated cell differentiation. The expression of CRABP1 was hard to detect on the Western blot membrane in the control clones where the band intensity was notably weak. However, CRABP1 expression was consistently upregulated across all SPTSSA KO

clones and bands were nicely visible especially in 5C11 and 4D10. The least fold increase was calculated in clone 5D8, approximately 2.4-fold, whereas clone 4D10 exhibited a 21-fold increase, and the highest upregulation was observed in clone 5C11 with 35-fold increase (Figure 32A and B). However, due to variability in CRABP1 expression among SPTSSA KO clones, statistical significance was not achieved.

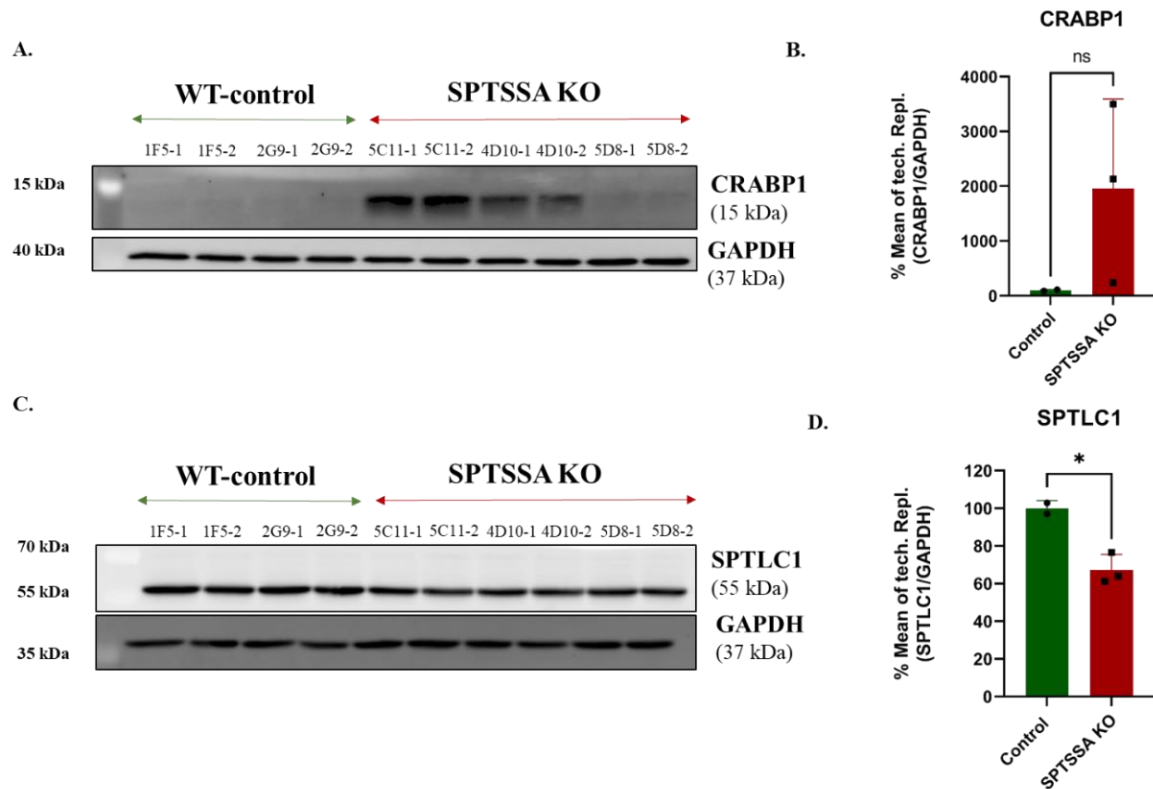


Figure 32. (A, C) Expression analysis of CRABP1 and SPTLC1 proteins via Western blot (B, D) Quantification of the protein bands of Western blot. Expression of CRABP1 and SPTLC1 proteins were normalized to GAPDH. Subsequently, the average expression values of proteins in the knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant, * $p \leq 0.05$).

I performed a second Western blot to evaluate the expression level of SPTLC1 in both control and SPTSSA KO cells (Figure 32C and D).. In the proteomic analysis, SPTLC1 did not passed the $\log_2$ fold change ≥ 1 or ≤ -1 filter as the calculated $\log_2$ fold change was -0.37. However, it still satisfied the criteria for adjusted p-value and ContrastMaxScore. Western blot analysis showed a similar trend with proteomics data and confirm the statistically significantly downregulation of SPTLC1 in SPTSSA KO clones.

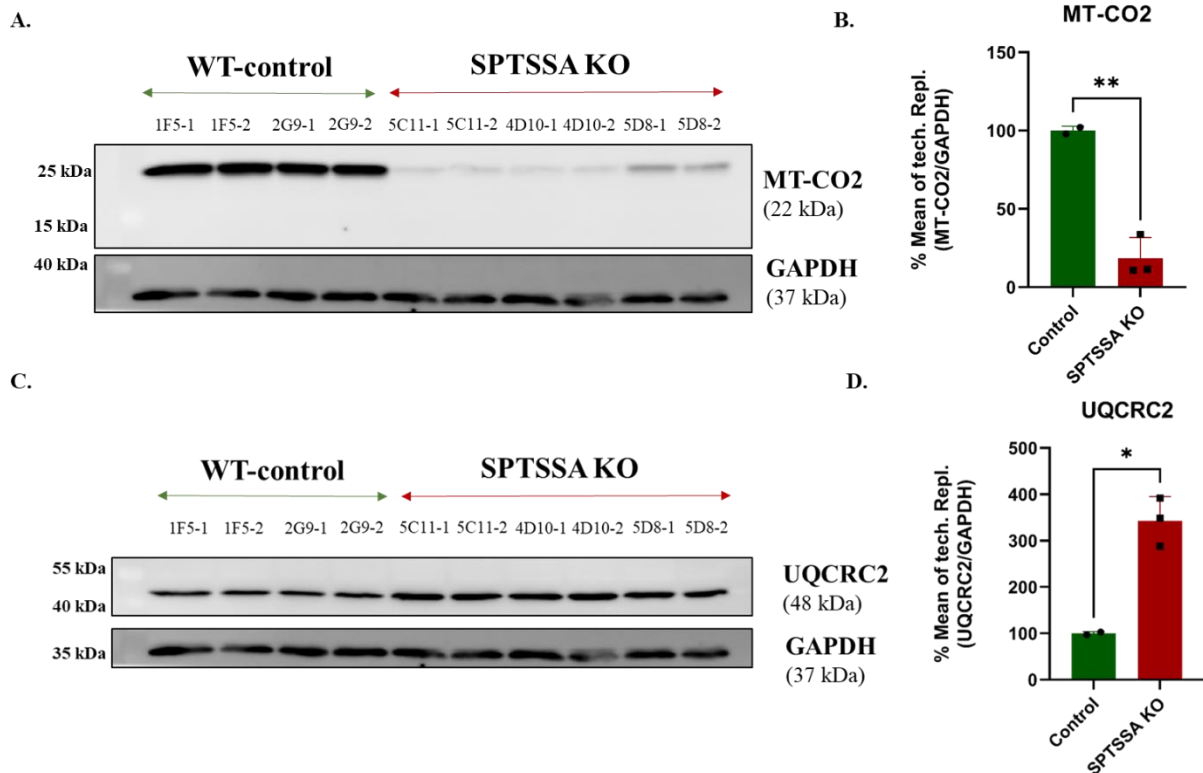


Figure 33. (A, C) Expression analysis of MT-CO2 and UQCRC2 proteins via Western blot (B, D) Quantification of the protein bands from Western blot. Expression of MT-CO2 and UQCRC2 proteins were normalized to GAPDH. Subsequently, the average expression values of proteins in the knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant, * $p \leq 0.05$, ** $p \leq 0.01$).

Lastly, I performed Western blot experiments on proteins which are part of the electron transport chain of mitochondria, focusing specifically on Complex III and IV proteins. There was a dramatic decrease in SPTSSA KO in the expression of MT-CO2, which is one of the two catalytic subunit of Complex IV (Figure 33A). This decrease was statistically significant, with consistent findings across all SPTSSA KO clones with relatively less variation compared to other Western blot analysis (Figure 33B). On the other hand, UQCRC2, a core protein subunit of Complex III, showed a significant upregulation in expression in SPTSSA KO clones compared to controls (Figure 33C). This upregulation was consistent across both SPTSSA KO and control clones, with small variations in expression patterns (Figure 33D). The validation of the changes in the expression pattern of these two proteins hints at a potential dysregulation in the electron transport chain of the mitochondria.

3.4.2 Phosphoproteome Analysis of SH-SY5Y SPTSSA KO and Control Clones

In order to understand the alternations in the cellular signaling at the steady state level, I conducted a phosphoproteomic analysis together with Proteomics Core Facility. My aim was to understand if the signaling pathways were affected by SPTSSA KO, and if so, to identify which pathways were affected. I followed a similar approach for the analysis as in the case of proteomics to obtain an initial overview. I made a collective comparison where I treated KO clones as one group and compared against the collective control groups by setting the following filters: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. In total 61 protein and 75 differentially phosphorylated sites were detected (Supplementary Table 7).

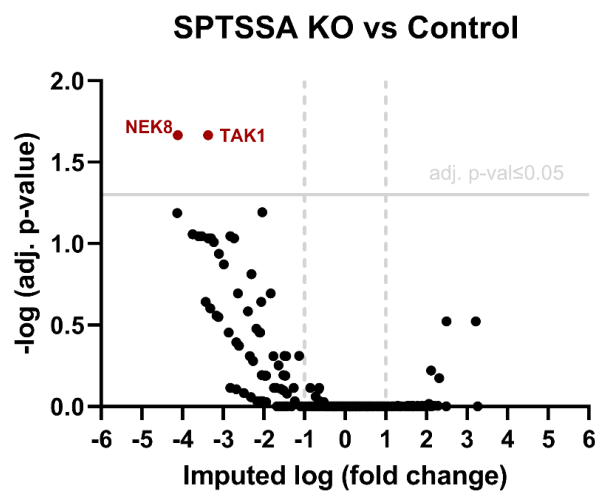


Figure 34. Differential phosphoproteome analysis of SPTSSA KO and control clones showing in a volcano plot. Kinases potentially activated or inhibited based on the patterns of phosphorylation analysis in phosphoproteomic data. Proteins meeting significance threshold were depicted in red.

I performed kinase enrichment analysis using online tool (<https://kinase.library.phosphosite.org>) (Johnson et al., 2023). I used the proteins and the associated phosphosites that were differentially regulated in project AK0800-1 (only passing the filter of $\text{ContrastMaxScore} \geq 0.7$, no filters were set on fold change or p-value) for the analysis. The online tool calculated the enrichment of known substrates for specific kinases present in the database. In other words, it took the proportion of proteins (along with their associated phosphosites) which are linked to certain kinase substrate in my dataset and compared it to the expected proportion in the background distribution. I ranked the kinases based on their enrichment with $\log_2$ fold change ≥ 1 or ≤ -1 , with an adjusted p-value ≤ 0.05 considered significant. Only two kinases passed the thresholds (Figure 34): NEK8 (Serine/threonine-

protein kinase Nek8, EC 2.7.11.1 (Never in mitosis A-related kinase 8, NimA-related protein kinase 8) (Nima-related protein kinase 12a)) and TAK1 (Mitogen-activated protein kinase kinase 7, EC 2.7.11.25 (Transforming growth factor-beta-activated kinase 1, TGF-beta-activated kinase 1)). Both of these kinases were predicted to be downregulated, with TAK1 showing a decrease of approximately 3.4 fold and NEK8 exhibiting a decrease of around 4.1 fold. These kinases were estimated to be functionally linked to the changes in the phosphorylation pattern of proteins as a consequence of the SPTSSA KO.

3.5 EGF Stimulation

The phosphoproteomic analysis yielded no significant changes related to EGFR phosphorylation at different phosphosites in SPTSSA KO cells under normal physiological condition. This enabled us to explore if EGFR signaling could be fine tuned by the sphingoid base chain length in the microdomain surrounding the EGFR at the plasma membrane. Any observed changes could be attributed to the altered lipid composition rather than pre-existing alterations in phosphorylation status of the signaling components. Based on the existing knowledge that EGFR signaling interacts with cellular microdomains, my aim was to understand the effect of EGF stimulation on EGFR phosphorylation over a specified treatment period, ranging from minutes to hours. Furthermore, I tried to explore its effect on downstream target proteins which is expected be altered either in time or intensity due to a potentially different EGFR-mediated signaling dynamics in the absence of SPTSSA

3.5.1 Optimization of EGF Concentration and Stimulation Time using SH-SY5Y wt (parental) Cells

3.5.1.1 Time-Dependent Effect of EGF Stimulation on EGFR Phosphorylation

To establish the optimal conditions for achieving a peak in EGFR phosphorylation, I conducted a series of experiments. Given the potential for variations in control clones regarding the conditions to reach maximum EGFR phosphorylation, I decided use SH-SY5Y wt (parental) cells for the optimization. Initially, I incubated the cells in serum free media for four hours. Following starvation, I stimulated the cells with 100 ng/ml EGF for various durations, (0, 5, 10, 15, 20, and 30 minutes). Additionally, I carried out direct stimulation without prior starvation (in normal growth media supplemented with 10% FBS). I tracked the changes of the phosphorylation status of EGFR using phosphosite tyrosine 992 (Y992) as a marker over time.

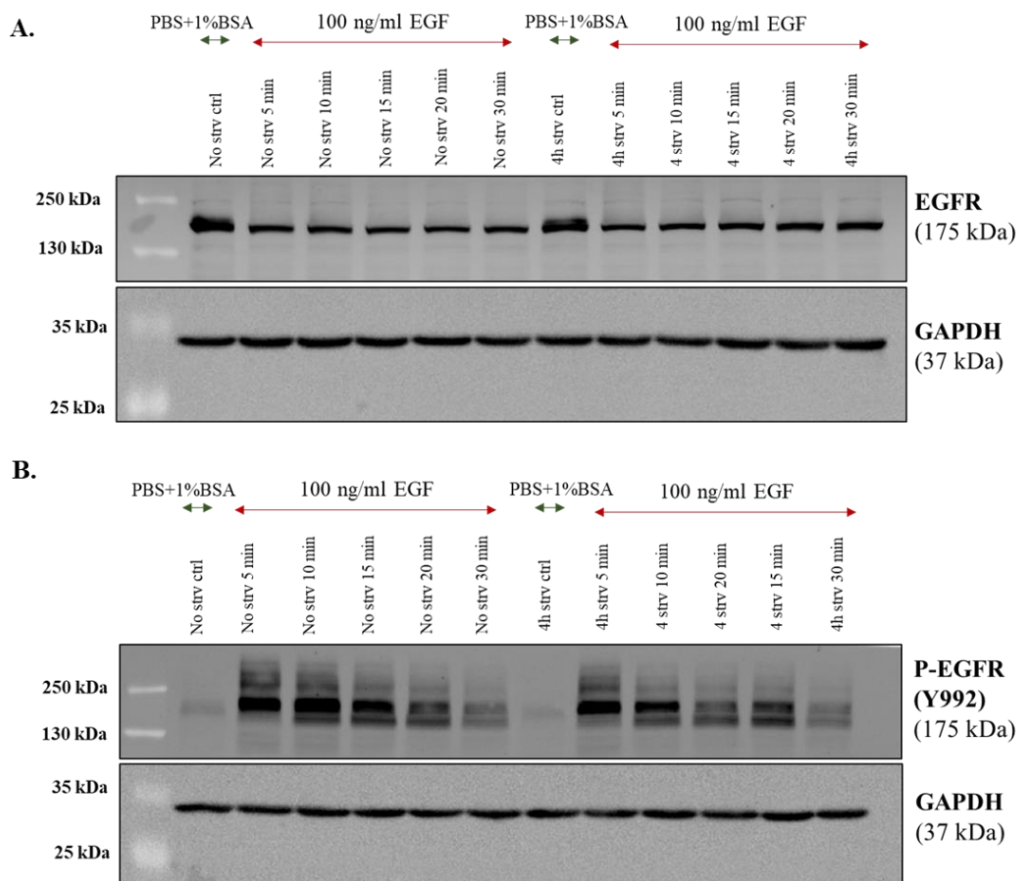


Figure 35. Changes in (A) expression status of EGFR and (B) the phosphorylation status of EGFR using phosphosite Y992 as marker under starvation (strv) and no starvation conditions with 100 ng/ml EGF stimulus for various durations in SH-SY5Y wt (parental) cells

Already 5 min after stimulation the total amount of EGFR decreased and stayed low for the next 25 minutes (Figure 35A). Concomittant strong Y992-phosphorylation appeared already 5 minutes after stimulation of starved cells, which progressively degraded over the next 25 min. In line with these results, EGFR activation and phosphorylation (Figure 35B), was reported to result in internalization and partial degradation of the active receptors (Sigismund et al., 2008). In summary, treatment with 100 ng/mL EGF resulted in a strong EGFR.

When the cells were treated directly without prior starvation, the phosphorylation of Y992 residue reached its maximum 5 minutes later compared to the starved scenario. In other words, while 5 minutes of starvation was sufficient for the starved cells, 10 minutes were required for the non-starved cells. However, the overall pattern of how the phosphorylation status of the Y992 residue developed and faded away over time was similar in both the starvation and non-starvation cases. Therefore, we decided to select the condition of no starvation and to record activation 10 minutes after stimulation (Figure 35B).

3.5.1.2 Dose-Dependent Effect of EGF on EGFR Phosphorylation

After determining the medium and stimulation time conditions, I proceeded to evaluate the effect of varying doses of EGF on EGFR phosphorylation using the same phosphosite. I treated the cells 10 minutes with EGF with concentrations of 0, 12.5, 25, 50 and 100 ng/ml in complete media without prior starvation.

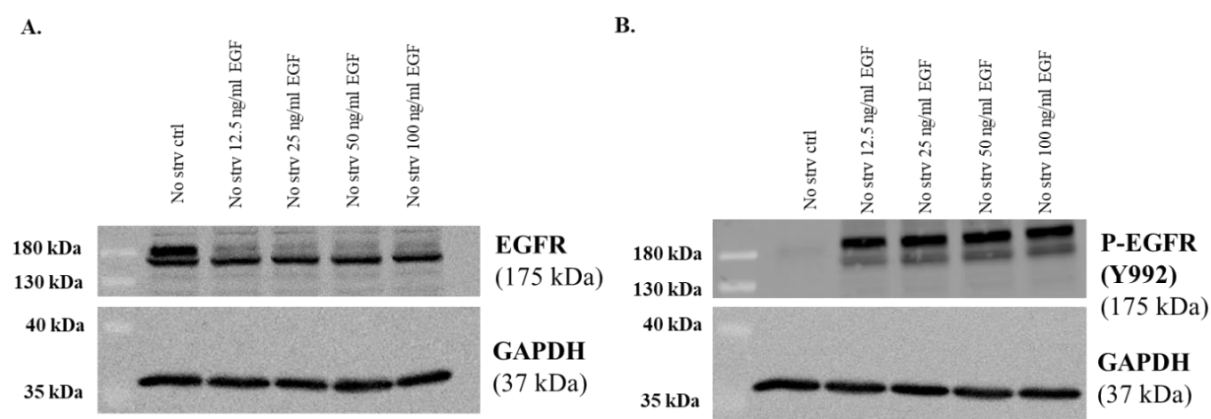


Figure 36. Changes in (A) EGFR expression status and (B) phosphorylation status of EGFR using phosphosite Y992 as a marker in SH-SY5Y wt (parental) cells cultured in complete media with various concentrations of EGF

As shown in Figure 36A, changing the concentration of EGF did not alter the reduction in the amount of EGFR due to internalization and degradation or recycling upon stimulation. This pattern was also observed in the phosphorylation of EGFR at Y992 (Figure 36B), where all concentrations resulted in similar levels of phosphorylation. Therefore, we selected 12.5 ng/ml EGF concentration as the optimal working concentration. The following experiments were conducted by stimulating the cells with 12.5 ng/EGF for 10 minutes with normal growth media without prior starvation.

3.5.2 Evaluation of Changes in Phosphorylation of EGFR in SPTSSA KO and Control Cells

To investigate the impact of SPTSSA KO and alterations in the length of the sphingoid base chain on the intensity of phosphorylation, I treated both SPTSSA KO and control cells with 12.5 ng/mL of EGF for 10 minutes. Subsequently, I examined the phosphorylation levels at various sites on EGFR including tyrosine 992 (Y992), tyrosine 1173 (Y1173) and tyrosine (Y1069), which are associated with different signaling pathways via Western blot (Figure 37).

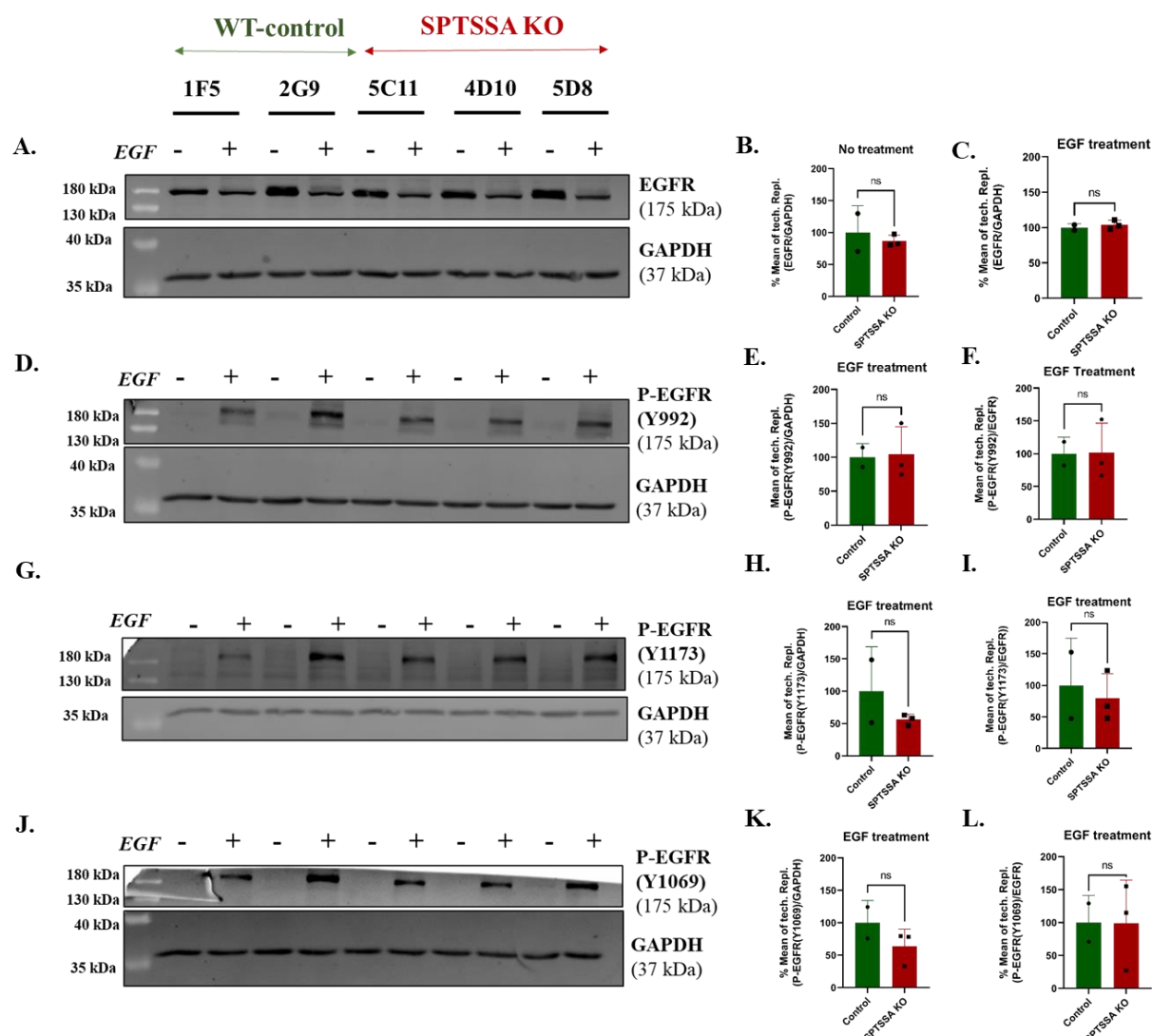


Figure 37. Alterations in the phosphorylation status of EGFR at different sites following 10 minutes of stimulation with 12.5 ng/ml EGF in SPTSSA KO and control clones. Protein expressions were normalized to loading control GAPDH (B, C, E, H, and K) and phosphorylation was normalized thereafter to total EGFR (F, I, and L). For statistical analysis, the average percent mean values of knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism (ns= not significant). Note: Due to high variability within the groups, no significant difference was observed between SPTSSA KO clones and control clones for this time point.

First of all, I checked the baseline expression of EGFR. The Western blot results showed that SPTSSA KO clones exhibited relatively similar EGFR expression levels. In contrast, there was variability in EGFR expression among the two control clones, with 2G9 having the highest expression and 1F5 having the expression level close to those of SPTSSA KO cells (Figure 37A-C). When both SPTSSA KO and control clones were stimulated with

12.5 ng/ml EGF, the amount of EGFR detected via Western blot decreased in all clones. Interestingly, thereafter the detected protein levels were comparable among all clones.

Phosphorylation of Y992 on EGFR is associated with the binding and activation of phospholipase C-gamma 1 (PLC γ 1 or PLCG1). Activated PLCG1 catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) which results in the production of 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 serves as an important secondary messenger and triggers calcium release from intracellular stores. DAG act as a cofactor and mediates the activation of serine/threonine kinase protein kinase C (Jorissen et al., 2003). I compared the phosphorylation status of EGFR on Y992 and its associated docking partner protein PLCG1 on Y783. At the steady-state level, phosphorylation of EGFR at Y992 was hardly detectable in all clones (Figure 37D). After EGF stimulation, the average phosphorylation levels of SPTSSA KO and control clones were similar, except for 5D8, which exhibited around 50% higher phosphorylation than the average (Figure 37E). Phosphorylation levels of EGFR (Y992) were normalized to their total EGFR level; however, normalization did not alter the overall observation (Figure 37F).

Before proceeding to analyze the phosphorylation of PLCG1, I initially determined the baseline level of total PLCG1 in both SPTSSA KO and control clones (Figure 38A-E). The results revealed that at the steady state level, the amount of total PLCG1 in KO clones was significantly lower compared to the control group, with minimal variation observed within each group (Figure 38B). However, following EGF treatment, the expression of PLCG1 in SPTSSA KO clones reached a level similar to that of the control group, while no change was observed in control clones (Figure 38C). It suggested that EGF treatment equalized total PLCG1 expression levels in both groups. Additionally, I performed a paired analysis where I paired GAPDH- normalized protein expression values of each untreated clones with treatment value of that clone. The results showed that the treatment did not induce a significant change in total PLCG1 expression within neither the SPTSSA KO and the decrease observed for both control clones was statistically not significant, likely as a result of n = 2 group (Figure 38D-E). Overall, results indicated that SPTSSA KO led to a decrease in total PLCG1 expression compared to control under steady state conditions.

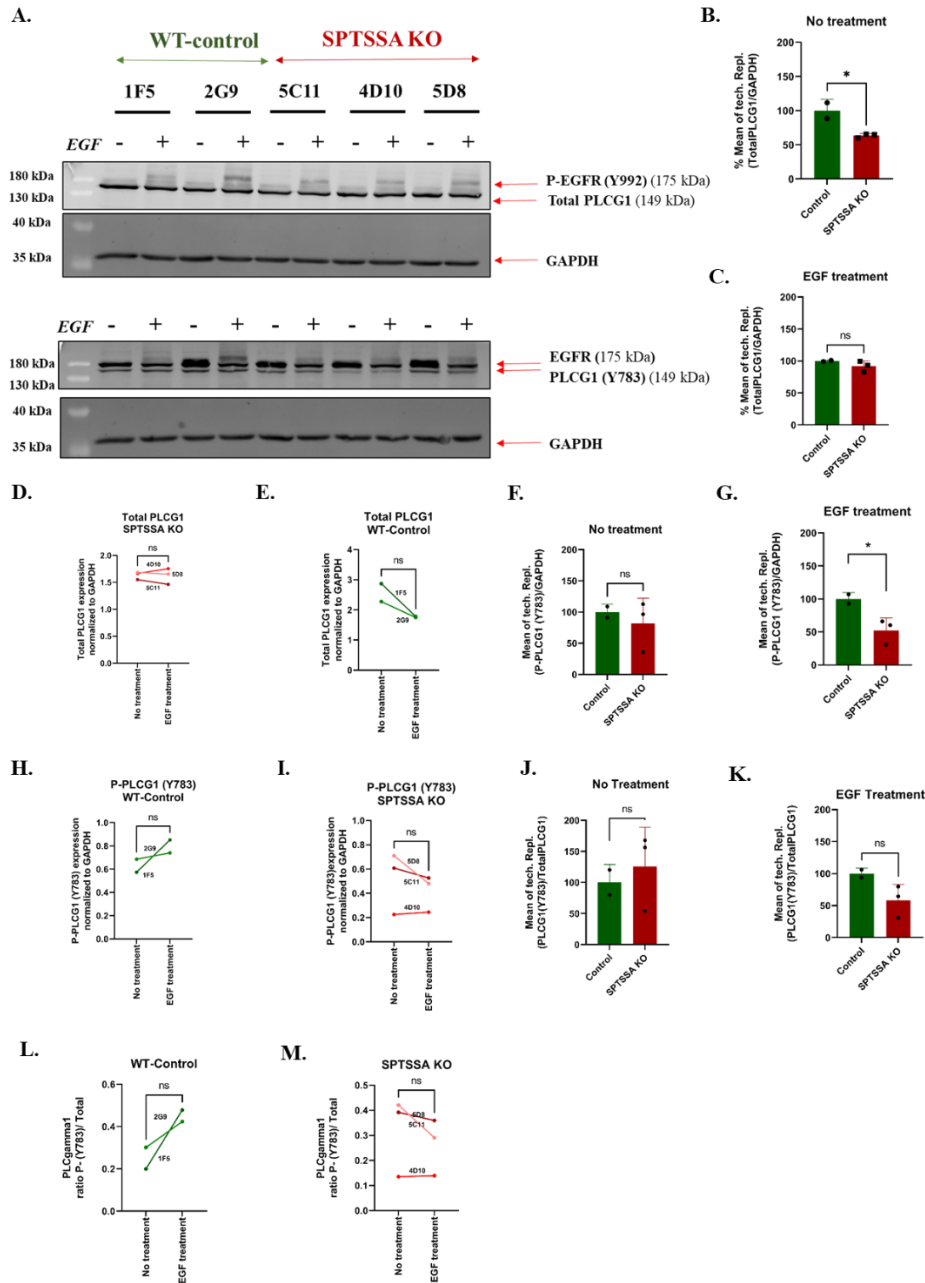


Figure 38. (A) The expression pattern of PLCG and P-PLCG1 at baseline and following 10 minutes of stimulation with 12.5 ng/ml EGF in SPTSSA KO and control clones. Protein expressions were normalized to loading control GAPDH (B, C, F, G) and phosphorylation was normalized thereafter to total PLCG1 (J and K). For statistical analysis, the average percent mean values of knockout (KO) group were compared to those in the control group using unpaired t-test with Welch's correction (B, C, F, G, J, K). GAPDH normalized protein expression values of each untreated clones were paired with its corresponding treatment value and paired t-test was performed (D, E, H, I, L, M) Statistical analysis was performed using GraphPad prism using (ns= not significant, * $p \leq 0.05$). Note: Although reduced phosphorylation of PLCG1 was observed in SPTSSA KO clones, this reduction was not significant anymore, when normalizing the total amount of PLCG1.

I examined the phosphorylation status of PLCG1 at Y783 (Figure 38A, F-I). Before treatment, all groups exhibited baseline phosphorylation. Generally, there was not a significant variation within the groups, except for 4D10, which displayed approximately 50% less phosphorylation compared to the other clones in the SPTSSA KO group (Figure 38F). Upon stimulation, the KO group showed a significantly decreased phosphorylation compared to the control group. However, the changes in 4D10 were not as drastic as in the other KO clones (Figure 38G). Also, I performed a paired analysis where I paired GAPDH- normalized protein expression values of each untreated clones with its corresponding treatment value. The results showed that the treatment did not induce a significant change in P-PLCG1 (Y783) expression within neither the SPTSSA KO nor control group (Figure 38H-I). The results showed that within each group, there was no significant change in P-PLCG (Y783) before and after treatment. This suggested that the treatment did not have consistent effect on P-PLCG (Y783) expression within either control or KO groups.

I normalized P-PLCG1 (Y783) to total PLCG1 to eliminate the impact of baseline total PLCG1. The variation within KO clones at steady state did not change, and the general view of control clones remained the same (Figure 38J). However, the average value of total PLCG1 for all clones increased, making the expression level higher than the average value of control clones. This change was due to the initial difference in the basal level of total PLCG1, which was lower. The pattern for the EGF treatment remained unchanged, but the significance at $p=0.05$ was lost; however, with $p=0.1$ it remained significant at $p\leq 0.1$ (Figure 38K). Additionally, I performed paired analysis in the same manner as I did for total PLCG1 and P-PLCG1 (Y783). The results indicated that treatment did not have consistent effect within either control or KO groups (Figure 38L- M)

In section 3.4.1.4, I examined the proliferation rate of both SPTSSA KO and control clones, discovering that SPTSSA KO cells initially proliferated at a slower rate compared to the controls. Phosphorylation of Y1173 provides a binding site for the Shc and GRB2 adaptor proteins. This phosphorylation event is associated with the stimulation of Erk MAPK pathway signaling pathways, which in turn regulate processes like proliferation and cell survival (Gerritsen et al., 2023). Therefore, I examined the alternation in phosphorylation of Y1173 of EGFR (Figure 37G- I) and docking protein GRB-2 (Figure 39D, G-J)).

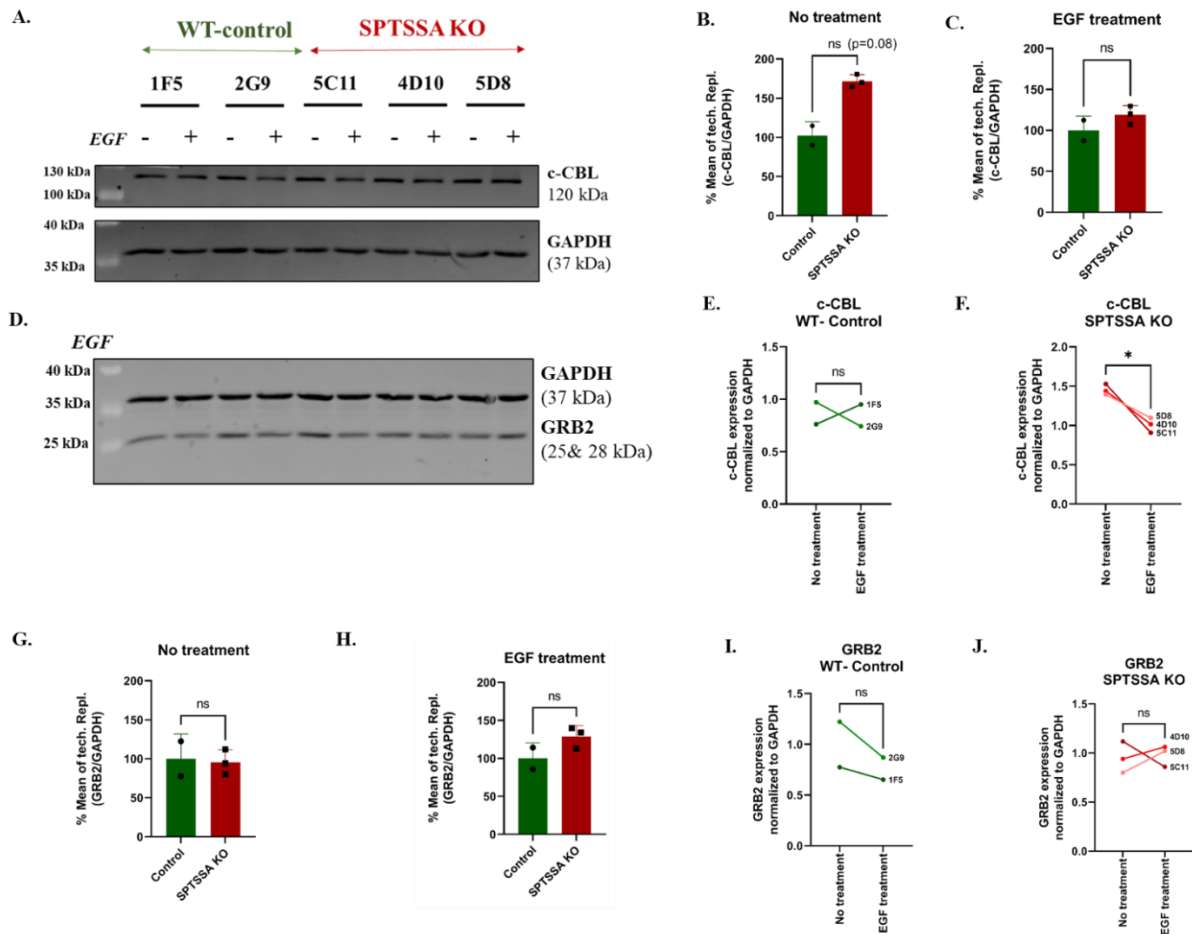


Figure 39. The expression pattern of c-CBL (A) and GRB2 (B) proteins at baseline and following 10 minutes of stimulation with 12.5 ng/ml EGF in SPTSSA KO and control clones. Protein expressions were quantified and normalized to loading control GAPDH (B-F: c-CBL and G-J: GRB2). For statistical analysis, the average percent mean values of knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant). GAPDH normalized protein expression values of each untreated clones were paired with its corresponding treatment value and paired t-test was performed (E, F, I, J)) Statistical analysis was performed using GraphPad prism using (ns= not significant, * $p < 0.05$)).

Figure 37G revealed that there was minimal detectable phosphorylated EGFR (Y1173) in all clones at baseline level. The phosphorylation levels of Y1173 were similar in all SPTSSA KO clones following EGF stimulation (Figure 37H). Control clone 1F5 exhibited expression levels similar to the SPTSSA KO clones, while control clone 2G9 showed nearly threefold higher phosphorylation of EGFR (Y1173). To eliminate the impact of baseline total EGFR levels, all P-EGFR (Y1173) expressions were normalized to the baseline total EGFR levels (Figure 37I). Normalization did not alter the phosphorylation levels of control clones; however, it resulted in variations among SPTSSA KO clones. SPTSSA KO clone 5D8 displayed the

highest expression, equivalent to the mean expression level of both control clones. Despite the average percent mean values of the SPTSSA KO group being lower compared to those in the control group, the difference was not statistically significant. This results became meaningful when I reevaluated the cell proliferation in SPTSSA KO and control cells as explained in section 3.5.3.

I also evaluated the baseline expression level and changes over stimulation in GRB2 expression, which is the first downstream target of the Y1173 phosphosite. At the baseline level, without any stimulus, all clones exhibited relatively similar levels of GRB2 protein (Figure 39D and G). Following EGF stimulation, the amount of GRB2 increased in SPTSSA KO compared to control (Figure 39H). Overall, the results indicated that the average percent mean values of the SPTSSA KO group were higher compared to those in the control group after stimulation, but the difference was not statistically significant. I also performed paired analysis where I paired GAPDH- normalized protein expression values of each untreated clones with its corresponding treatment value. The results indicated that the treatment did not induce any significant changes in GBR expression within each KO and control groups (Figure 39I- J)

After 10 minutes of 12.5 ng/ml EGF stimulation, a reduction in the amount of EGFR becomes visible on Western blot analysis (Figure 37A). This indicated the initiation of downregulation of active EGFR via internalization. Therefore, I investigated another phosphorylation site, tyrosine 1069 (Y1069, also known as Y1045, Figure 37J, K and L)) and changes in expression of c-CBL (Figure 39A-C, E, F), which is recognized for its role in attenuating EGFR signaling. Phosphorylation of Y1069 on EGFR creates a binding site for c-CBL (E3 ubiquitin ligase CBL). Here, it serves as a negative regulator of EGFR by ubiquitinating and targeting the activated EGFR for endocytosis and eventual lysosomal degradation (Wee & Wang, 2018).

When I examined the phosphorylation status of EGFR at Y1069 in both SPTSSA KO and control clones at the steady state, I observed a faint band that was hardly detectable on Western blot (Figure 37J). After EGF stimulation, two SPTSSA KO clones (4D10 and 5D8) and one control clone (1F5) showed comparable levels of phosphorylation. However, clone 2G9 exhibited the highest phosphorylation levels (almost two times higher than the average of 1F5, 4D10, 5D8), while clone 5C11 had the lowest levels (50% less than the average of 1F5, 4D10, 5D8). Although the average of percent mean values of the SPTSSA KO group were

lower compared to those in the control group after stimulation, the difference was not statistically significant) (Figure 37K). I normalized P-EGFR (Y1069) to total EGFR to minimize the influence of baseline total EGFR levels. This adjustment led to an increase in variation within KO clones and resulted in the average ratio of P-EGFR (Y1069) over EGFR for both SPTSSA KO and control clones being equal (Figure 37L).

As the final step, I analyzed the protein level of c-CBL at steady state and upon EGF stimulation (Figure 39A-F). The results revealed that the expression of c-CBL is higher in SPTSSA KO clones compared to control clones, with minimal variation. This difference was found to be statistically significant at the $p \leq 0.1$ level (Figure 39B). However, upon stimulation, the level of c-CBL decreased in SPTSSA KO clones, bringing the protein level closer to that of the control clones (Figure 39C). This indicated that EGF treatment equalized c-CBL expression levels between the control and SPTSSA KO groups. Additionally, I performed paired analysis where I paired GAPDH- normalized protein expression values of each untreated clones with its corresponding treatment value. The results indicated that c-cbl was only downregulated significantly in the KO clones (Figure 39E- F).

3.5.3 Cell Proliferation

I investigated the effect of EGF stimulation on control and SPTSSA KO clones over 24 hours treatment. Following 24 hours of cell seeding, they were treated with 12.5 ng/ml EGF or vehicle solution (1X PBS+ 1%BSA, referred to as “no treatment”) for an additional 24 hours. The proliferation rate of all clones showed a slight increase with the addition of EGF, with the most significant effect observed in the 5D8 clone (Figure 40A-E).

To evaluate cell proliferation, I quantified the cell numbers just before EGF addition using the crystal violet assay, as the staining intensity correlates with cell count. After 24 hours of treatment, cell numbers were measured again, and the fold change was calculated by dividing the initial crystal violet measurements by the subsequent ones. These calculations were done for both non treated and EGF treated clones (Figure F, column 2 and 3).

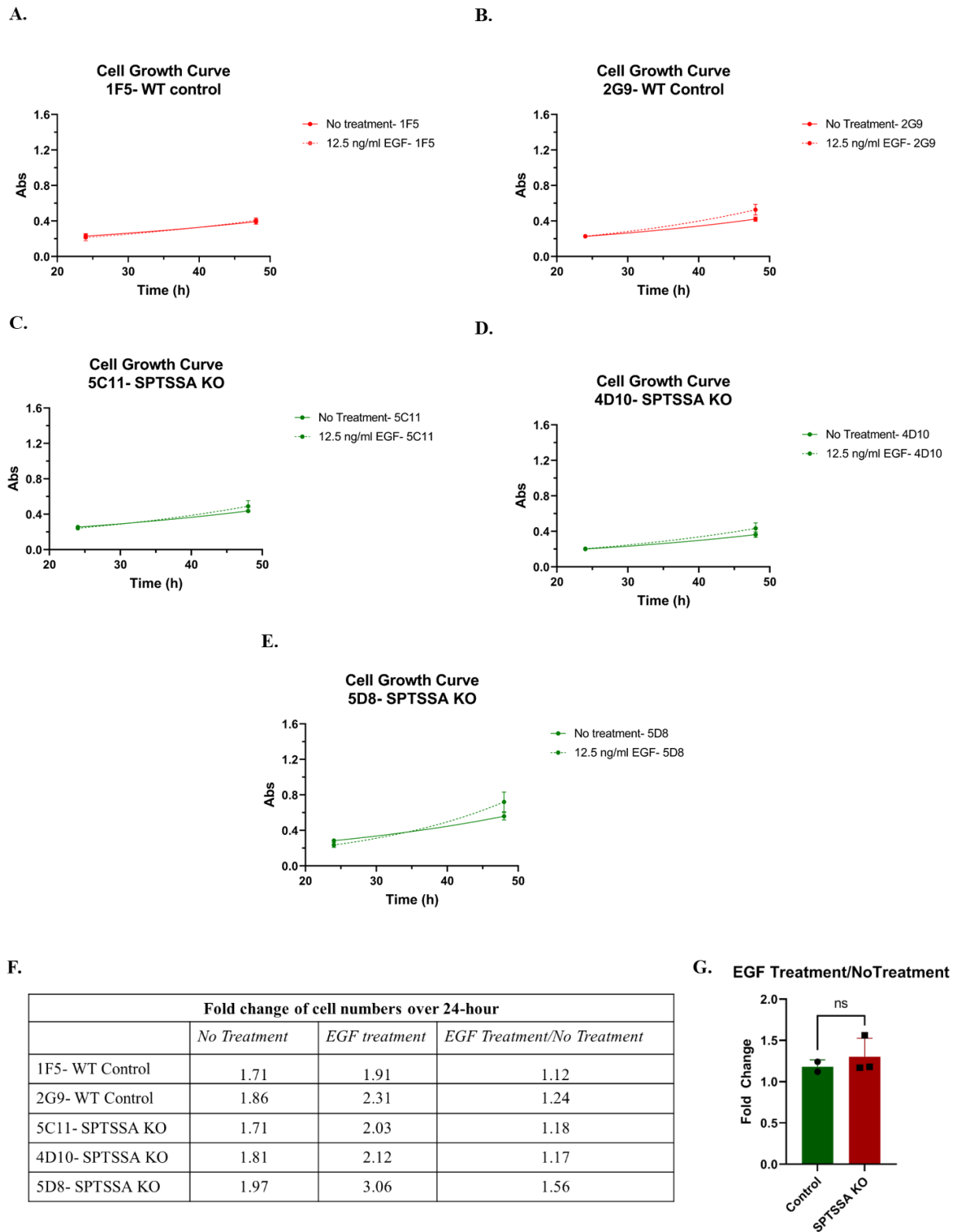


Figure 40. (A-E) Effect of EGF treatment on proliferation of control and SPTSSA KO cells. (F,G) Quantification of cell numbers over 24 hours of EGF treatment. For statistical analysis, the average fold change values of knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant,* $p \leq 0.05$).

The overall effect of EGF stimulation compared to nontreated samples were calculated by dividing the fold change values of no treatment to EGF treatment for each clone and shown in Figure 40F, column 4. The analysis indicated that the observed increase in proliferation after EGF treatment in the SPTSSA KO clones was not statistically significantly different from that of WT clones (Figure 40G).

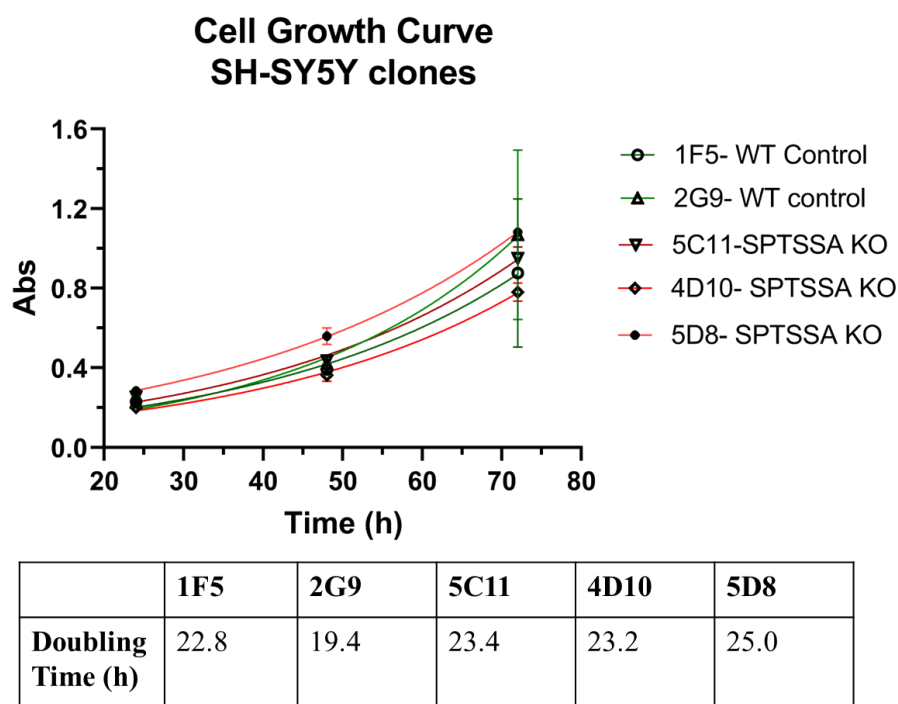


Figure 41. Proliferation rate of SPTSSA KO and control clones upon adaptation

In previous section 3.4.1.4, I had evaluated the proliferation rate of SPTSSA KO and control clones. That analysis had revealed a greater doubling time for SPTSSA KO clones. While investigating the impact of EGF stimulation on cell proliferation, I noticed that now untreated cells showed similar increase in the amount of cells over 24 hours. This led me to repeat the experiment from section 3.4.1.4, yielding an interesting and unexpected finding: the doubling time of KO clones decreased, indicating an increase in their rate of division (Figure 41). The most notable change was seen in 4D10 clones, which previously they were dividing every 72.9 hours but now doing so in just 23.2 hours, similar to control clones.

3.6 All-Trans Retinoic Acid (ATRA) Induced Differentiation of SPTSSA KO and Control Clones

3.6.1 Optimization of Differentiation Protocol using SH-SY5Y WT (parental) Cells

SH-SY5Y cells have the capacity to differentiate into mature-neuron like cells. Various methods have been documented, explaining induction media with diverse compositions. One of the extensively studied approaches involves treatment with all-trans retinoic acid. Thus, for this project, I adopted the method outlined by Kovalevich and Langford (Kovalevich & Langford, 2013). I tried the method initially in SH-SY5Y wt (parental cells) to evaluate the establishment of neuronal differentiation by analyzing the transcript level of neuronal markers.

In order to do this, I cultured SH-SY5Y wt (parental) cells in 3 different media: DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12) or Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only) or Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA) for 7 days before harvesting (Figure 42).

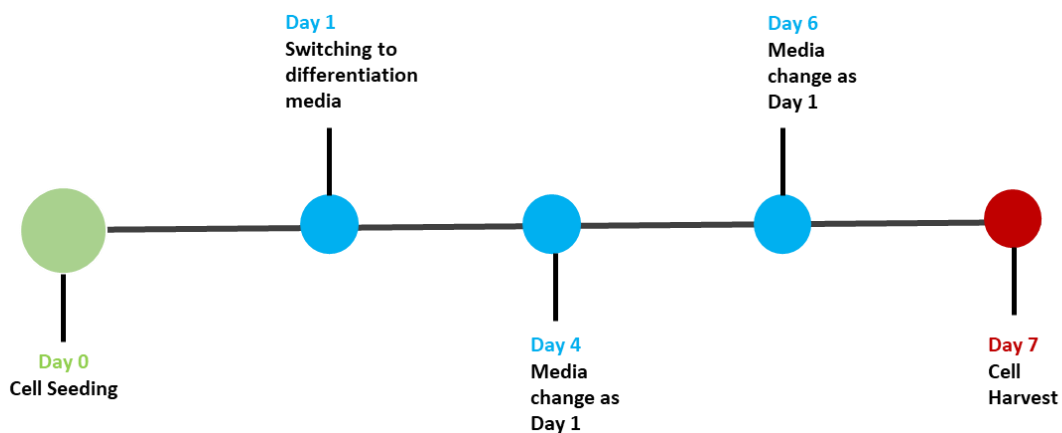


Figure 42. Outline of the experimental setup for the differentiation of SH-SY5Y wt (parental cells). Cells were seeded and 24 hours later the medium of the cells was switched to either control (DMEM/F12 supplemented with 10% FBS and %1 P/S) or differentiation media (Neurobasal media supplemented with 1X B27 and 1X Glutamax) without (B27 only) or with 10 μ M ATRA (B27+ATRA). The cells were cultured in this specified media for 6 days. The media of the cells were changed at day 4 and day 6.

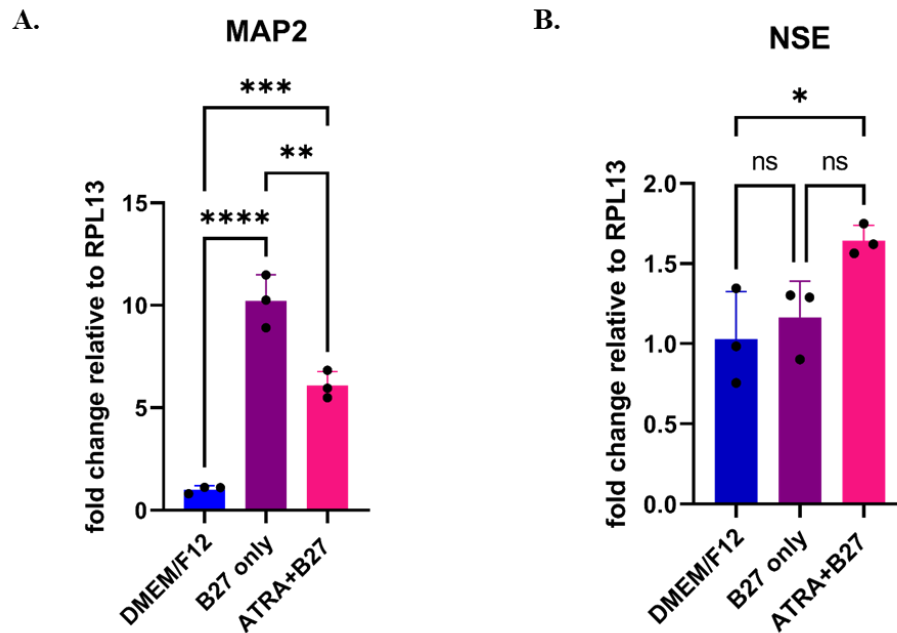


Figure 43. Relative fold change in the mRNA level of the two neuronal differentiation markers MAP2 and NSE were measured in SH-SY5Y wt (parental) cells after treating the cells with either control (DMEM/F12 supplemented with 10% FBS and %1 P/S) or differentiation media (Neurobasal media supplemented with 1X B27 and 1X Glutamax) without (B27 only) or with 10 μ M ATRA (B27+ATRA) for 7 days. mRNA levels were measured by qPCR and normalized to the housekeeping gene ribosomal protein 13 (RPL13). Each data point represents means of three replicates. Statistical analysis was performed using ordinary one-way ANOVA by GraphPad prism using (ns= not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

At day 7, I harvested the cells and performed qPCR analysis for two neuronal markers: microtubule-associated protein-2 (MAP2) and neuron specific enolase (NSE), whose expressions were shown to be upregulated in response to differentiation (Lopes et al., 2010; Murillo et al., 2017). On mRNA level, MAP2 was significantly upregulated in cells which were treated either with B27 alone (around 10-fold) or B27 together with ATRA (around 6-fold) media compared to DMEM/F12 control. There was a significant difference in MAP2 expression between cells treated with B27 alone and those treated with B27 along with ATRA. Interestingly, B27 alone increased MAP2 levels even more compared to when B27 was combined with ATRA (Figure 43A). On the other hand, cells treated with B27 alone showed no significant difference in the expression of NSE compared to the DMEM/F12 control. However, treatment with B27 along with ATRA significantly increased the expression of NSE in cells compared to the DMEM/F12 control (Figure 43B). Overall, increase in the expression

of both MAP2 and NSE in B27 along with ATRA indicated that the conditions for differentiation were suitable for further experiments.

3.6.2 Ceramide Analysis of Neuron-Like SH-SY5Y WT (Parental) Cells

I differentiated SH-SY5Y wt (parental) cells following the same protocol in section 3.6.1 to investigate the changes in distribution of NS-ceramides containing 18- and 20- carbon sphingoid bases taking all acyl chains into account. Lipid analysis was performed by LC-MS/MS analysis in targeted MRM mode. SH-SY5Y wt (parental) cells incubated in DMEM/F12 had around 94% NS-ceramide containing C18-sphingosine and around 6% NS-ceramide containing C20-sphingosine on average (Figure 44, blue bar). Switching the media to B27 alone (Figure44, purple bar) or B27 along with ATRA (Figure44, pink bar) led to 4-fold decrease of NS-ceramide with C20- sphingosine, comprising then only around 1.5% of the total ceramide on average, compared to DMEM/F12 control.

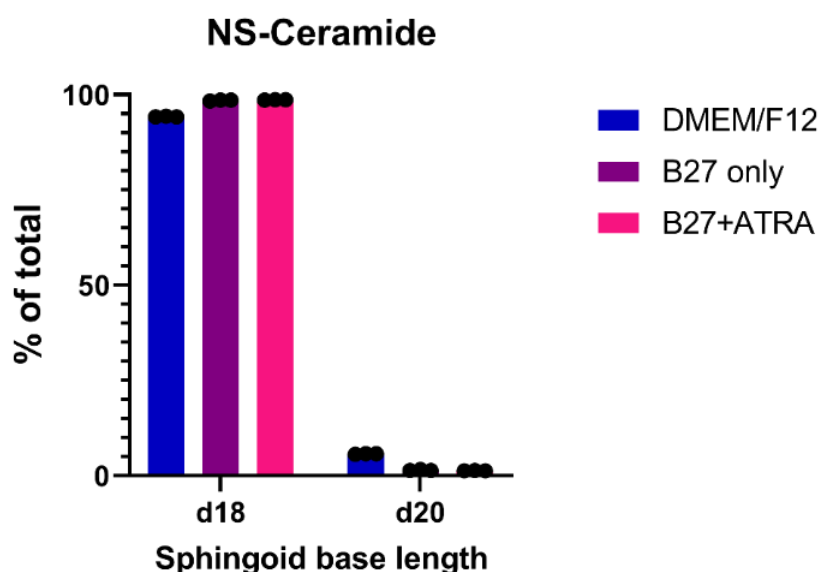


Figure 44. Distribution of NS-ceramides containing 18- and 20-carbon sphingosine as determined by LC-MS/MS analysis in MRM mode in SH-SY5Y wt (Parental) in DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). The cells were cultured for 7 days in relevant media before harvesting and proceeding with the lipid extraction. Around 4 fold decrease in the proportion of NS-ceramides with C20-sphingosine was observed in B27 only and B27+ATRA compared to DMEM/F12 treated cells (purple and pink bars).

I analyzed the NS-ceramides to determine the relative contribution of each NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) to the total NS-ceramides under different media conditions (Figure 45). The dominant species in all conditions appeared to be NS-Cer(18:1; 24:1), with around 45% of the total on average. When the medium was switched from DMEM/F12 to either B27 alone or B27 containing ATRA, amount of NS-Cer(20:1; 24:1) decreased more than 4 fold (from 3.5% to 0.8%) in SH-SY5Y wt (parental cells) in both media conditions compared to DMEM/F12. Another notable decrease was seen in NS-Cer(20:1; 24:0). It declined from 1.1% in DMEM/F12 to 0.1% in SH-SY5Y cells treated with either B27 alone or B27 containing ATRA. However, the corresponding increase was not observed neither in NS-Cer(18:1; 24:1) nor in NS-Cer(18:1; 24:0). Strikingly, the level of NS-Cer(18:1; 24:0) also decreased in both B27 alone or B27 containing ATRA treated SH-SY5Y wt (parental) cells. Overall, the amount of ceramides with long acyl chains (C16 – C20) increased over those with very long acyl chains (C22 – C24) under conditions of cell differentiation.

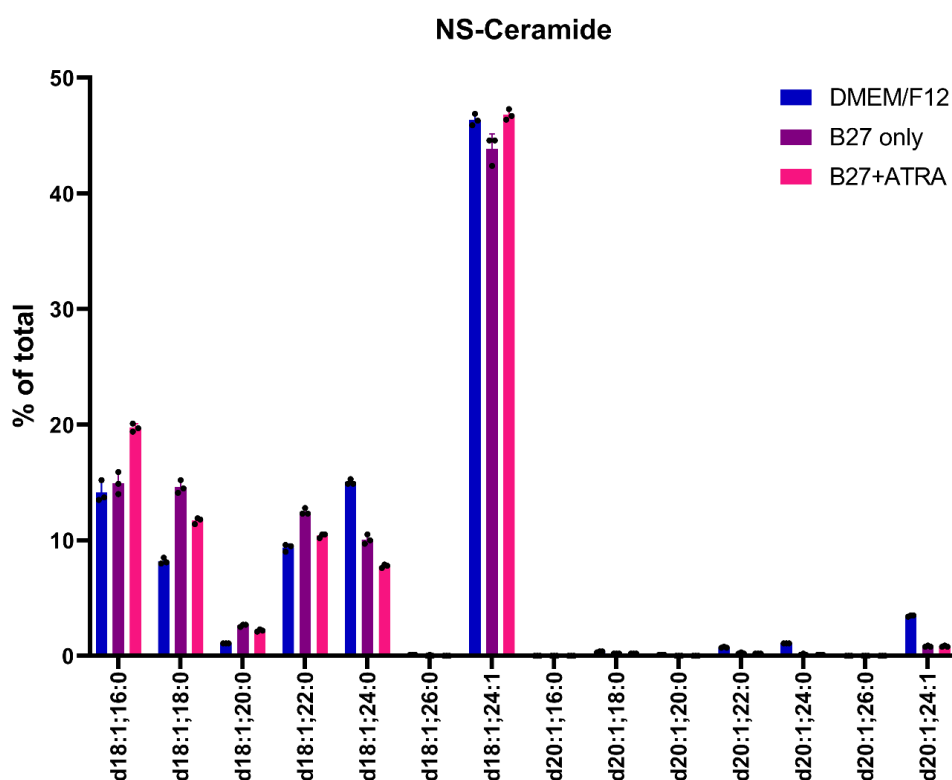


Figure 45. Relative abundance of NS-Cer (d18:1; X:Y) and NS-Cer (d20:1; X:Y) in SH-SY5Y wt (Parental) cells incubated in DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA) as calculated from the targeted LC-MS/MS data

3.6.3 Analysis of SPT Complex Expression and Activity Assay

The alteration in NS-ceramide profile of SH-SY5Y wt (parental) cells upon changing the media from DMEM/F12 to differentiation media suggested a potential impact on sphingolipid metabolism. To investigate whether SPT complex was affected by the media change, I evaluated the mRNA levels of SPT subunits by qPCR. The experimental setting was used as in section 3.6.2.

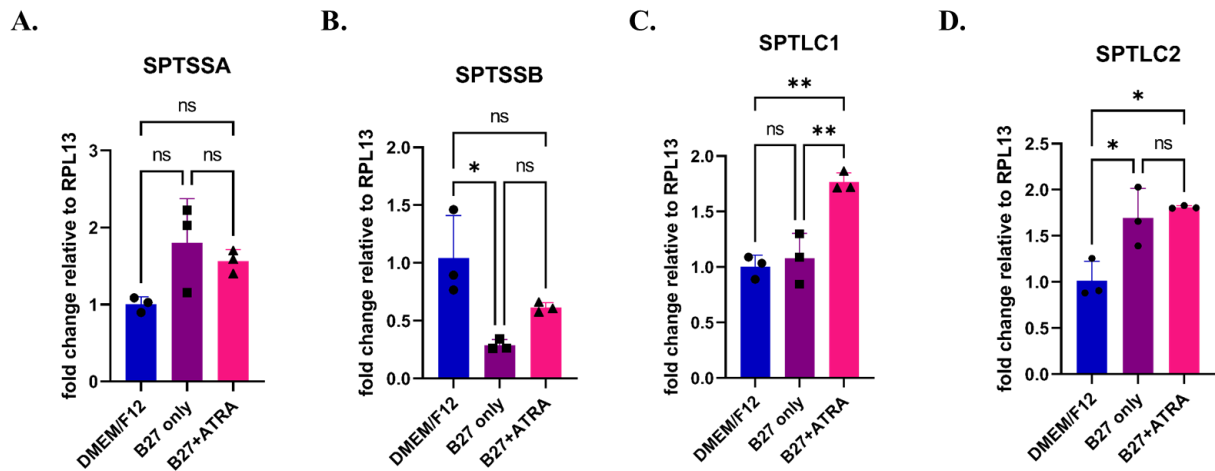


Figure 46. Relative fold change in the mRNA level of SPT complex subunits were measured in SH-SY5Y wt (parental) cells after treating the cells with either control (DMEM/F12 supplemented with 10% FBS and %1 P/S) or differentiation media (Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only) or Neurobasalmedia supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA) for 7 days. mRNA levels were measure by qPCR and normalized to the housekeeping gene ribosomal protein 13 (RPL13). Each data point represents a mean of three technical replicates. Statistical analysis was performed using ordinary one-way ANOVA by GraphPad prism using (ns= not significant, * $p \leq 0.05$, ** $p \leq 0.01$).

Although statistically not significant, higher SPTSSA levels were recorded in cells cultured with either B27 $\pm$ ATRA (Figure 46A). On the other hand, a decreased expression of SPTSSB was determined in when cells were treated B27 $\pm$ ATRA as groups compared to the DMEM/F12 controls (Figure 46B). Notably, the effect was most pronounced and significant in medium without ATRA. These findings align with the observed decrease in the relative amount of C20-sphingosine in both the B27-only and B27+ATRA groups compared to the controls.

While changing the medium from DMEM/F12 to B27 alone did not affect the expression of SPTLC1, combining B27 with ATRA resulted in a significant increase in

SPTLC1 expression compared to both the DMEM/F12 and B27-only groups (Figure 46C). Meanwhile, the expression of SPTLC2 increased when cells were cultured in B27 with or without ATRA, with both conditions showing statistically higher expression levels compared to the control (Figure 46D).

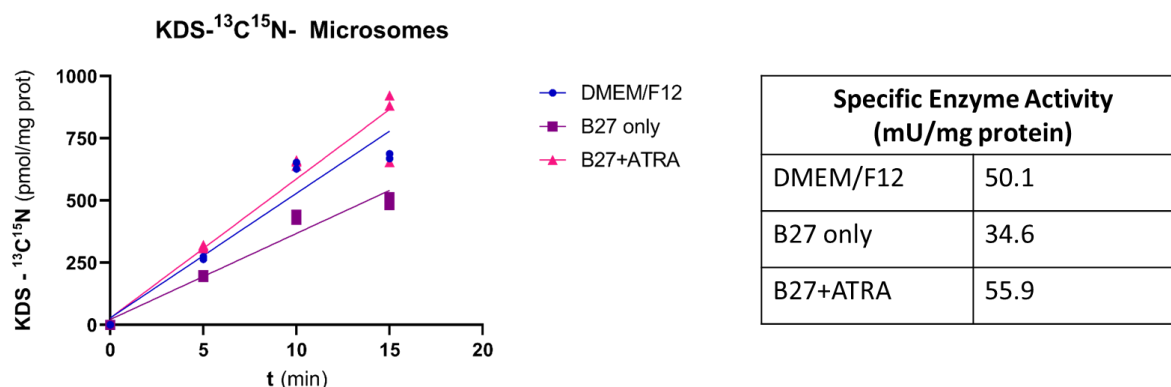


Figure 47. Determination of the specific SPT activity of SH-SY5Y wt (parental) cells incubated in DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA) for 21 hours. Statistical analysis performed with Graphpad prism indicated that the slopes are under different treatments significantly different ($p=0.0008$).

The alterations observed in the expression pattern of SPT complex subunits led us to measure the SPT activity. To be able to capture rapid changes in cellular responses, I cultured the cells with 3 different media (as in section 3.6.2) for a relatively short period of time (21 hours) and harvested the cells for SPT activity assay. The SPT activity was performed as in section 3.3.3 and the linear increase of product over time (slope of the regression curve in the graph of Figure 47) was used to calculate the SPT activity. The results showed that the enzymatic activity of SPT was similar in SH-SY5Y wt (parental) cells which were cultured either in DMEM/F12 or differentiation medium with ATRA (B27 + ATRA). However, the cells showed a significantly reduced SPT activity compared to DMEM/F12 control when they were cultured in differentiation medium without ATRA (B27 only).

3.6.4 Cell Viability

I tested the whether SH-SY5Y cells retained the ability to differentiate into neuron-like cells even in the absence of functional SPTSSA, resulting in longer sphingoid bases. I treated them with either 1X B27 alone or in combination with 10 μ M ATRA in neurobasal media for 5 days. I used normal growth media as control and monitored the cell growth/proliferation and differentiation via two approaches: crystal violet staining and live cell imaging by using the IncuCyte[®] system .

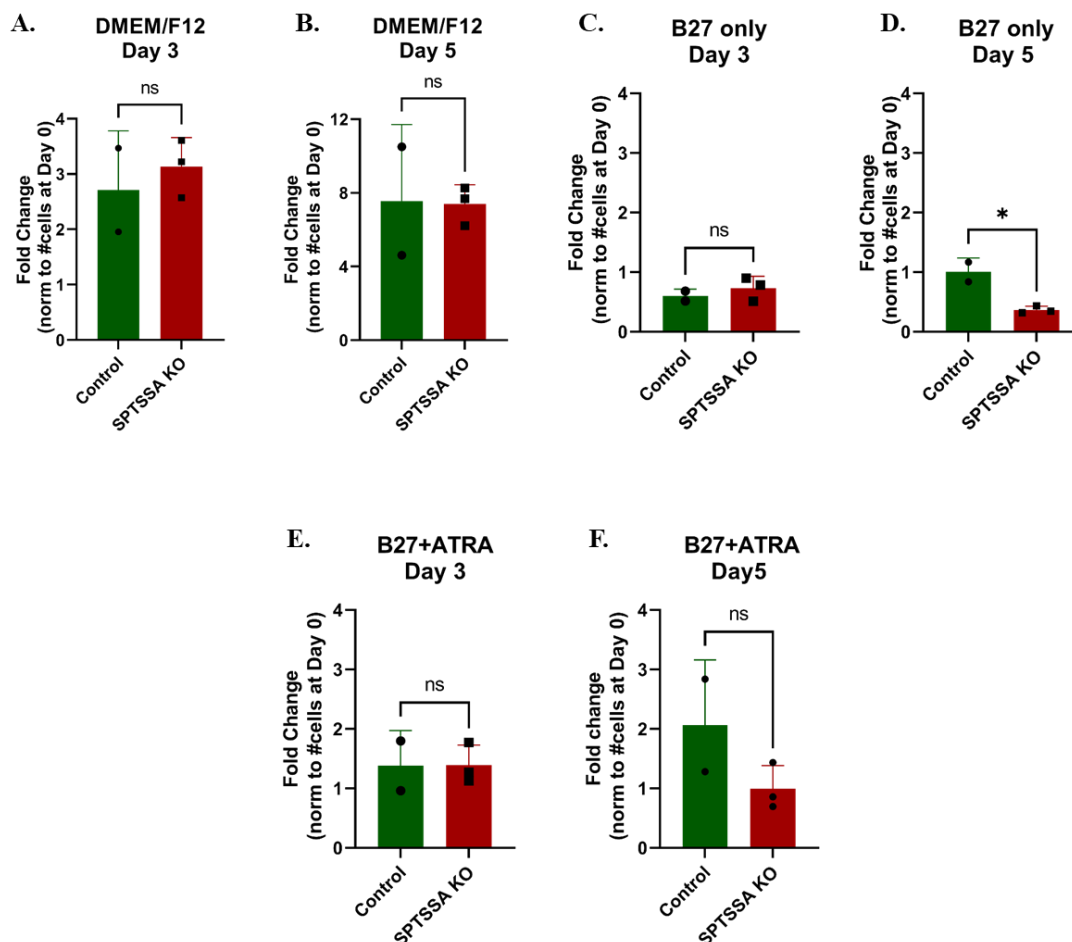


Figure 48. Cell viability of SPTSSA KO and controls cells was quantified via crystal violet staining in (A-B) DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), (C-D) Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or (E-F) Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). The cell numbers for each day were normalized to that of day 0. Subsequently, the average fold change in cell numbers of knockout (KO) group were compared to those in the control group (n=4) using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant, * p $\leq$ 0.05).

In the first approach, the cell growth/proliferation was determined by crystal violet staining. I stopped the experiment at day 0, 3 and 5. In DMEM/F12 media (DMEM/F12 supplemented with 10% FBS and 1% P/S), the cell growth was normal and cell number increased over days both in SPTSSA KO and control clones (Figure 48A-B).

However, the cell number of cells started decrease in B27 only (Neurobasal media supplemented with 1X B27 and 1X Glutamax) as the cells died over time in SPTSSA KO cells (Figure 48C-D). The addition of ATRA to the media mitigated this decrease, only to a limited extent, suggesting a partial recovery of the phenotype (Figure 48E-F). Concurrently, the proliferation rate of control cells in both B27 only and B27+ATRA decreased. This reduction in proliferation rate is typical in SH-SY5Y cells as cells exit from the cell cycle during the differentiation (Kovalevich & Langford, 2013).

In order to monitor cell death over time, the cells were imaged by IncuCyte® S3-Live Cell Images System and the pictures of the cells were taken every 6 hours for 5 days. The growth of the cells was quantified by calculating the area covered by the cells in 96-well plate. In DMEM/F12, SPTSSA KO and control cells grew and proliferated normally (Figure 49A-B) and at day 5, the cells were almost confluent (Supplementary Figure 13).

When SPTSSA KO clones were treated only with 1X B27 supplement, they showed a trend consistent with that observed in the crystal violet experiment (Figure 49C-D). Over time, the cells began to undergo cell death, characterized by obtaining round shape and detachment from the plate surface. The most severe phenotype was observed in KO clones 4D10 and 5C11. While KO clone 5D8 still exhibited cell death, although its condition was slightly improved (Supplementary Figure 14). The cell death was still seen in SPTSSA KO cells in the presence of 1X B27 supplement in combination with 10 μ M ATRA, however, there were still living cells at Day 5 (Supplementary Figure 15).

Overall, two different experimental settings for cell viability yielded similar results and reached the same conclusion. When the cells were cultured in B27-only media, SPTSSA KO cells began to undergo cell death. This difference in cell viability between SPTSSA KO and control cells reached significance by day 5. The addition of 10 μ M ATRA to the media partially restored the phenotype.

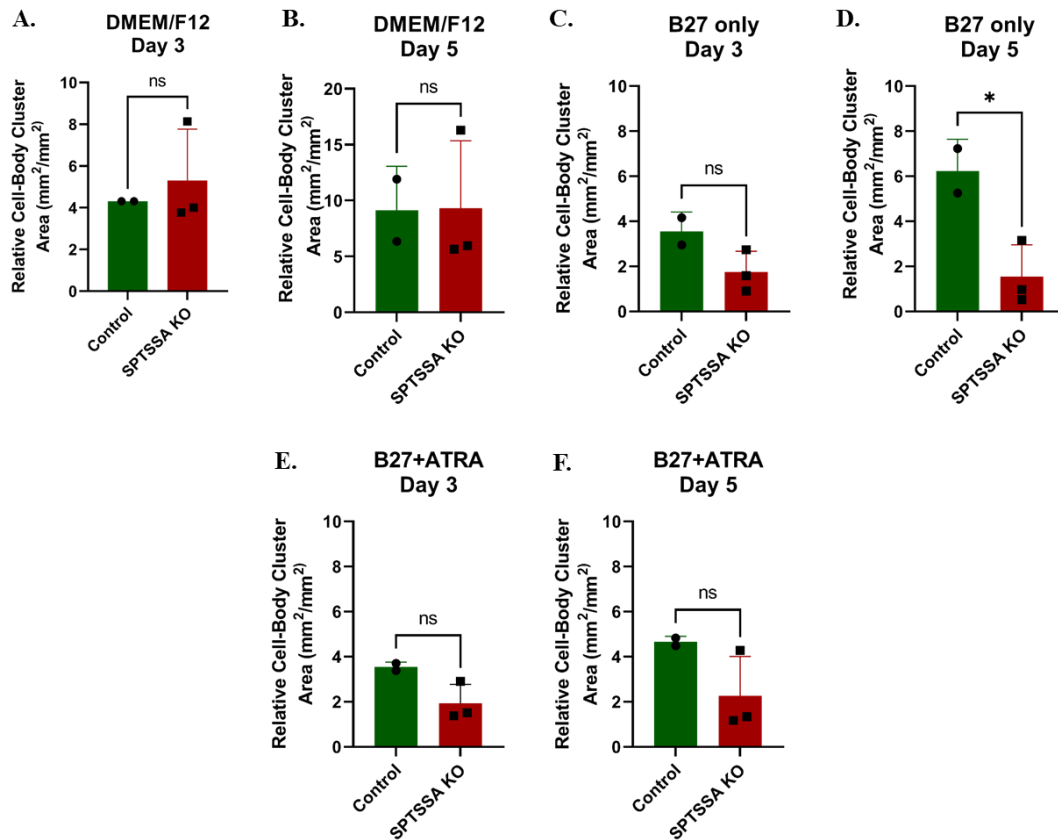


Figure 49. Confluency of SPTSSA KO and controls cells on 96-well plate was quantified via IncuCyte® Neurotrack Analysis Software Module in (A-B) DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), (C-D) Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or (E-F) Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). The cell-body cluster area for each day were normalized to that of day 0. Subsequently, the average cell body cluster area values of knockout (KO) group were compared to those in the control group (n=4) using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant, * $p \leq 0.05$).

3.6.5 Mitochondrial assays

The differentiation of SH-SY5Y cells is known to alter mitochondrial biogenesis and energy metabolism (Matrella et al., 2024; Schneider et al., 2011). Therefore, I thought about my previous findings in proteome and Western blot analysis about changes between KO and control clones in the expression of MT-CO2 and UQCRC2, which are the components of oxidative phosphorylation (OXPHOS) system (Table 34 and Figure 33). These two findings provided us with a clue about potential changes in mitochondrial function under steady-state conditions which might get relevant in response to stimuli of cell differentiation. Therefore, I

examined the effect of 1X B27 alone or in combination with 10 μ M ATRA in neurobasal media on mitochondrial membrane potential and mitochondrial mass in SPTSSA KO and control cells. In the previous functional analyses, variation in the behaviour of control clones led to inconclusive results. In order to minimize the clonal effect, SH-SY5Y WT (parental) cells were added as an additional control.

3.6.5.1 Optimization of Mitochondrial Assays using SH-SY5Y wt (parental) Cells

3.6.5.1.1 Changes in Mitochondrial Membrane Potential, Mitochondrial Mass and Total ATP Level in SH-SY5Y wt (parental) Cells upon Treatment

Based on the images obtained from the IncuCyte® S3-Live Cell Imaging System, cell death was initiated approximately 18-24 hours after treatment in SPTSSA KO cells. Therefore, in the following experiments, I tested whether treatment with 1X B27 alone or in combination with 10 μ M ATRA in neurobasal media induces alterations in mitochondrial membrane potential or mass after a 21-hour treatment period in SH-SY5Y wt (parental) cells. Selecting the correct time point was crucial to establish a consistent starting point for testing under the same time frame and conditions with SH-SY5Y KO and control clones. I also tested the changes in the total ATP level upon treatment in SH-SY5Y wt (parental) cells.

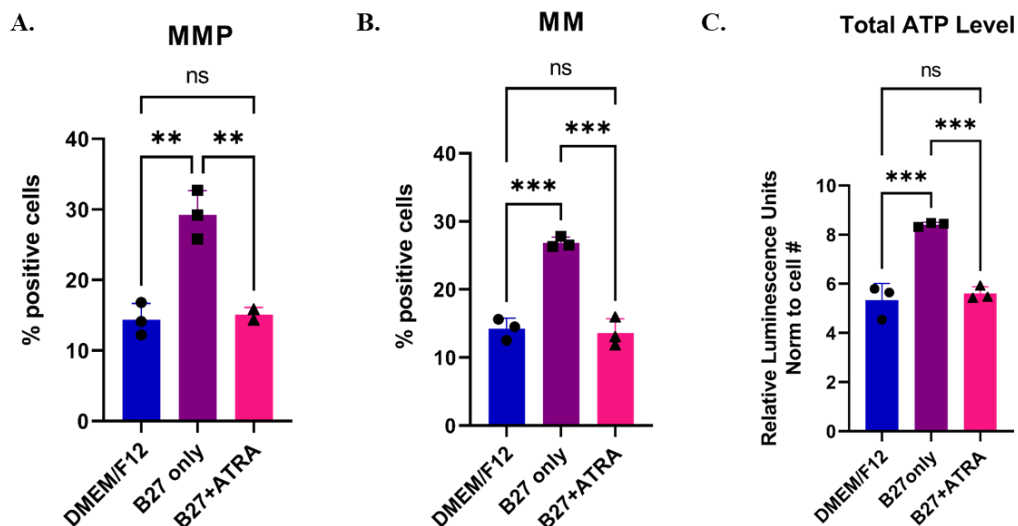


Figure 50. Changes in (A) mitochondrial membrane potential (MMP), (B) mitochondrial mass (MM) and (C) total ATP levels in SH-SY5Y wt (parental) cells, when the cells were treated with DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X

Glutamax (B27+ATRA). Percent positive cell population was calculated for each measurement in A and B. The cells were labelled with (A) MitoTracker Red CMXRos or (B) MitoView Green and analyzed by flow cytometry. Total ATP level was determined by measuring luminescent signal by plate reader and normalizing the values to the total number of cells (A-C). Subsequently, values (n=3) in each treatment were compared using ordinary one-way anova. Statistical analysis was performed using GraphPad prism using (ns= not significant, ** $p \leq 0.01$, *** $p \leq 0.001$).

Treatment of cells with 1X B27 in neurobasal media caused a significant increase in mitochondrial membrane potential (MMP) and also mitochondrial mass (MM). Interestingly, when the cells were treated with 1X B27 in combination 10 μ M ATRA in neurobasal media, the levels of MMP and MM were nearly equivalent to those observed when the cells were in DMEM/F12 media (Figure 50A and B). This trend continued with total ATP level analysis, where the total ATP level peaked in the presence of 1X B27 supplement alone in neurobasal media (Figure 50C). This data indicates that a 21-hour treatment is sufficient to observe changes in MMP, MM, and total ATP level.

3.6.5.2 Evaluation of Mitochondrial Membrane Potential of SPTSSA KO and Control Clones at Baseline and upon Treatment

I treated parental, SPTSSA KO and control clones with either 1X B27 supplement alone or in combination with 10 μ M ATRA in Neurobasal media for 21 hours. I used normal growth media as control. The mitochondria of the cells were labelled with MitoTracker Red CMXRos and the changes were quantified by flow cytometry.

When the cells were in their normal growth media, at the steady state, SPTSSA KO cells exhibited a slight increase in their mitochondrial membrane potential (MMP), although this change was not statistically significant (Figure 51A). An interesting observation is that control clone 1F5 showed a similar behavior to the parental cells, while control clone 2G9 was similar to SPTSSA KO clone 5C11, which behaved differently from the other two SPTSSA KO clones (Figure 51D).

Treatment of cells with 1X B27 supplement in neurobasal media led to an increase in MMP in all cells compared to DMEM/F12 condition, with an average around 2-fold increase in control and 3-fold increase in SPTSSA KO clones (Figure 51B). The mitochondrial membrane potential was significantly higher in SPTSSA KO clones compared to controls at a significance level of $p \leq 0.1$. Again, the same trend was observed in clones, where 1F5 behaved similarly to parental cells, while 2G9 exhibited similar behavior to KO clones (Figure 51D).

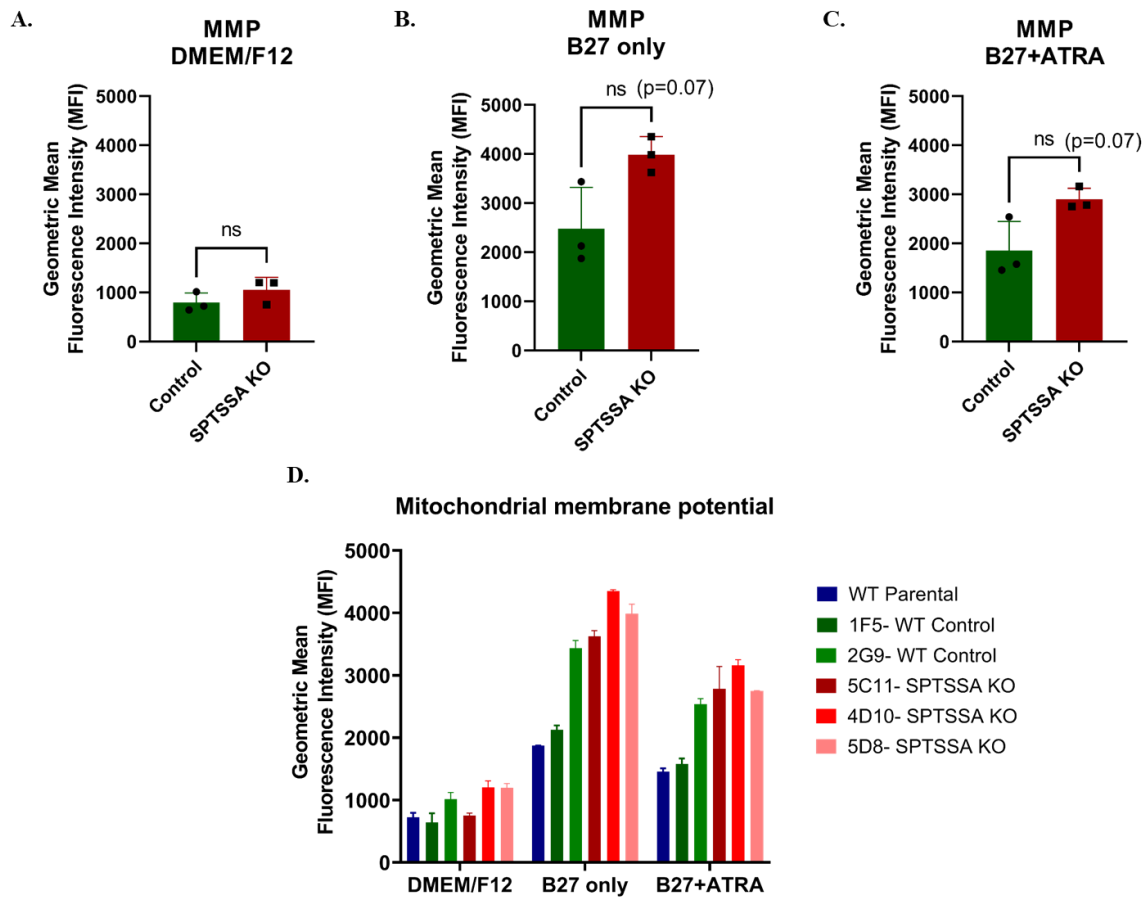


Figure 51. Changes in mitochondrial membrane potential (MMP) of SPTSSA KO and controls cells. The cells were treated with (A) DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), (B) Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or (C) Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). (D) Average geometric mean fluorescence intensity values for each cell line. The cells were labelled with MitoTracker Red CMXRos and analyzed by flow cytometry. (A-C) Subsequently, the average cell numbers of knockout (KO) group were compared to those in the control group (n=2) using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant).

The addition of 10 μ M ATRA to B27 supplement in neurobasal media maintained the same trend observed with B27 alone (Figure 51C). There was an increase in MMP; however, this increase was not as pronounced as observed with B27 alone in all cells. The mitochondrial membrane potential was again significantly higher in SPTSSA KO clones compared to controls at a significance level of $p \leq 0.1$.

3.6.5.1 Evaluation of Mitochondrial Mass of SPTSSA KO and Control Clones at Baseline and upon Treatment

The experimental settings used to measure changes in mitochondrial membrane potential were also employed for measuring mitochondrial mass (MM). The mitochondria of the cells were labelled with MitoView Green and the changes were quantified by flow cytometry.

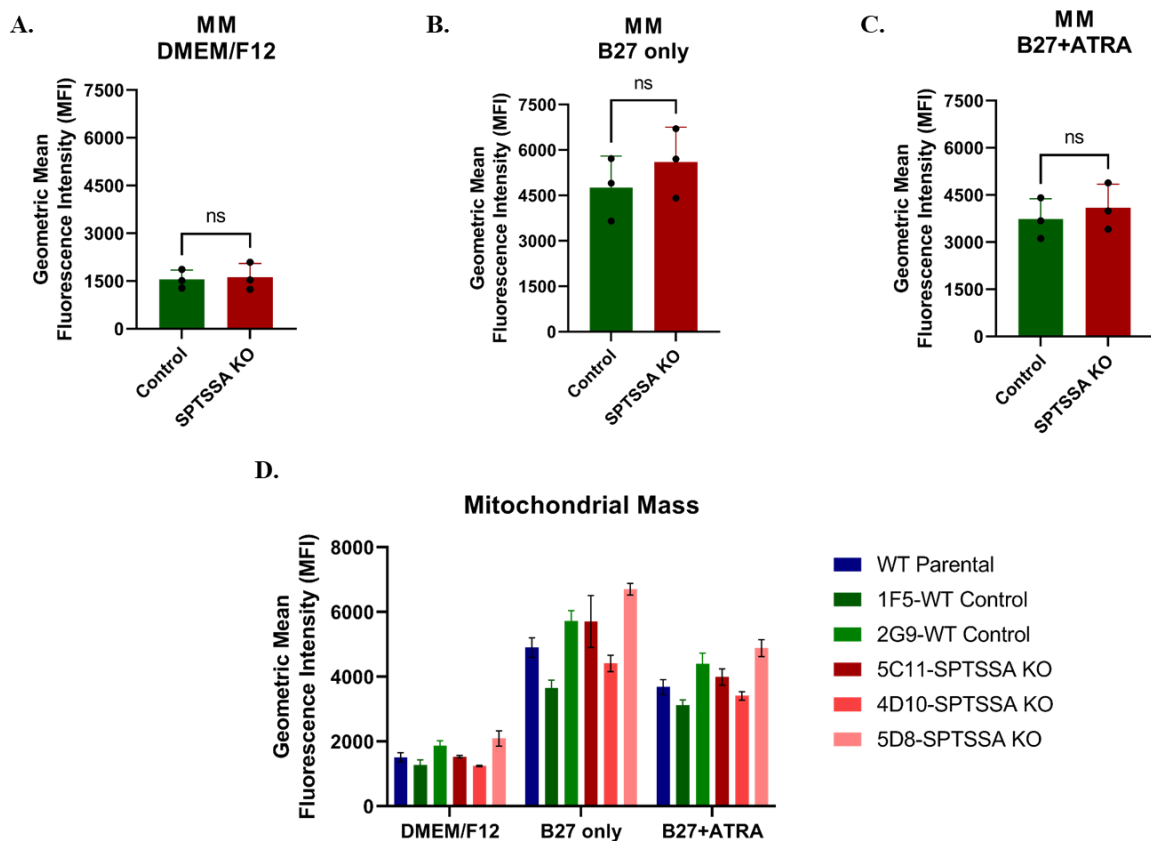


Figure 52. Changes in mitochondrial mass (MM) of SPTSSA KO and controls cells. The cells were treated with (A) DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12), (B) Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only), or (C) Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). (D) Average geometric mean fluorescence intensity values for each cell line. The cells were labelled with MitoView Green and analyzed by flow cytometry. (A-C) Subsequently, the average cell numbers of knockout (KO) group were compared to those in the control group (n=2) using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant).

Under normal growth conditions, at a steady state, KO cells showed nearly the same amount of MM as control cells (Figure 52A and 52D). However, upon transitioning the media

to 1X B27 supplement in neurobasal media, MM increased across all cell lines (Figure 52B). Among the KO clones, 5D8 displayed the most significant increase in mitochondrial mass, while 1F5 showed the least increase (Figure 52D). Although there was variation among all clones, the average geometric mean fluorescence intensity values for mitochondrial mass in KO clones were higher than those of control clones, though this difference was not statistically significant (Figure 52B).

Compared to control DMEM/F12 medium, cells cultured in 10 μ M ATRA with 1X B27 supplement in neurobasal media increased mitochondrial mass similar to the trend observed with B27 alone, but weaker and with no significant difference between control and KO cells (Figure 52C and 52D).

3.7 Polar Metabolite Analysis

To gain deeper insights into the effects caused by SPTSSA KO (and consequently alterations in sphingoid bases), we conducted polar metabolite analysis. For this analysis, I cultured the cells in their normal growth media (DMEM/F12 supplemented with 10% FBS and 1% P/S) and harvested them 48 hours after seeding to allow them sufficient time to adapt to their environment and reach their normal growth state. Our aim was to see the changes on a steady state condition.

Hierarchical clustering based on polar metabolite analysis showed that SPTSSA KO and control clones were neatly separated into two main clusters and technical replicates behaved the same way as they clustered in the same branch (Figure 53).

Polar metabolite analysis was conducted using a targeted approach, quantifying a total of 82 metabolites. Among this selection, as much as 63 were found to be significantly altered, as depicted in Figure 54. Despite variations in the changes of certain metabolites within control and SPTSSA KO clones, distinct blocks of metabolites showed clear separation between KO and control groups in terms of consistent alteration. For instance, amino acids and derivatives (L-asparagine, L-aspartic acid, L-cysteine, phosphoserine, and L-cystathionine) were notably downregulated, while metabolic intermediates and cofactors (glyceraldehyde 3-phosphate, L-lactic acid, and NADH) as well as carnitine derivatives (acetyl carnitine and butanocarnitine) were upregulated in SPTSSA KO cells compared to controls. Surprisingly, also L-serine levels were on average reduced in SPTSSA KO clones, although this is one of the direct substrates of the SPT.

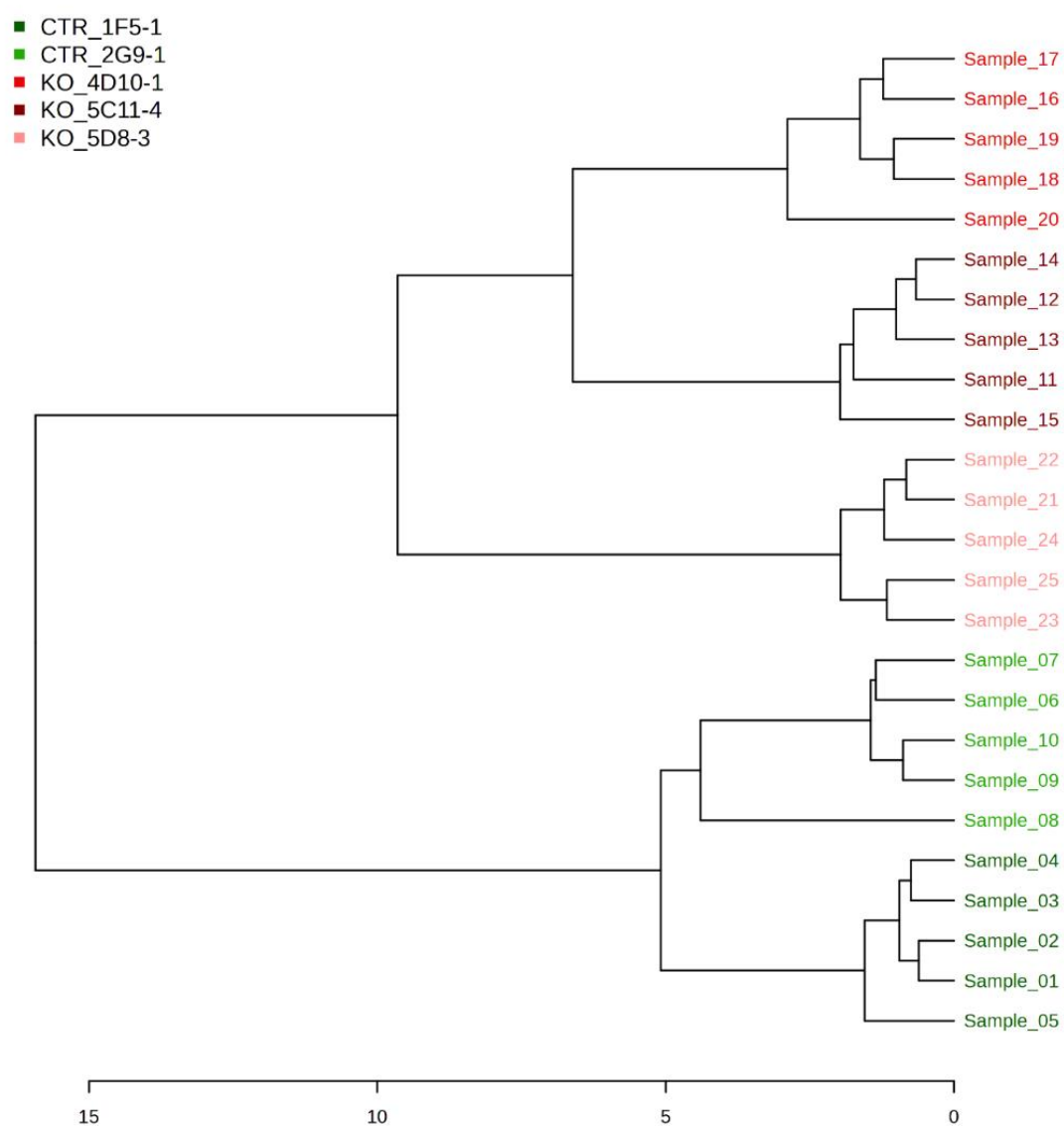


Figure 53. Hierarchical clustering of control and SPTSSA KO clones based on polar metabolite analysis

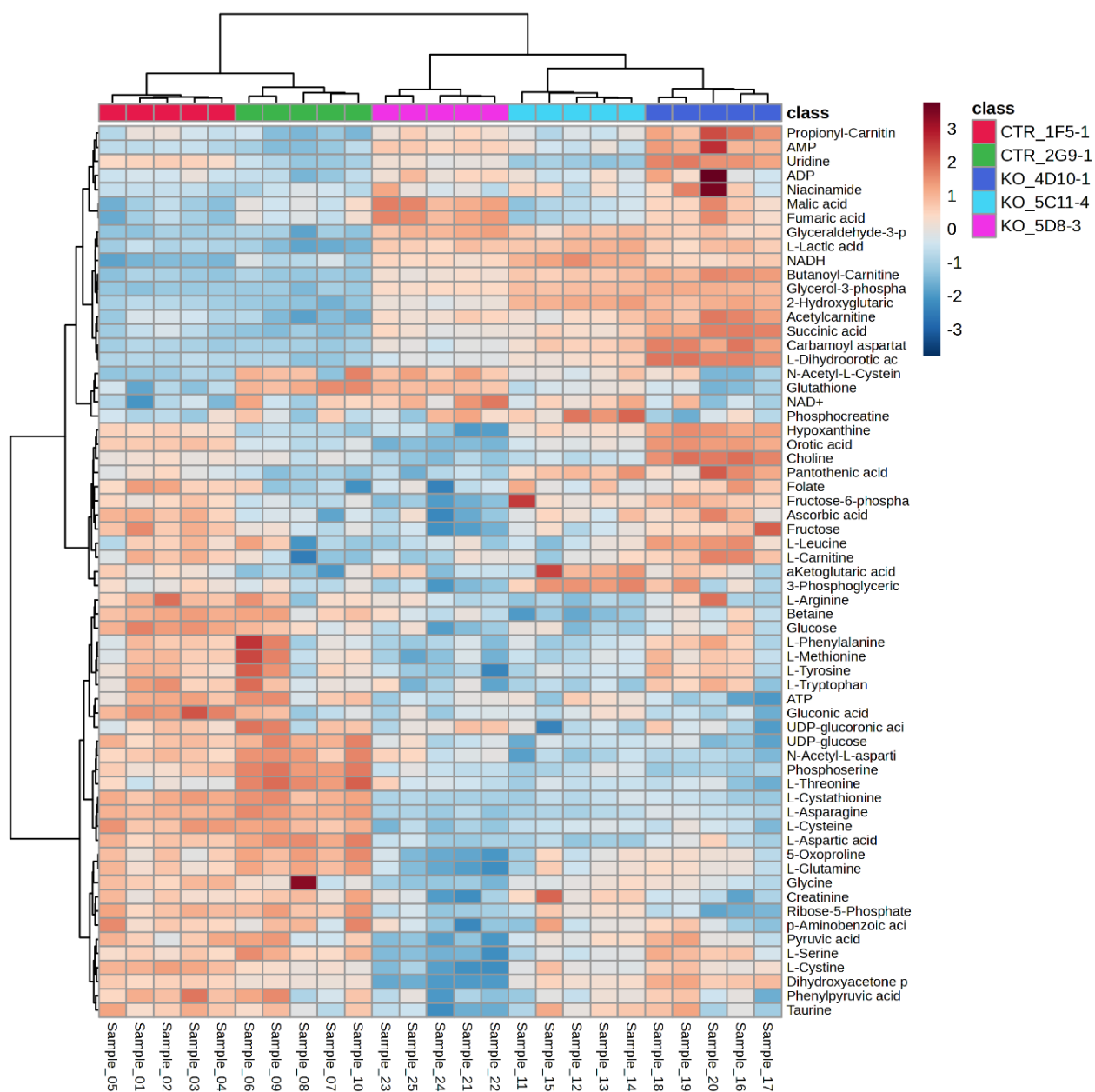


Figure 54. A heatmap analysis was generated to display the 63 significantly altered metabolites between SPTSSA KO and control clones, as determined by polar metabolite analysis. Significance was assessed by performing one-way ANOVA on a total of 82 metabolites, with a p-value threshold set to ≤ 0.05 . Representing z-scores, upregulated metabolites in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.

3.8 Lipidome Analysis

To understand whether SPTSSA KO induced any general changes in the overall lipidome, we conducted a semi-untargeted lipidome analysis. We used the same samples that we used in polar metabolites to extract lipids.

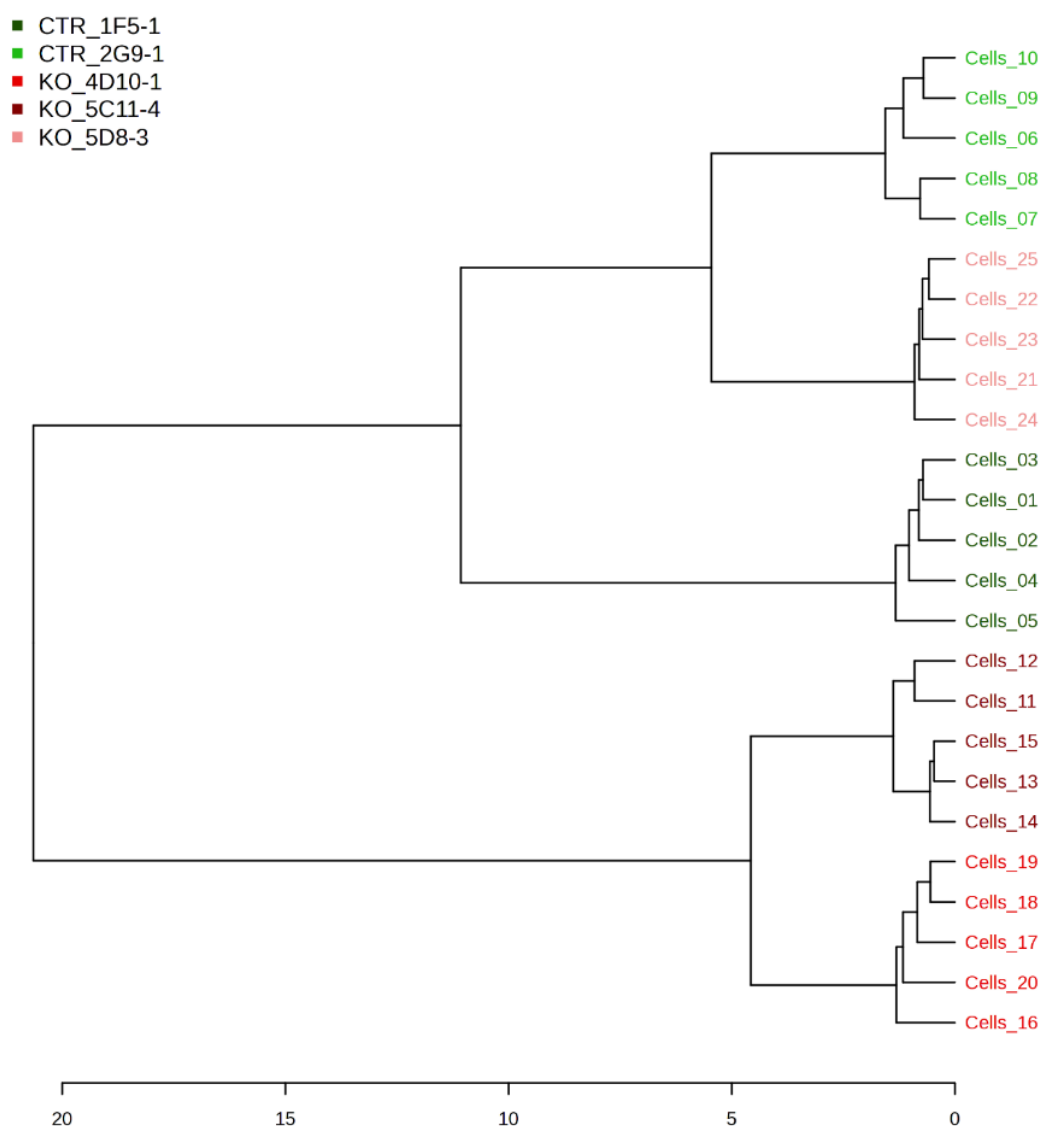


Figure 55. Hierarchical clustering of control and SPTSSA KO clones based on semi-untargeted lipidome analysis

Hierarchical clustering based on lipidome analysis revealed two main branches (Figure 55). In the first branch, two control clones (1F5 and 2G9) clustered together with SPTSSA KO 5D8. This was followed by a further branching, where 2G9 and SPTSSA KO 5D8 grouped

together. Interestingly, KO clone 5D8 was more closely related to control clone 2G9 than 2G9 was to control clone 1F5. In the second branch, the other two SPTSSA KO clones 4D10 and 5C11 were grouped together as expected.

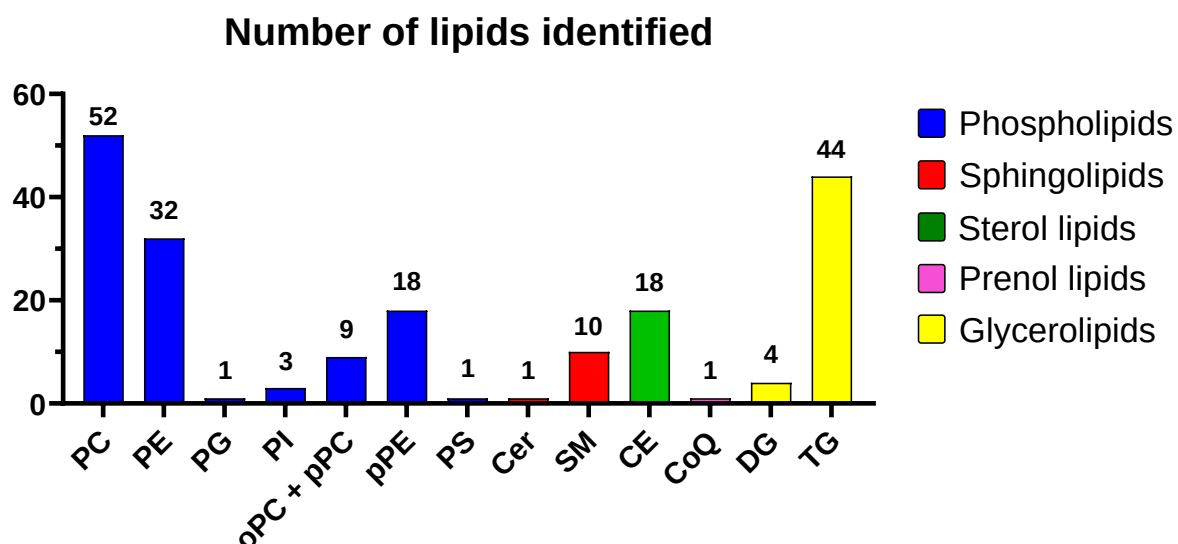


Figure 56. Number of lipids identified in all SH-SY5Y cells by untargeted DDA-measurement and targeted lipidome analysis. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; Cer, ceramide; SM, sphingomyelin; CE, cholesteryl ester; CoQ, coenzyme Q; DG, diacylglycerol, TG, triacylglycerol. The “p” or “o” before phospholipids (PC and PE) indicate plasmeyl or plasmany, respectively.

Lipidome analysis was conducted using a targeted data search approach, quantifying a total of 194 lipids (Figure 56). Among these lipids, as much as 172 were found to be significantly altered. Heat maps for each lipid groups are shown in supplementary data (Supplementary Figure 16-19).

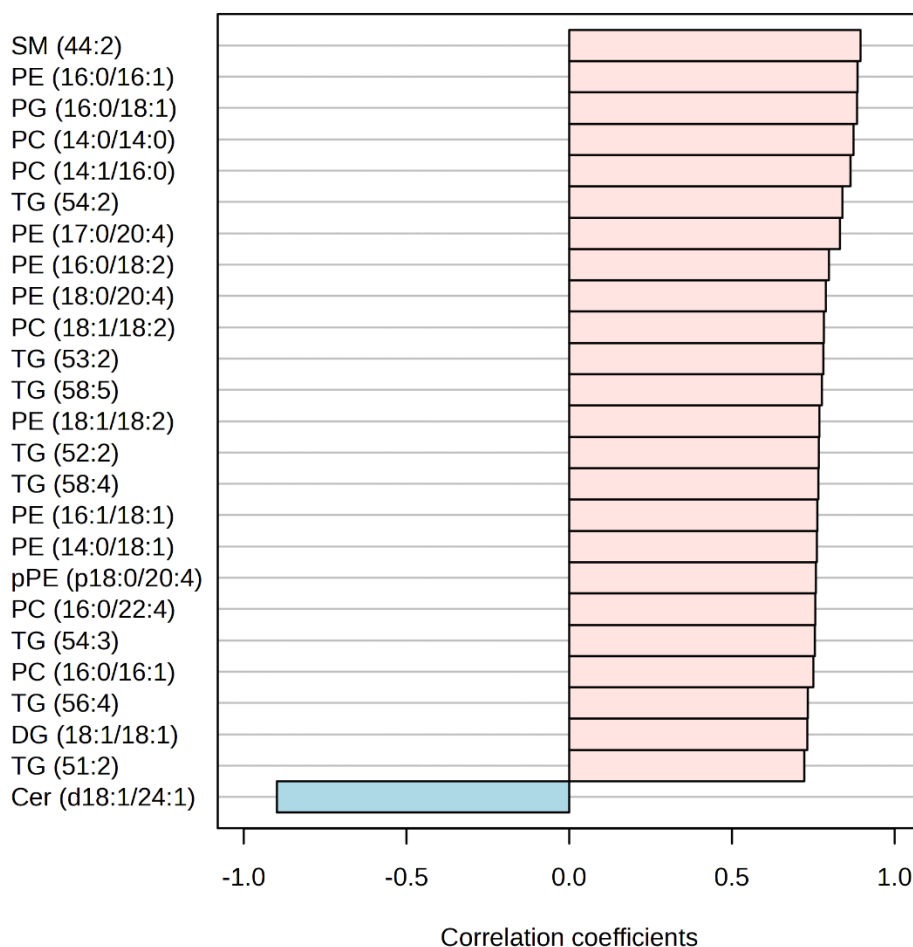


Figure 57. The top 25 lipids out of 172, which were statistically significantly altered and demonstrated consistent differential expression patterns between SPTSSA KO groups and controls. Upregulated metabolites in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.

However, there was considerable variation within the lipid composition of SPTSSA KO groups, as 5D8 exhibited similarities to the control groups. Despite these variations, we were still able to identify 25 lipids, which were differentially expressed between SPTSSA KO clones on one side and control clones on the other side (Figure 57).

4. DISCUSSION

4.1 CRISPR-Cas9 Mediated SPTSSA KO in SH-SY5Y Led to Increase in the Level C20-sphingosine, Decreased the SPT Activity and Altered Lipidome

Mammalian cells have been shown to produce a wide range of sphingoid bases with lengths spanning from 14 to 22 carbons (Pons et al., 2002). Although, there are other contributing factors for the determination of the diversity in sphingoid bases such as local substrate availability, one of the main factors is the components of the SPT complex. SPT complex is a heterotrimeric protein composed of two large and one small subunits. The catalytically active SPT complex contains SPTLC1 which binds to SPTLC2 or SPTLC3. The basal activity of the complex is dramatically increased by the binding of one of the two small subunits (SPTSSA or SPTSSB) to the complex (Wang et al., 2021). SPTSSA typically prefers 16C-CoA as a substrate and has limited activity with shorter or longer acyl-CoAs. However, the presence of SPTSSB allows SPT to utilize 16C-CoA or 18C-CoA thereby modifying its substrate preference in mammalian cells. Therefore, the complex formed with SPTLC1/SPTLC2/SPTSSA leads to production of 18 carbon sphingoid bases. However, when SPTSSB replaces SPTSSA in the complex, complex utilizes stearoyl-CoA (18 carbon) alongside palmitoyl-CoA (16-carbon), resulting in the additional generation of 20 carbon sphingoid bases in addition to 18 carbon ones (Harmon et al., 2013).

In this project, our initial aim was to generate a cell line producing longer sphingoid bases in addition to 18 carbon sphingoid bases and follow the functional changes induced by alternation in sphingoid base profile. This shift of chain length is observed in neural gangliosides upon aging (Sonnino & Chigorno, 2000). However, the functional consequences for the nerve system remain so far unknown. Here, I used neuroblastoma cell line (SH-SY5Y) cell line due to its versatility. SH-SY5Y cells can exist in tumor tissue state (neuroblastoma) or in mature-neuron like state when they are cultured in different media conditions (Feles et al., 2022). Furthermore, by using various cocktails of supplements, extracellular matrix proteins, and neurotrophic factors, they can differentiate into different types of neurons and express specific neuronal markers (Shipley et al., 2016; Teppola et al., 2016). This diversity provides us with a wide range of opportunity to explore the effect of changes in sphingoid base length on cell signaling and cellular function both, when the cells are in tumor tissue state or differentiated into mature like-neurons.

To be able to find the correct strategy to further pursue on generating cell line producing longer sphingoid bases, I first investigated the expression profile of SPT subunits in wild type SH-SY5Y (parental) cells. The results showed that the large subunits of the SPT complex are SPTLC1 and SPTLC2 in these cells. Although, the cells expressed both small subunits, the expression of SPTSSA was stronger compared to the SPTSSB. This information led us to further investigate their ceramide profile to better understand the distribution and ratio of ceramide species with different sphingoid base chain length. The ceramide profile showed that only % 5 of the NS-ceramides contained the C20-sphingoid bases while 95% of NS-ceramide had C18-sphingoid bases. We did not detect longer sphingoid bases in these cells (Figure 8). This result aligns with the observation that SPTSSA is the dominant small subunit compared to SPTSSB in terms of mRNA expression in SH-SY5Y cells.

The small subunit SPTSSA exhibited stronger expression than SPTSSB in SH-SY5Y wt (parental) cells, making it more likely for SPTSSA to form a complex with the other two large subunits compared to SPTSSB. Therefore, my hypothesis was that if we knocked out SPTSSA in the cells, they would need to rely on the expression of SPTSSB, leading to the formation of an active SPT complex consisting of SPTLC1/SPTLC2/SPTSSB. This, in turn, would result in increased production of C20-sphingoid bases. To accomplish this, I used Crispr-Cas9 mediated gene editing using pSpCas9(BB)-2A-GFP plasmid. The presence of GFP as an insert in the plasmid allowed us to identify the cells that successfully had incorporated the plasmid and distinguish them from the non-transfected cells. This method facilitated the FACS sorting of cells for single cell cloning, thereby enhancing the likelihood of obtaining cells with desired modifications and producing monoclonal cells. In addition to single cell sorting, we utilized bulk sorting via FACS. During bulk sorting, we sorted as many GFP-positive cells as possible without any threshold for GFP fluorescence intensity levels. This approach enabled us to cover a wide spectrum of the cell population, encompassing various expression levels for the Crispr-Cas9 construct.

I validated the gene editing at DNA level through Sanger sequencing (only in clonal cells), by investigating the changes in SPT activity (only in clonal cells) and ceramide profile (both in clonal and bulk population). Sanger sequencing of SPTSSA KO clones demonstrated that generated indels in their SPTSSA sequences were not a multiple of three. Therefore, Crispr-Cas9 mediated gene editing resulted in frameshift mutation (Figure 11). The frameshift mutation likely resulted in an altered reading frame, leading to a completely different amino

acid sequence being translated. Unfortunately, due to the unavailability of commercially available antibodies against SPTSSA, I was unable to perform Western blot analysis to further observe its potential effects on the protein level.

The study conducted by Han et. al showed that in the absence of small subunits, SPT complex had a extremely low basal activity (Han et al., 2009). In contrast, overexpression of SPTSSA or SPTSSB together with the large subunit combination of SPTLC1/SPTLC2, the SPT activity reached to its maximal activity. In the same study, they also showed that even though both small subunits boosted up the SPT complex, SPTSSA increased the SPT activity around three times more compared to SPTSSB. This could explain that with a SSA:SSB ratio of about 3:1 I did not detect a 7:1 ratio of C18- over C20-bases in ceramides but a ratio of 19:1. Given this information, we performed SPT activity assay for SPTSSA KO and control clones (Figure 17). The results showed that the enzymatic activity of SPT decreased at least 3-6 fold in SPTSSA KO clones 5C11 and 5D8 compared to control clones while one of the SPTSSA KO clone (4D10) had still 80% residual enzymatic activity as compared to the control clone with the lower activity. This result was a bit surprising, as a general strong decrease in SPT activity had been expected in all clones in the absence of SPTSSA. The paper published by Wang et al., showed that SPTSSA increases the catalytic activity of SPT complex by interacting exclusively with SPTLC2 subunit, which is the catalytic subunit of SPT complex. It keeps the hydrophobic surface of SPTLC2 away from the aqueous environment. Additionally, it helps SPTLC2 to stay attached to the ER membrane and prevent it from diffusing away. In that way, SPTSSA stabilizes SPTLC2 for the formation the active site (Wang et al., 2021). The same study showing the importance of small subunits for the activity of SPT complex mentioned that SPTSSB and SPTSSA are homologous to each other and shares a central domain including the acyl-CoA selection site (Han et al., 2009). This indicates that SPTSSB may bind to the SPTLC2 subunits in a similar way to increase SPT activity. Therefore, even in the absence of SPTSSA, cells might have had a compensatory mechanism to increase the SPTSSB production. Unfortunately, we were unable to detect the effect of SPTSSA KO absence on the expression of SPTSSB via Western blot due to unavailability of suitable antibody. Additionally, it is possible that the clones may have exhibited variation in small subunit expressions even prior to editing. This clonal variation could have contributed to the observed differences in SPT activity.

For further validation of SPTSSA KO in both monoclonal and polyclonal cells, I analyzed the changes in the NS-ceramides containing 18- and 20-carbon sphingoid bases with different fatty acyl chain lengths. Although, both polyclonal and monoclonal SPTSSA KO cells showed a similar acyl-chain composition of the ceramides, the increase in those with a C20-base as compared to controls was more than sevenfold in the monoclonal SPTSSA KO cells, whereas that of polyclonal SPTSSA KO cells was only around threefold compared to wt (parental) cells. This discrepancy may be explained by the bulk sorting procedure. There, the cells were sorted based on the GFP expression, which is linked to Cas9 expression, but not necessarily to frame shift mutation results. Events with precise repair of the modified region, leading to the formation of wild type cells would have been sorted together with potential KO-clones. Furthermore, with an initial retardation in cell proliferation real KO-clones were prone to be overgrown by GFP-positive non-KO clones in the bulk. To exclude these variables diluting the effect of real SPTSSA KO, I choose to continue the experiments using the monoclonal population.

In monoclonal SPTSSA knockout (KO) cells, an increase in ceramides with C20 sphingosine was predominantly observed in combination with C18-, C20-, C22-, and C24-acyl chains, but not with C16-acyl chain. This increase was counteracted in a decrease of ceramides with C18-sphingosine only for the very long acyl chains with C22 and C24 (Figure 14). One hypothesis to explain this effect is that CerS2, which is mainly responsible for the production of C22- and C24- ceramides (Laviad et al., 2008), might accept C20- sphingosine better than those ceramide synthases preferring to incorporate shorter acyl chains. Especially, CerS5 and/or CerS6, which strongly prefer palmitoyl-CoA (C16:0-CoA) to produce ceramides (Mizutani et al., 2005) may disfavor C20- over C18-sphingosine, which could prevents the entry of C16-acyl CoA into ceramides with C20-sphingosine.

In total, 37% of the ceramides contained a C20-sphingosine in the SH-SY5Y SPTSSA-KO clones. This ratio is a bit lower than expected from the specificity and activity of SPTSSB in yeast, which showed a slightly better activity to produce C20-sphingosine than C18-sphingosine (Harmon et al., 2013). However, other factors such as substrate availability and the downstream incorporation of sphingosines with different chain length into ceramides may contribute to these differences.

In addition to sphingolipids analysis, we performed lipidome analysis via semi targeted approach. Lipidome of SPTSSA KO clones showed a considerable variation within their lipid

composition. Despite variation within the clones, we found an altered lipidome in response to SPTSSA KO and detected 25 lipids which were commonly differentially expressed. However, we have yet to fully comprehend the function of altered lipids and the mechanisms behind why and how their concentrations change in response to SPTSSA knockout.

4.2 Characterization of the Effect of SPTSSA Knockout and Corresponding Changes in Sphingoid Base Length on Cell Signaling and Cell Functionality in SH-SY5Y Cell

4.2.1 Proteome and Phosphoproteome of SH-SY5Y Cells were Altered in SPTSSA KO Compared to Control Clones at Steady-State Level

CRISPR-Cas9 mediated SPTSSA KO successfully resulted in generation of SH-SY5Y cells producing increased C20-sphingosine concentrations. To further characterize the effect of changes in LCB chain length on steady state cell status, we conducted three proteome analyses. The first analysis was performed 2-3 passages after the initial generation of SPTSSA KO cells (AK0735), and the second one was conducted after more than 30 passages. In the latter analysis, data were acquired using both data-dependent acquisition (DDA) (AK0800-1) and data-independent acquisition (DIA) (AK0800-2) methods. As data from both AK0735 and AK0800-2 measurements were acquired by DIA, I compared the lists of differentially expressed proteins from the two measurements. This approach allowed me to identify proteins with consistent differential expression between the KO and control groups across multiple measurements, providing valuable insights into the biological effects of the knockout. Out of more than eight thousand proteins, which were identified and quantified, I found five proteins with a persistent differential expression between the KO and control groups across both measurements. All five were upregulated compared to the control group, indicating that their differential expression- as compared to control clones- did not change after passaging cells almost 30 times (Table 30). One of the protein detected was AHNAK (Neuroblast differentiation-associated protein, also known as desmoyokin), which is an exceptionally large scaffolding protein and known to be located mostly in cytosol. However, it is recruited to the plasma membrane via annexin A2/S100A10 complex, where annexin A2 has been suggested to take part in the formation of cholesterol-rich membrane microdomains (Babiychuk & Draeger, 2000; Oliferenko et al., 1999). The study by Benaud et al., highlighted the role of AHNAK in membrane rearrangement, its association with microdomains and possible role in cell signaling (Benaud et al., 2004). The paper followed by Lim et al., in 2013, showed that indeed AHNAK act as an scaffolding protein connected the Erk and Rac activation in response

to platelet-derived growth factor (PDGF) and involved in migration of mouse aortic smooth muscle cells (ASMCs) (Lim et al., 2013). Until today, AHNAK has not directly been associated with sphingolids (i.e. when searching for (AHNAK OR desmoyokin) AND (sphingolipid OR ceramide OR sphingomyelin OR ganglioside OR glycosphingolipid)). However, it interacts with the dysferlin C2A domain in coronary arterial endothelial cells, which is required for FasL-induced lysosome fusion to the cell membrane, ASMase translocation and membrane raft clustering (Han et al., 2012). ASMase translocation may result in production of ceramides at the plasma membrane, which are together with sphingomyeline affected in my cell model by the SPTSSA KO. Previously, it has been suggested that the length and the saturation level of sphingoid bases play an important role in the arrangement and ordering of the lipids. Longer and more saturated chains typically result in increased membrane rigidity, whereas shorter and more unsaturated chains tend to promote membrane fluidity (Quinville et al., 2021). The observed higher levels of AHNAK expression in knockout (KO) cells compared to control cells in the proteome analysis dataset suggests that AHNAK protein may be sensitive to changes in chain length of sphingoid bases or corresponding microdomain stability. Since AHNAK functions at the cellular membranes, participating in microdomain formation and cell signaling, alterations in lipid membrane composition leading eventually to more stable microdomains due to SPTSSA KO could potentially impact its function and prolong its half life.

Another protein that showed a consistent differential expression between the KO and control groups across both measurements was Contactin-associated protein 1 (CNTNAP1). CNTNAP1 has been shown to be one of the γ -secretase-associated proteins (GSAP) which interacts with γ -secretase to form a complex for processing of amyloid precursor protein (APP). It is enriched in the detergent resistant membranes fraction and, can lead to the formation of oligomeric amyloid β -peptide (A β). Accumulation of A β oligomers are suggested to be the main player in starting the cascades of events causing synaptic dysfunction, neuronal death and progression of neurodegenerative disorder of Alzheimer's diseases (Hur et al., 2012). In 2015, Zhao et. al reported a *Sptssb* mutation, referred to as *Stellar* (*Stl*) in mice. This mutation in *Sptssb* was caused by the substitution of histidine with leucine at the 56th residue (H56L) (Zhao et al., 2015). As a consequence, there was an increased production of C20 sphingosine observed in the brains and eyes of mutant mice leading to increased ceramides with C20 sphingosine next to unchanged ceramides with C18 sphingosine. The alteration in sphingoid base length in the mutant mouse brain was associated with neurodegenerative effects. Interestingly, the

change in ceramides with C20-sphingosine pattern observed in that study resembled the data in our project, where significant changes were observed in C18:0, C20:0, C22:0, and C24:0 acyl chain containing ceramides with C20 sphingosine, but not with C16:0 acyl chain. It would be worth while to analyze if changes in CNTNAP1/y-secretase complex contribute to the phenotype of these mice.

A few passages after gene editing, KO cells showed around 1.5 to 3-fold decrease in proliferation rate (Figure 25). The string interaction network analysis and clustering of proteins using the MCL (Markov Cluster) algorithm performed for AK0735 data pointed out the potential alterations occurring during the mitotic cell cycle, especially in G2/M transition of mitotic cell cycle (Figure 23). The proteomics data associated with the proteins, which were part of the cluster related to cell cycle, were downregulated 2-3 fold in SPTSSA KO cells compared to control (Table 30). This finding aligns with the possibility that the downregulation of cell cycle-related proteins could have contributed to the observed changes in proliferation rate. Building on these data, I initiated a cell cycle analysis, revealing that a significant portion of the KO cells were in the G0/G1 phase, indicating that these cells were not actively preparing to divide. This suggested that more cells might have been arrested in either the G0/G1 or G2/M phases. Ceramides are known to play crucial roles in apoptosis, proliferation, and cell growth. The shift in the equilibrium between ceramides of various fatty chain lengths has been demonstrated to alter cellular physiology, leading to either inhibition of cell proliferation followed by induction of apoptosis or stimulation of cell proliferation (Hartmann et al., 2012). The SPTSSA KO did not cause a significant impact on the total amount of ceramides with various fatty chain lengths with the exception of palmitic acid. It did not appear in ceramides with C20 sphingosine. Instead, it resulted in a shift in the sphingoid base profile. Even though we did not fully understand the mechanism by which the shift in sphingoid base chain length induced alterations in proliferation, we once again confirmed the importance of maintaining ceramide homeostasis at equilibrium. Additionally, it was interesting to observe that cellular proliferation was the first induced and noticeable phenotypical change in response to the SPTSSA KO. However, after over 30 passages, the SPTSSA KO cells adapted to the KO and evolved towards a more proliferative phenotype (Figure 41). Our chosen cell culture model was a neuroblastoma cell line. Cancer cells demonstrate a notable metabolic flexibility and can readily adapt to changes in nutrient availability (Grasmann et al., 2021). The SPTSSA KO clones grew in FBS supplemented media, which was rich in survival signals such as growth

factors and cytokines. This might have helped KO cells to persist and continue to live. The sustained survival signals could have eventually led to epigenetic changes and/or alterations in associated signaling events, enabling KO cells to transition from a slow proliferating state to a rapidly proliferating cancerous state.

After performing the second proteome analysis (AK0800-1 and AK0800-2), the cell cycle-related proteins were no longer among the top differentially regulated proteins between knockout and control cells. This was one of the first indication that the cell had diverged from their initial phenotype over time. However, despite differences in the ranking of proteins in the top differentially regulated list, we still observed the same proteins being regulated in the same direction (e.g., consistently downregulated or upregulated). One of the protein, Integrin alpha-2 (ITGA2), was upregulated in both measurements in SPTSSA knockout (KO) cells. Despite our inability to validate the changes obtained from proteome analysis via Western blotting due to statistical insignificance caused by clonal variation, we observed initially and after 30 passages an increases ITGA2 expression in KO clones by Western blot, consistent with the proteomics findings. Of note the relative expression of ITGA2 within SPTSSA knockout (KO) clones also changed over time with several passages (Figure 22A and Figure 28). This also supported the fact that the cells underwent changes.

ITGA2 is part of the transmembrane integrin transmembrane receptors called called Integrin alpha-2/beta-1. This integrin receptor binds both to extracellular matrix components like collagen or fibronectin and brings the large focal adhesion protein complex together. By linking extracellular matrix to the actin cytoskeleton, integrins convey the signals inside the cell to mediate cell processes including spreading and migration. Membrane microdomains play a crucial role in integrin mediated cell adhesion to the extracellular matrix by both affecting the integrin structure and providing a large signaling platforms to concentrate large protein complexes (Lietha & Izard, 2020). The STRING analysis of proteome data (AK0800-2) provided additional clues about potential changes in cell adhesion, as it revealed two related Gene Ontology (GO) terms: "Cadherin binding" and "Cell adhesion molecule binding". Therefore, I conducted a cell adhesion assay as show in Figure 29. The results, however, remained inconclusive due to variation within both control and KO clones.

The proteome of the SPTSSA knockout (KO) and control cells were reanalyzed in project AK0800-1 using a different acquisition method compared to AK0800-2. The first

employed Data-Dependent Acquisition (DDA), while the second utilized Data-Independent Acquisition (DIA). I conducted the same analysis I did for AK0800-2. However, the top differentially regulated proteins in the collective analysis for both projects were different, except for AHNAK which appeared in both lists (Table 32 and 33). From the list of proteins that we differentially regulated between SPTSSA KO and control groups, I picked two proteins (MT-CO2 and CRABP1) for further validation via Western blot. Unlike other potential candidates that were selected from project AK0735, I successfully confirmed the observed protein expression changes of these two proteins through Western blot analysis. The potential significance of changes in MT-CO2 expression is discussed in a later section.

In addition to proteome analysis, I performed phosphoproteome analysis in project AK0800-1. Subsequently, I performed kinase enrichment analysis to find the kinases potentially activated or inhibited based on the patterns of the proteins and the associated phosphosites that were differentially regulated in project AK0800-1. Only two kinases (TAK1 and NEK8) passed the predefined filters as explained in section 3.4.2 and they were predicted to be downregulated. TAK1 is a mitogen-activated protein kinase kinase kinase (MAP3K) and TAK1 signaling has been known to influence cell fate by integrating its multiple pathways involved in both cell survival and cell death (Mihaly et al., 2014). Its activation has been shown to be stimulated by C2-ceramides and it has been suggested that TAK1 acts as mediator of ceramide signaling to SAPK/JNK (Shirakabe et al., 1997). A study by Nica et. al., showed that C6-ceramide promoted JNK activation and induced its major downstream target transcription factor AP-1 (c-JUN) phosphorylation in K562 cells (Nica et al., 2008). Similarly, C2-ceramide treatment of the mouse embryonic cortical neurons was shown to induce JNK pathway and increase phosphorylation of c-Jun at serine 73 (Willaime-Morawek et al., 2003). The common observation in the literature is that short chain ceramides lead to the induction of JNK pathway. However, our phosphoproteome dataset showed that phosphorylation of c-Jun at serine 63 was downregulated at steady state level without any external stimuli in KO clones (Supplementary Table 7). Additionally, the signaling pathway starting from TAK1 was predicted to be downregulated in response to SPTSSA KO at steady state. Taken the literature into account, this observation might have resulted from changes in the proportion of C20- over C18-long-chain bases in ceramides and other sphingolipids.

4.2.2 Effect of SPTSSA KO on EGFR Signaling

The EGF receptor belongs to the family of receptor tyrosine kinases (RTKs). Gangliosides, which are sialic acid containing glycolipids with a hydrophobic ceramide anchor, have been described to fine tune the signaling strength of such tyrosine kinase receptors in the plasma membrane (Julien et al., 2013). In case of the EGFR, GM3 suppresses EGFR signaling through an interaction of the oligosaccharide head group of GM3 with a N-glycosylation of the EGFR close to the membrane (Yoon et al., 2006). Whether such modulations can be fine tuned by the composition of the gangliosides' ceramide anchor, i.e. the length of its sphingoid base, has not been investigated until now and was a question raised in this thesis. The phosphoproteomic analysis revealed no significant alterations in EGFR phosphorylation at various phosphosites in SPTSSA KO cells under normal conditions. This opened up the opportunity to investigate whether EGFR signaling through addition of EGF could potentially be altered by the length of sphingoid base chains within the microdomain encircling the EGF receptor in the plasma membrane. Any observed changes could be attributed to the altered lipid composition rather than pre-existing alterations in phosphorylation status of the signaling components. I tested and refined the EGF stimulation protocol selecting phosphosite tyrosine 992 (Y992) of EGFR as a marker, focusing on optimizing both the concentration of EGF and the duration of stimulation to achieve maximum phosphorylation levels. This optimization was carried out using SH-SY5Y wt (parental) cells, as we observed high clonal variability within both SPTSSA KO and control groups. I selected the working condition for EGF stimulation as 12.5 ng/ml concentration and 10 minute stimulation period. I chose different phosphorylation site including tyrosine 992 (Y992), tyrosine 1173 (Y1173) and tyrosine (Y1069), which are associated with different signaling pathways and their two downstream targets as explained in section 3.5.2. Overall, we did not observe any differences in strength of the induced phosphorylation patterns of the selected EGFR phosphosites and their two associated downstream targets after normalization to their corresponding untreated values. Due to high variability within the groups, small differences of EGF stimulation at this specific time point between KO and control clones can not be excluded. I used the same protocol which is optimized for tyrosine 992 (Y992) in SH-SY5Y wt cells for exploring the changes in different EGFR phosphorylation sites including tyrosine tyrosine 1173 (Y1173) and tyrosine (Y1069). However, the initiation and termination of signaling cascades do not happen in a single event but rather occur with individual time periods (Salazar-Cavazos et al., 2020). Moreover, the timing of phosphorylation for each phosphosite on EGFR may vary in SPTSSA KO clones

compared to control clones, highlighting the need for enhanced EGFR-mediated time course experiments using both SPTSSA KO and control clones. While we focused on only three phosphosites in this study, it is important to note the presence of numerous other phosphosites on EGF receptors (Wee & Wang, 2017). Therefore, utilizing EGFR phosphorylation arrays in future studies may provide valuable insights. Therefore, it is possible that in our work, we may have missed the specific phosphosite affected by SPTSSA KO. Further optimization of experimental parameters or exploration of alternative treatment strategies may be necessary to fully understand the potential effects of SPTSSA KO on EGF signaling.

4.3 Differentiation of SH-SY5Y wt (parental), SPTSSA KO and Control Clones into Mature Neuron-Like Cells

4.3.1 Alterations in NS-Ceramide Profile and SPT Activity upon Differentiation of SH-SY5Y wt (Parental) Cells into Mature Neuron-Like State

Our main goal was to understand if SPTSSA KO would affect differentiation of the neuroblastoma cells into neuron-like cells. After successfully optimizing the differentiation protocol with the parental cell line, I analyzed the distribution of NS-ceramides containing 18- and 20-carbon sphingosine with various acyl-chain length in these cells at the end (7 days of induction) of the differentiation protocol. In contrast to the aging phenomenon of increased brain ganglioside with C20-sphingosine content over life time (Rosenberg & Stern, 1966; Sonnino & Chigorno, 2000) NS-ceramides with C20-sphingosine decreased more than 4 fold in differentiation media with or without 10 μ M ATRA as compared to controls (Figure 44). Additionally, the quantity of ceramides with long acyl chains (C16 – C20) increased compared to those with very long acyl chains (C22 – C24) cell differentiation in cell culture (Figure 45). This effect is the opposite of what is observed upon deleting SPTSSA, where the production of C22- and C24- ceramides with C20-sphingosine increased. Neurons preferentially express CerS1, which facilitates production of ceramides with C18:0 acyl chains, whereas oligodendrocytes rather express CerS2 leading to incorporation of C22- and C24-acyl chains (Tidhar & Futerman, 2013). The acyl pattern shift of ceramides during cell culture differentiation as well as upon deletion of SPTSSA might be explained by the relative expression of CerS2 over other CerS, if CerS2 would accept ceramides with C20- sphingosine better than those ceramide synthases preferring to incorporate shorter acyl chains.

The differentiation dependent change in the ratio of NS-ceramides with C20- over C18- indicated a potential shift in expression the composition of the SPT complex and its activity.

Therefore, I analyzed the mRNA levels of SPT subunits by qPCR (Figure 46). When the cells were treated with B27 containing neurobasal media, the expression of SPTSSA increased while SPTSSB expression decreased. B27 treatment alone did not affect the SPTLC1 expression while it led to an increase in SPTLC2 expression. A similar trend was observed in cells treated with B27+ATRA, except for SPTLC1 whose expression was increased only upon B27+ATRA treatment. The alteration in the expressions of SPT complex subunits led me to determine the SPT activity. In 21 hours differentiation experiment, the SPT activity was decreased only in the cells treated with 1X B27 supplemented neurobasal media without ATRA (Figure 47). On the other hand, addition of 10 μ M ATRA to B27 supplement brought the SPT activity level similar to that of the cells cultured in normal growth media (DMEM/F12). This decreased SPT activity may not be explained solely by the changes in large subunits expressions over B27 $\pm$ ATRA treatment. This is because several studies have demonstrated that SPTLC1 is not the limiting factor for SPT activity, and its primary role has been shown to be supportive rather than catalytic (Gable et al., 2002; McCampbell et al., 2005; Wang et al., 2021). Even though treatment with B27 $\pm$ ATRA resulted in increased expression of SPTLC2, which is the catalytic subunit of SPT complex, the observed increase of SPTLC2 mRNA was similar in both conditions, B27 alone and in combination with 10 μ M ATRA treatment. This left us with two possibilities. Firstly, the SPTSSA to SPTSSB ratio might not be the same after treatment with 1X B27 with or without ATRA. Secondly, the different treatment methods might have modulated negative regulators, such as ORMDLs of SPT, in different ways leading to a decrease in activity of SPT.

4.3.2 Induction of Differentiation in SPTSSA KO Cells Led to Reduced Cell Viability

I tried to differentiate SPTSSA KO cells with the same protocol that I used for SH-SY5Y wt (parental) cells. My aim was to understand whether they still have the ability to differentiate into mature neuron-like state. I monitored the cell viability and differentiation of SPTSSA KO and control cells by crystal violet staining and live cell imaging by using the IncuCyte[®] system. The results for two different experimental settings reached the same conclusion. When SPTSSA KO cells were treated with 1X B27 only, the cell viability decreased over time in contrast to the control cells. This difference in cell viability between SPTSSA KO and control cells reached significance five days after initiation of differentiation (Figure 48 and 49). The addition of ATRA to the media partially alleviated this decrease, but only to a limited degree, indicating a partial restoration of the phenotype by 10 μ M ATRA.

Even though treatment conditions induced cell death in all SPTSSA KO cells, one (clone 5D8) out of three KO clones showed a slightly milder phenotype. This led me to investigate the underlying cause of the induced cell death. An hypothesis was that the cells were undergoing cell death due to reduced SPT activity, resulting from SPTSSA KO, potentially leading to an inadequate environment for normal growth under the treatment condition. However, SH-SY5Y wt (parental) cells treated with 1X B27 alone exhibited decreased SPT activity but continued to grow and proliferate. This led me to consider that the extent of reduction in SPT activity might have been the cause of cell death. In other words, SPTSSA KO cells already displayed decreased SPT activity, and treatment with B27 further reduced the activity to a level where the cells can no longer cope with the changes, ultimately died. This hypothesis, however, can not explain the fact that 4D10, which had the highest SPT activity among other SPTSSA KO clones under control conditions showed the most severe phenotype in terms of cell viability while 5D8 cells, which had one of the lowest SPT activity resist to B27 treatment and showed a better phenotype (Figure 17). While we cannot rule out the possibility that reduced SPT activity may contribute to cell death, we cannot rely on solely this explanation, and there may be other factors at play as well.

B27± ATRA treated SH-SY5Y wt (parental) cells showed 4 fold decrease in the level of NS-ceramides with C20-sphingosine compared to DMEM/F12 control. Also, differentiating cells tended to increase the level of ceramides with long acyl chains (C16 – C20) compared to those with very long acyl chains (C22 – C24). The main reduction in the level of NS-ceramides with C20-sphingosine came from the ceramides with C22-C24 chain. However, the impact of SPTSSA knockout resulted in the opposite effect, leading to an increase in C22 and C24 ceramides with C20-sphingosine. Hence, the already elevated concentration of C22-C24 ceramides with C20-sphingosine resulting from SPTSSA knockout might be a contributing factor to cell death. Additionally, with B27 induced media, cells may not be able to reduce C22-C24ceramides with C20-sphingosine levels to a point where proper neuronal cell function can occur. Moreover, we observed an increase in the mRNA expression of SPTSSA and a decrease in SPTSSB with 1X B27 treatment. However, SPTSSA KO cells lack functional SPTSSA, thereby this hinders their ability to increase SPTSSA levels where necessary. Overall, the exact reason why the SPTSSA KO cells choose cell death over proliferation is still unclear, but the concentration and eventually subcellular localization of certain ceramide species might be a contributing factor.

4.3.3 Mitochondrial Function was Altered in SPTSSA KO Clones Compared to Control

4.3.3.1 Expression of Proteins Associated with Mitochondrial Function Decreased in SPTSSA KO Cells

In section 3.4.1.6, I performed collective comparison for the proteome analysis obtained from project AK0800-1. This analysis yielded four proteins which were differentially expressed between KO and controls. One of the top differentially downregulated protein was Mitochondrially Encoded Cytochrome C Oxidase II (MT-CO2) (Table 33), I could validate by Western blot that the expression of MT-CO2 indeed significantly decreased in SPTSSA KO clones compared to control. The variation among the SPTSSA KO clones still visible as clone 5D8 showed relatively strong expression compared to other two KO clones (Figure 33). However, overall, the difference in MT-CO2 expression between control and KO clones was around 80% on average. I also performed the string interaction network analysis and clustered protein using the MCL (Markov Cluster) algorithm. The results indicated potential alterations occurring within the mitochondrial electron transport chain (Figure 31). The proteins associated with this clustering revealed that mitochondrial respiratory chain complexes (I, III and IV) appeared to be affected in different direction. Specifically, proteins within complexes I and III exhibited upregulation, while complex IV related proteins showed downregulation (Table 34). Based on this knowledge, I further tried to validate the expression of proteins which appeared in proteome analysis and were involved in mitochondrial respiration through Western blot. I chose UQCRC2 as a second candidate since it is a core protein subunit of Complex II. Consistent with proteome data, Western blot analysis showed the upregulation of UQCRC2 expression in KO cells compared to control.

These results led me to think the mitochondria related changes as the main cause of differentiation induced cell death. The study conducted by Schneider et al. showed that retinoic acid induced differentiation of SH-SY5Y change the mitochondrial bioenergetics. They revealed that differentiated SH-SY5Y cells exhibited an increased spare respiratory capacity, suggesting that Complex IV contributed to this enhancement (Oliva et al., 2010; Schneider et al., 2011). Increased reserve capacity is important as it contributes to the defense mechanism against oxidative stress (Dranka et al., 2010). They also proposed that complex IV might enhance the efficiency of the electron transfer chain (Schneider et al., 2011). As in SPTSSA KO cells, the expression of MT-CO2, which is the core catalytic subunit of complex IV, was downregulated, KO cells may lack sufficient reserve capacity to rapidly increase ATP

production via oxidative phosphorylation when induced for differentiation. Therefore, I suggest to further investigate the mitochondrial oxygen consumption rates including the spare respiratory capacity of the SPTSSA KO clones upon differentiation in an Seahorse experiment.

4.3.3.2 Mitochondrial Membrane Potential Increased in SPTSSA KO Cells upon Induction of Differentiation

The proteome and Western blot analysis pointed out the mitochondrial dysfunction in SPTSSA KO cells at steady state level. In order to test potential changes in mitochondrial function upon induction of differentiation, we performed assays to measure mitochondrial potential and mitochondrial mass. Although both SPTSSA KO and control cells had relatively similar membrane potential when they were cultured in DMEM/F12, induction of differentiation with or without 10 μ M ATRA caused an increase in the membrane potential after 21 hours. The increase was around 1.5-2 fold higher in KO cells compared to control (Figure 51). The results were statistically significant ($p=0.07$) at a significance level of $p\leq 0.1$. Likewise, induction of differentiation caused a slight increase in mitochondrial mass in control and SPTSSA KO cell clones without any statistically significance between the two groups.

These results suggested that the increased mitochondrial potential observed in SPTSSA KO cells was not only caused by an increase in mitochondrial mass. One explanation might be an impaired electron transport chain in KO clones. As discussed in previous section that clustering of proteins using MCL (Markov Cluster) algorithm revealed that mitochondrial respiratory chain complexes (I, III and IV) might have been affected in different direction. Table 34 shows all the proteins present in the clustering analysis. Based on that the proteins involved in complex I and III were upregulated and complex IV were downregulated. When the cells are in need of high energy like in the case of neuronal differentiation, they increase ATP production via oxidative phosphorylation which relies on active functioning of OXPHOS complexes. Even though, in SPTSSA KO cells, complex IV protein (MT-CO2) was downregulated, they still have other components of the electron transport chain which were upregulated. This means that other complexes still continue to pump protons into the intermembrane space and can generate a higher proton gradient across the inner mitochondrial membrane. Therefore, increased membrane potential detected in SPTSSA KO cells compared to the control after induction with differentiation media might have been caused by the increased expression and activity of complex I and III proteins.

However, these finding still do not explain the reason why SPTSSA KO cells undergo cell death upon induction of differentiation and how this might be connected to the changes in the sphingoid base chain length. The partial explanation would be as following. The mitochondrial lipids are mostly synthesized in the ER and transferred to other organelles through vesicles or transport proteins for example at the ER-mitochondria contact site. This process is especially important for organelle communication and regulation of cell signaling. The study by Aaltonen et. al., showed that SPTLC2 on mitochondrial outer membrane interacts in trans and makes a complex with SPTLC1 on the ER membrane. They showed that this interaction is important to supplement mitochondria with sphingolipids (Aaltonen et al., 2022). In this paper, they did not investigate the role of small subunits and how they possible contribute to SPT complex formation in ER-mitochondria contact site. As SPTSSA KO changed the distribution of total NS-ceramides with C20-sphingosine within the cells, these produced ceramides may have been transferred to the mitochondria and changed the distribution of lipid composition of mitochondria. The change in the composition of mitochondrial sphingolipids eventually could have caused mitochondrial dysfunction which could be investigated in future by subcellular fractionation under differentiation conditions.

5. CONCLUDING REMARKS AND FUTURE DIRECTION

In this project, I followed the SPTSSA KO cells over 30 passages and investigate the cellular changes and their adaptation to the KO over time. Although, I could be able to catch the changes at an early stage after SPTSSA KO, I still had to wait until single SPTSSA KO cells grew into monoclonal population to start experiments. Therefore, I could not detect the changes happening in a short period of time after the editing of SPTSSA gene, which I think is a major drawback. Therefore, I suggest in the future studies to use an inducible CRISPR-Cas9 systems, which would allow to capture rather instant changes occurring in the cells. This is especially important to follow the alternations in the cell signaling events. Additionally, throughout the project, I faced the challenge that there was a variation within both SPTSSA KO and control clones which might have made me miss significant changes in cellular events. Therefore, to minimize clonal effect in the future, wild type cells can be sorted as single cells to generate monoclonal population prior to genomic manipulation. This might help to reduce phenotypic variability after gene editing.

I characterized the effect of SPTSSA KO and associated changes in the sphingoid bases on cellular events by utilizing multiomics approaches and assays for cell signaling, cell adhesion, proliferation and mitochondrial function. However, there are still open questions that need to be addressed. We still have to characterized the changes in mitochondrial function and bioenergetics by other methods, for example by Seahorse assay both at steady state and especially after induction of differentiation. Likewise, evaluating the mitochondrial size, cristae ultrastructure and the changes in mitochondria-ER interactions via electron microscope would enhance our knowledge about possible effects of SPTSSA KO. In this study, we did not investigate the changes in ER structure or function, but ER is the main site where active SPT complex forms and sphingolipid synthesis starts. Also, the cell death we observed in SPTSSA KO upon stimulation with differentiation media might be initially cause by ER stress in SPTSSA KO clones. Therefore, it would be interesting to investigate the effect of SPTSSA KO on ER function and morphology in both steady state and upon induced differentiation. In line with this, finding out the type of cell death that SPTSSA KO cells undergo upon induced differentiation, adds an invaluable information for understanding the mechanism.

In summary, experiments performed in this thesis strongly indicate that differentiation of neuroblastoma cells into neuron-like cells and mitochondrial homeostasis requires fine tuned

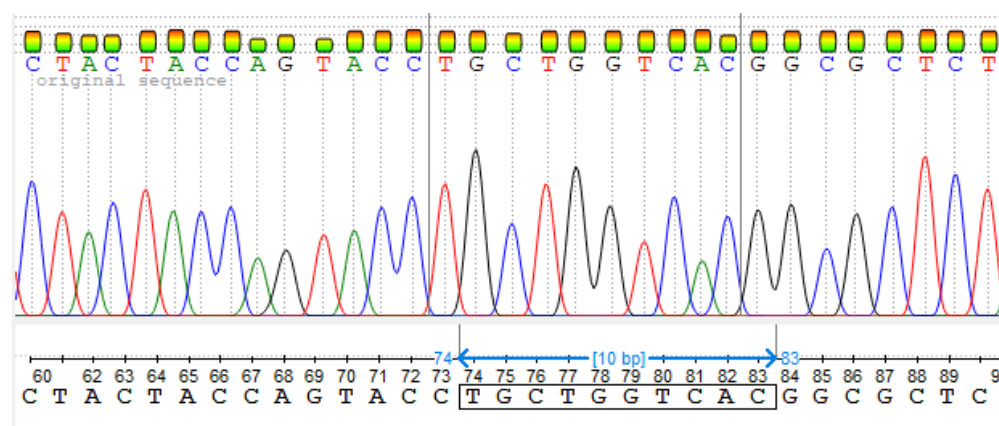
sphingolipid metabolism and a proper ratio of sphingolipids containing sphingosine with C18- and C20-sphingosine.

6. CONTRIBUTION

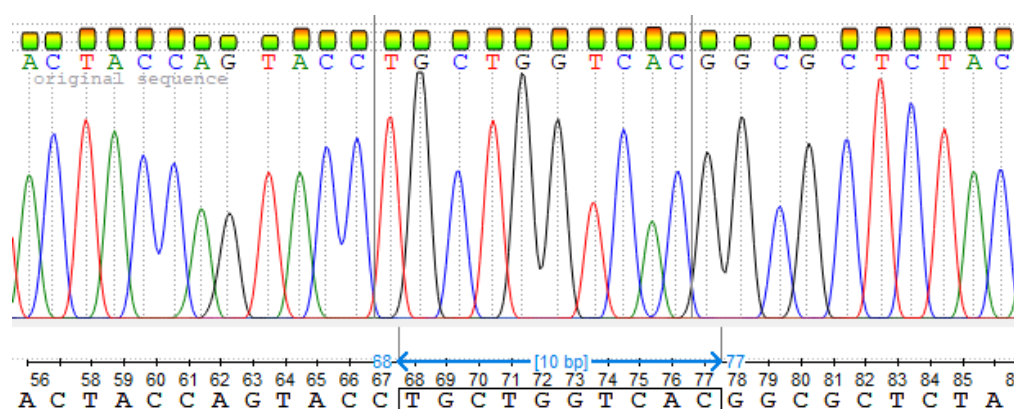
The project involved extensive experimentation, data analysis, and quantification, all of which were conducted by myself unless stated hereafter. Dr. Mariona Rabionet (Research Group Lipid Pathobiochemistry, DKFZ) led the reactions for the SPT activity and collaborated with Martina Volz (Research Group Lipid Pathobiochemistry, DKFZ) on the lipid extraction process. Additionally, Dr. Rabionet and Dr. Robert Engel performed the LC-MS/MS analysis, contributing significantly to data acquisition and interpretation. Martin Schneider (Proteomics Core Facility, DKFZ) played a key role in bioinformatics analysis, particularly for proteomics studies, enhancing the depth of data analysis. Dr. Dominic Helm (Proteomics Core Facility, DKFZ) provided valuable guidance throughout the experimental setup and interpretation of results, ensuring the project's coherence and direction. FACS measurements were performed by Richard Jennemann (Research group Lipid Pathobiochemistry, DKFZ), contributing essential data to the overall analysis and conclusions. Dr. Adriano B. Chaves-Filho (Research group Tumour Metabolism and Microenvironment, DKFZ) meticulously conducted the lipidome and polar metabolite analysis presented in this research. His expertise in data acquisition and evaluation significantly enriched our study, enabling us to comprehensively explore the intricate metabolic pathways. Last but not least, PD Dr. Roger Sandhoff (Research group Lipid Pathobiochemistry, DKFZ) actively influenced the scientific discussion and data interpretation. His insightful comments and expertise were pivotal in directing the course of our research and ensuring a comprehensive and robust analysis of the findings. Together, these contributions advanced the project's objectives effectively.

7. SUPPLEMENTARY DATA

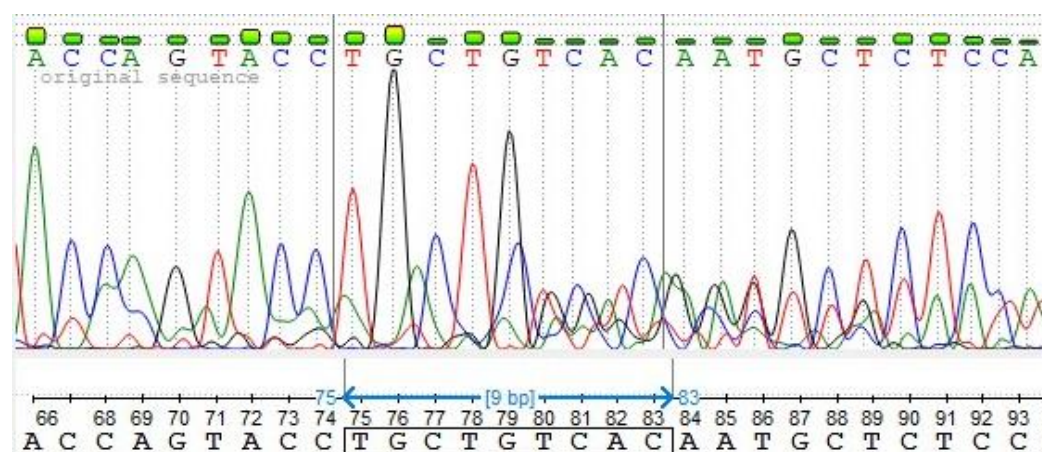
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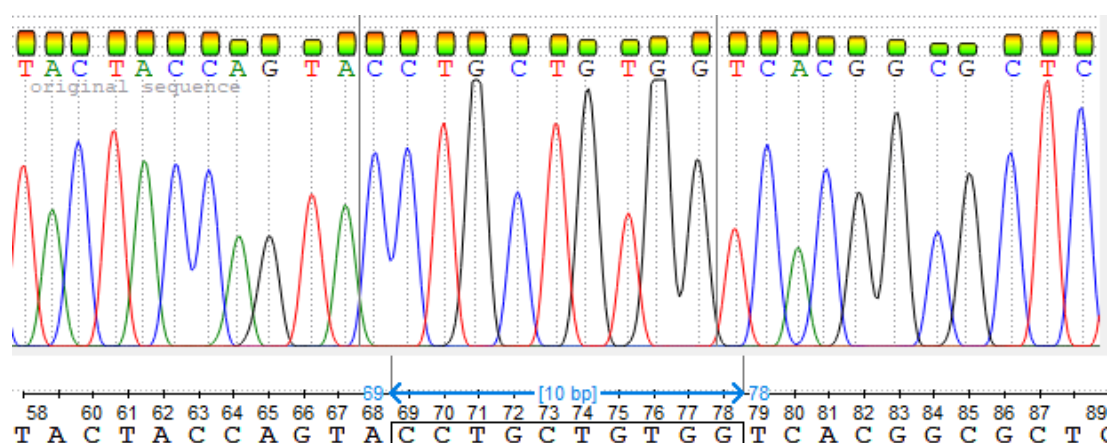
Supplementary Figure 1. Chromatogram analysis of amplified PCR product of SPTSSA gene from clone 1F5



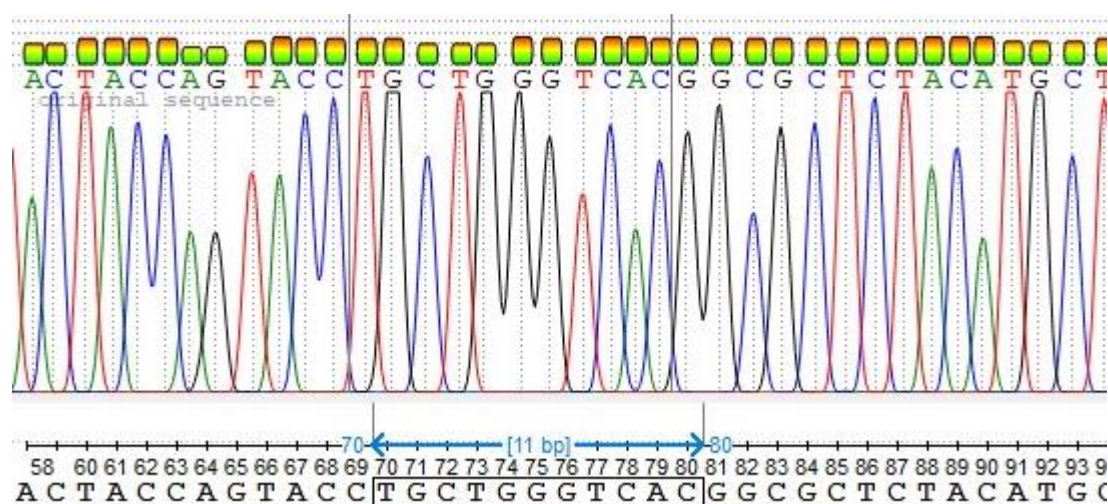
Supplementary Figure 2. Chromatogram analysis of amplified PCR product of SPTSSA gene from clone 2G9



Supplementary Figure 3. Chromatogram analysis of amplified PCR product of SPTSSA gene from clone 5C11



Supplementary Figure 4. Chromatogram analysis of amplified PCR product of SPTSSA gene from clone 4D10



Supplementary Figure 5. Chromatogram analysis of amplified PCR product of SPTSSA gene from clone 5D8

7.2 Proteome Analysis

7.2.1 AK0735

Supplementary Table 1. The list of upregulated proteins from the AK0735 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Log fold change and adjusted p-values were calculated based on imputed data

<i>Upregulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
ITGA2	Integrin alpha-2	2.47	4.57E-07
MVP	Major vault protein	2.43	1.93E-04
AHNAK	Neuroblast differentiation-associated protein AHNAK	2.17	2.27E-04
PLEKHA5	Pleckstrin homology domain-containing family A member 5	2.13	3.79E-05
DCLK1	Serine/threonine-protein kinase DCLK1	2.08	3.06E-06
SEMA3C	Semaphorin-3C	1.99	5.92E-05
PRKCA	Protein kinase C alpha type	1.98	6.41E-05
EPPK1	Epiplakin	1.85	2.82E-03
RELN	Reelin	1.83	7.63E-03
AKR1C1	Aldo-keto reductase family 1 member C1	1.81	1.10E-03
FAM114A1	Protein NOXP20	1.74	5.39E-06
TMTC2	O-mannosyl-transferase	1.71	3.93E-06
OGDHL	2-oxoglutarate dehydrogenase-like, mitochondrial	1.68	4.58E-04
PBXIP1	Pre-B-cell leukemia transcription factor-interacting protein 1	1.67	1.78E-03
PYGL	Glycogen phosphorylase, liver form	1.67	3.10E-05
ASL	Argininosuccinate lyase	1.56	3.10E-05
TPM1	Tropomyosin 1 (Alpha), isoform CRA_m	1.53	6.94E-04
UGGT2	UDP-glucose:glycoprotein glucosyltransferase 2	1.52	1.08E-02
MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	1.50	1.74E-02
ISOC1	Isochorismatase domain-containing protein 1	1.47	6.65E-05
TP53I3	Quinone oxidoreductase PIG3	1.43	1.83E-04

(continued)

Supplementary Table 1 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	1.40	3.68E-05
MB21D2	Nucleotidyltransferase MB21D2	1.39	1.97E-03
AGL	Glycogen debranching enzyme	1.39	4.43E-06
FLNC	Filamin-C	1.38	4.67E-06
MAN2A1	Alpha-mannosidase 2	1.38	2.72E-04
CNTNAP1	Contactin-associated protein 1	1.35	3.47E-03
VAT1L	Synaptic vesicle membrane protein VAT-1 homolog-like	1.35	4.41E-04
RTN1	Reticulon-1	1.34	2.87E-03
IQGAP2	Ras GTPase-activating-like protein IQGAP2	1.34	2.78E-03
CPS1	Carbamoyl-phosphate synthase [ammonia], mitochondrial	1.32	3.12E-03
ARHGEF28	Rho guanine nucleotide exchange factor 28	1.30	5.00E-03
PTPRE	Receptor-type tyrosine-protein phosphatase epsilon	1.29	3.08E-03
SARDH	Sarcosine dehydrogenase, mitochondrial	1.29	9.70E-06
AKAP12	A-kinase anchor protein 12	1.29	3.52E-04
MYO1D	Unconventional myosin-Id	1.27	3.06E-06
PPFIBP1	Liprin-beta-1	1.26	5.66E-05
PLXNA4	Plexin-A4	1.26	1.15E-04
SLC27A6	Long-chain fatty acid transport protein 6	1.25	6.99E-08
SH3GLB1	Endophilin-B1	1.24	1.25E-04
PPFIBP2	Liprin-beta-2	1.22	2.36E-03
TJP2	Tight junction protein ZO-2	1.22	1.84E-04
ADAMTS19	A disintegrin and metalloproteinase with thrombospondin motifs 19	1.22	3.00E-03
ATP2B1	Plasma membrane calcium-transporting ATPase 1	1.21	1.66E-04
PRSS12	Neurotrypsin	1.21	3.60E-03
DOCK5	Dedicator of cytokinesis protein 5	1.20	1.33E-04

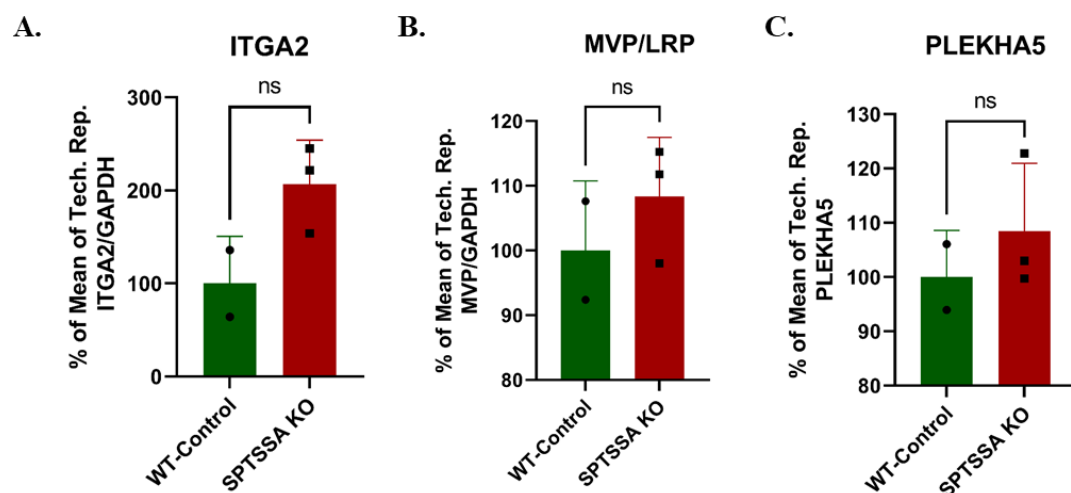
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Supplementary Table 1 (continued)

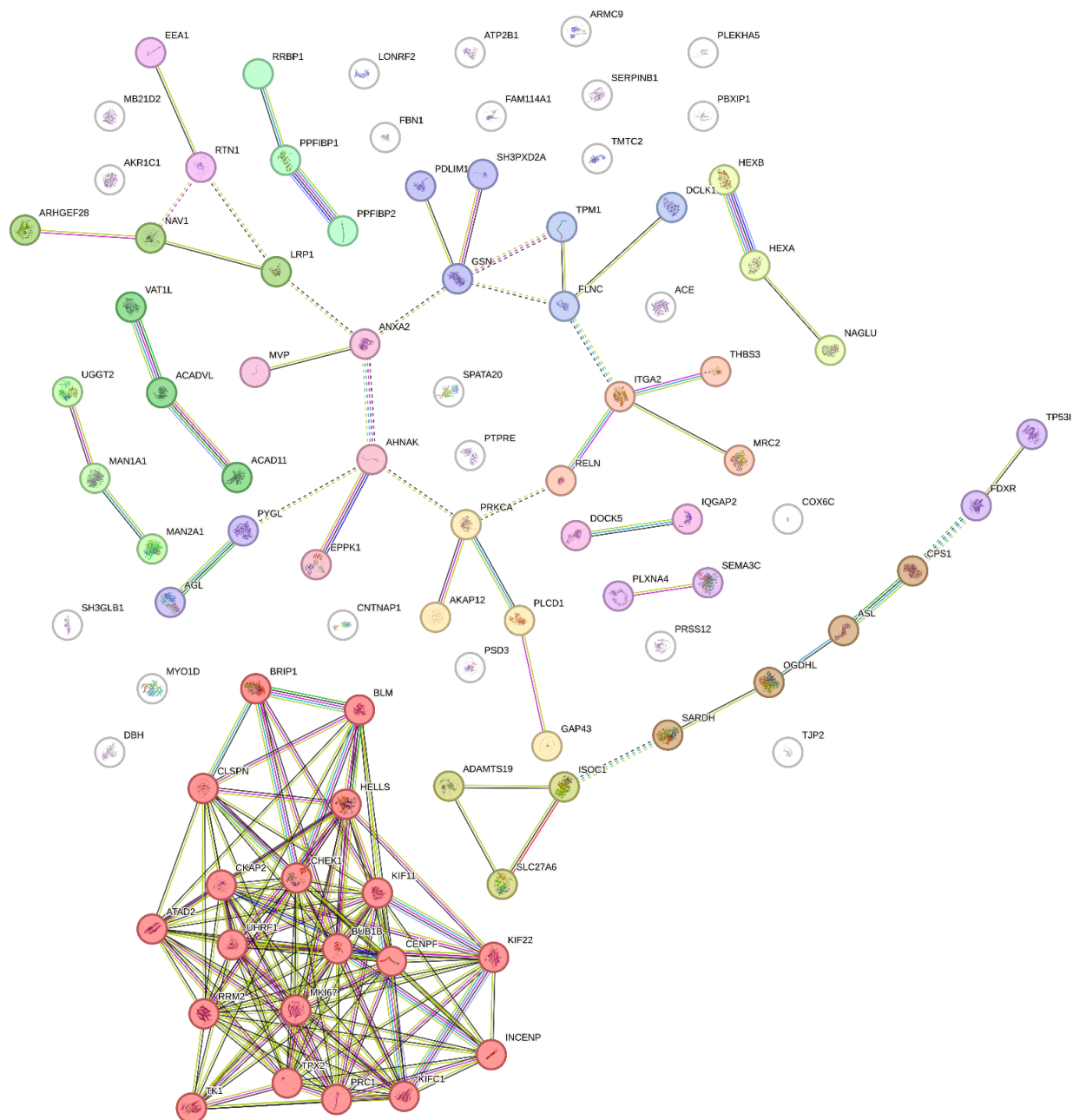
Gene names	Protein names	Log ₂ FC	Adj p-val
NAGLU	Alpha-N-acetylglucosaminidase	1.20	4.15E-03
HEXB	Beta-hexosaminidase subunit beta	1.18	3.53E-04
PLCD1	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1	1.17	4.25E-04
LONRF2	LON peptidase N-terminal domain and RING finger protein 2	1.17	2.04E-04
PDLIM1	PDZ and LIM domain protein 1	1.11	3.28E-03
GAP43	Neuromodulin	1.11	3.80E-02
FDXR	NADPH:adrenodoxin oxidoreductase, mitochondrial	1.09	2.22E-03
ACE	Angiotensin-converting enzyme	1.08	1.63E-03
HEXA	Beta-hexosaminidase;Beta-hexosaminidase subunit alpha	1.08	1.12E-03
SPATA20	Spermatogenesis-associated protein 20	1.07	5.12E-04
SERPINB1	Leukocyte elastase inhibitor	1.07	7.26E-05
RRBP1	Ribosome-binding protein 1	1.06	5.15E-03
THBS3	Thrombospondin-3	1.06	2.12E-02
NAV1	Neuron navigator 1	1.06	3.31E-02
ACAD11	Acyl-CoA dehydrogenase family member 11	1.06	2.77E-02
GSN	Gelsolin	1.05	2.07E-03
LRP1	Prolow-density lipoprotein receptor-related protein 1	1.05	3.47E-03
EEA1	Early endosome antigen 1	1.04	1.73E-04
ARMC9	LisH domain-containing protein ARMC9	1.03	3.65E-06
SH3PXD2A	SH3 and PX domain-containing protein 2A	1.03	1.82E-06
FBN1	Fibrillin-1	1.03	1.60E-03
ANXA2	Annexin A2	1.01	2.92E-04
PSD3	PH and SEC7 domain-containing protein 3	1.01	1.11E-03
MRC2	C-type mannose receptor 2	1.00	6.44E-03

Supplementary Table 2. The list of downregulated proteins from the AK0735 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq -1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Log fold change and adjusted p-values were calculated based on imputed data

<i>Downregulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
BUB1B	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	-1.01	3.78E-03
ATAD2	ATPase family AAA domain-containing protein 2	-1.01	1.20E-02
BRIP1	Fanconi anemia group J protein	-1.02	1.26E-02
KIF11	Kinesin-like protein KIF11	-1.05	1.45E-02
BLM	RecQ-like DNA helicase BLM	-1.07	7.15E-03
CHEK1	Serine/threonine-protein kinase Chk1	-1.08	1.16E-02
TK1	Thymidine kinase, cytosolic	-1.09	4.21E-02
MKI67	Proliferation marker protein Ki-67	-1.09	4.00E-02
UHRF1	RING-type E3 ubiquitin transferase	-1.11	1.30E-02
KIFC1	Kinesin-like protein KIFC1	-1.12	1.28E-02
CENPF	Centromere protein F	-1.14	1.69E-02
HELLS	Lymphoid-specific helicase	-1.21	9.18E-03
RRM2	Ribonucleoside-diphosphate reductase subunit M2	-1.28	6.07E-04
PRC1	Protein regulator of cytokinesis 1	-1.34	9.37E-03
KIF22	Kinesin-like protein KIF22	-1.34	1.44E-02
INCENP	Inner centromere protein	-1.39	1.45E-02
TPX2	Targeting protein for Xklp2	-1.43	1.68E-02
CLSPN	Claspin	-1.43	7.96E-03
CKAP2	Cytoskeleton-associated protein 2	-1.46	1.09E-02
DBH	Dopamine beta-hydroxylase	-1.77	1.33E-05
COX6C	Cytochrome c oxidase subunit 6C	-2.72	9.47E-12



Supplementary Figure 6. Quantification of Western blot results from Figure 18. Protein expressions were normalized to GAPDH expression. Each dot represents the average value of protein expression for clones. Technical replicates were performed in duplicate, and the average expression value was calculated for each protein in each clone of both KO and control. Subsequently, the average expression values of proteins in the knockout (KO) group were compared to those in the control group using Welch's t-test. Statistical analysis was performed using GraphPad prism using (ns= not significant).



Supplementary Figure 7. STRING interaction network generated data from supplementary table 1 and 2. Proteins were clustered using MCL algorithm. Each color represents different clusters with members of these clusters shown accordingly.

Network Stats

number of nodes: 91
 number of edges: 176
 average node degree: 3.87
 avg. local clustering coefficient: 0.507

expected number of edges: 40
 PPI enrichment p-value: $< 1.0e-16$
your network has significantly more interactions than expected (what does that mean?)

Functional enrichments in your network

[explain columns](#)

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:1903729	Regulation of plasma membrane organization	4 of 18	1.68	0.0225
GO:0090307	Mitotic spindle assembly	5 of 51	1.33	0.0305
GO:0014910	Regulation of smooth muscle cell migration	5 of 65	1.22	0.0394
GO:0098813	Nuclear chromosome segregation	8 of 229	0.88	0.0355
GO:0003018	Vascular process in circulatory system	8 of 262	0.82	0.0453
GO:1903047	Mitotic cell cycle process	13 of 537	0.72	0.0212
GO:0010564	Regulation of cell cycle process	13 of 716	0.59	0.0440
GO:0022402	Cell cycle process	14 of 835	0.56	0.0453
GO:0006996	Organelle organization	33 of 3470	0.31	0.0394
GO:0016043	Cellular component organization	45 of 5436	0.25	0.0305

[\(less ...\)](#)

Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0015929	Hexosaminidase activity	3 of 15	1.64	0.0382
GO:0004553	Hydrolase activity, hydrolyzing O-glycosyl compounds	6 of 93	1.15	0.0157
GO:1901981	Phosphatidylinositol phosphate binding	7 of 178	0.93	0.0382
GO:0035091	Phosphatidylinositol binding	8 of 270	0.81	0.0382
GO:0005543	Phospholipid binding	10 of 464	0.67	0.0382
GO:0008092	Cytoskeletal protein binding	15 of 1002	0.51	0.0382
GO:0043168	Anion binding	31 of 2404	0.45	0.00028
GO:0003824	Catalytic activity	44 of 5522	0.24	0.0382
GO:0043167	Ion binding	46 of 6033	0.22	0.0382
GO:0005515	Protein binding	53 of 7242	0.2	0.0382

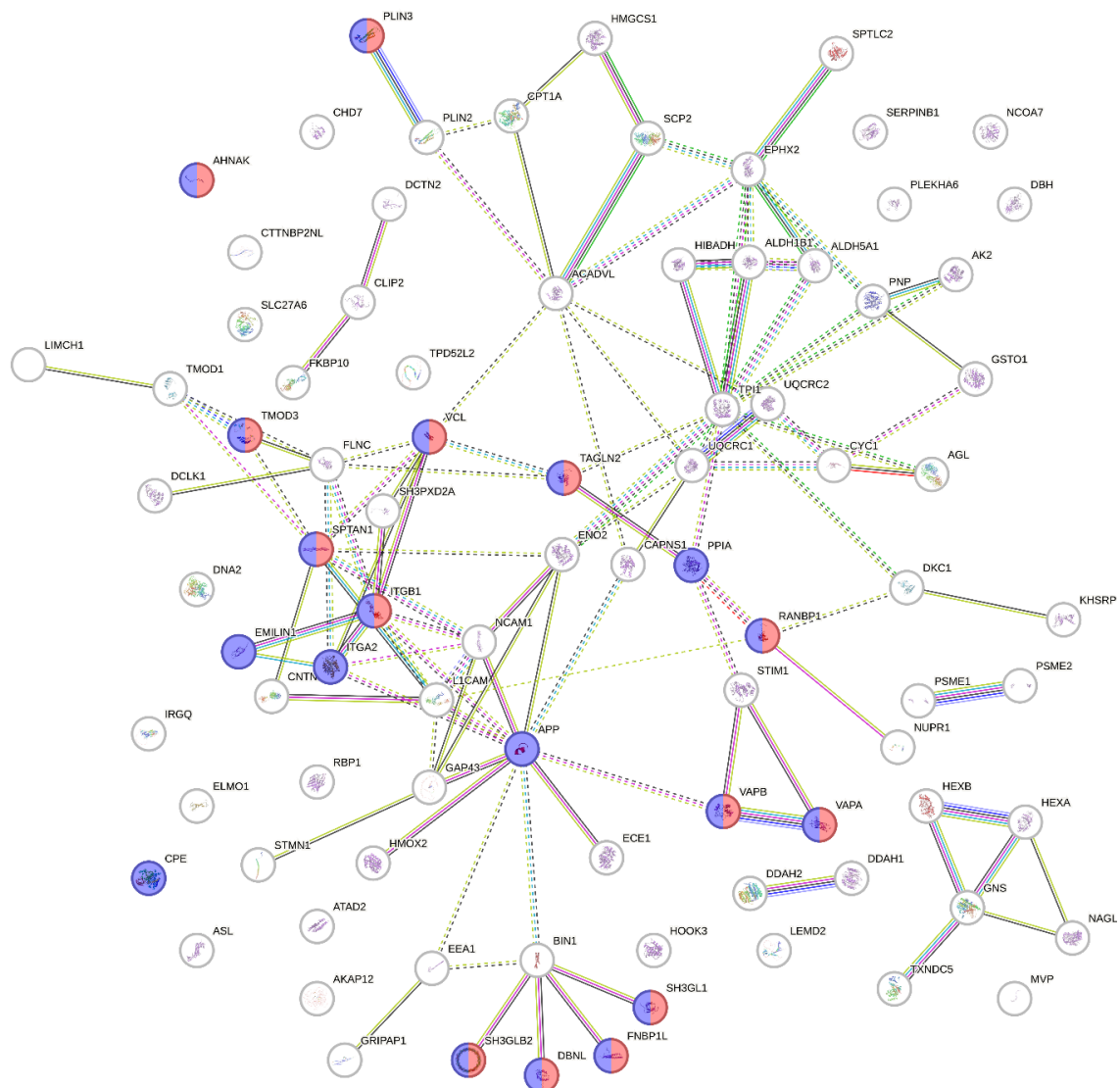
[\(less ...\)](#)

Cellular Component (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:1905379	beta-N-acetylhexosaminidase complex	2 of 2	2.34	0.0183
GO:0048786	Presynaptic active zone	4 of 78	1.05	0.0475
GO:0072686	Mitotic spindle	6 of 180	0.86	0.0208
GO:0045178	Basal part of cell	7 of 269	0.75	0.0252
GO:0034774	Secretory granule lumen	8 of 321	0.73	0.0186

[\(more ...\)](#)

Supplementary Figure 8. GO terms (Biological Process, Molecular Function, Cellular Component) associated with data from supplementary table 1 and 2 based on STRING analysis. Ontology terms were ranked based on strength in descendent order.

7.2.2 AK0800-2



Supplementary Figure 9. STRING interaction network generated data from supplementary table 3 and supplementary table 4. Proteins associated with the GO term "cadherin binding" are highlighted in red, while those associated with "cell adhesion molecule binding" are highlighted in blue.

Network Stats

number of nodes: 88	expected number of edges: 54
number of edges: 113	PPI enrichment p-value: 1.32e-12
average node degree: 2.57	<i>your network has significantly more interactions than expected (what does that mean?)</i>
avg. local clustering coefficient: 0.464	

Functional enrichments in your network

[explain columns](#)

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0006122	Mitochondrial electron transport, ubiquinol to cytochrome c	3 of 13	1.71	0.0286
GO:0006525	Arginine metabolic process	3 of 17	1.6	0.0353
GO:0019915	Lipid storage	4 of 41	1.34	0.0286
GO:0045429	Positive regulation of nitric oxide biosynthetic process	4 of 43	1.32	0.0286
GO:0045428	Regulation of nitric oxide biosynthetic process	5 of 59	1.28	0.0198
(more ...)				
Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0098634	Cell-matrix adhesion mediator activity	3 of 7	1.98	0.0070
GO:0015929	Hexosaminidase activity	3 of 15	1.65	0.0407
GO:0045296	Cadherin binding	14 of 334	0.97	1.02e-06
GO:0050839	Cell adhesion molecule binding	19 of 560	0.88	3.63e-08
GO:0008092	Cytoskeletal protein binding	17 of 1002	0.58	0.0017
(more ...)				
Cellular Component (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:1905379	beta-N-acetylhexosaminidase complex	2 of 2	2.35	0.0077
GO:0034668	Integrin alpha4-beta1 complex	2 of 3	2.17	0.0117
GO:0034666	Integrin alpha2-beta1 complex	2 of 3	2.17	0.0117
GO:0008537	Proteasome activator complex	2 of 3	2.17	0.0117
GO:0005750	Mitochondrial respiratory chain complex III	3 of 13	1.71	0.0042
(more ...)				

Supplementary Figure 10. GO terms (Biological Process, Molecular Function, Cellular Component) associated with data from supplementary table 5 and 6 based on STRING analysis. Each ontology ranked using strength in descendent order.

Supplementary Table 3. The list of upregulated proteins from the AK0800-2 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 0.5$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Log fold change and adjusted p-values were calculated based on imputed data

Upregulated proteins in SPTSSA KO compared to control clones			
Gene names	Protein names	Log ₂ FC	Adj p-val
CPE	Carboxypeptidase E	1.43	4.40E-02
AHNAK	Neuroblast differentiation-associated protein AHNAK	1.31	1.51E-02
SLC27A6	Long-chain fatty acid transport protein 6	1.22	3.34E-05
MVP	Major vault protein	1.13	2.17E-02
TAGLN2	Transgelin-2	1.08	5.14E-03
CNTNAP1	Contactin-associated protein 1	1.08	4.76E-03

(continued)

Supplementary Table 3 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
SH3PXD2A	SH3 and PX domain-containing protein 2A	1.05	2.05E-05
L1CAM	Neural cell adhesion molecule L1	1.05	3.54E-02
STMN1	Stathmin	0.99	2.08E-02
ITGA2	Integrin alpha-2	0.95	1.12E-02
CYC1	Cytochrome c1, heme protein, mitochondrial	0.90	3.72E-06
EEA1	Early endosome antigen 1	0.89	2.62E-03
DCLK1	Serine/threonine-protein kinase DCLK1	0.88	1.00E-03
GAP43	Neuromodulin	0.87	1.81E-02
RBP1	Retinol-binding protein 1	0.84	1.38E-02
SH3GLB2	Endophilin-B2	0.83	6.18E-04
ASL	Argininosuccinate lyase	0.79	5.02E-03
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	0.76	3.51E-05
AGL	Glycogen debranching enzyme	0.75	7.90E-04
CPT1A	Carnitine O-palmitoyltransferase 1, liver isoform	0.75	2.45E-03
RASA4B;RASA4	Ras GTPase-activating protein 4B;Ras GTPase-activating protein 4	0.75	3.91E-03
RANBP1	Ran-specific GTPase-activating protein	0.72	2.93E-02
PLEKHA6	Pleckstrin homology domain-containing family A member 6	0.71	1.73E-04
GNS	N-acetylglucosamine-6-sulfatase	0.71	1.21E-03
DDAH2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	0.71	1.02E-03
ITGB1	Integrin beta-1	0.70	9.62E-03
FKBP10	Peptidyl-prolyl cis-trans isomerase FKBP10	0.70	4.56E-03
TPD52L2	Tumor protein D54	0.70	2.45E-02
TMOD3	Tropomodulin-3	0.69	3.06E-03
PLIN2	Perilipin-2	0.69	1.30E-03
HEXB	Beta-hexosaminidase subunit beta	0.68	1.80E-05
PSME1	Proteasome activator complex subunit 1	0.68	5.55E-03
PSME2	Proteasome activator complex subunit 2	0.68	1.81E-03
SPTAN1	Spectrin alpha chain, non-erythrocytic 1	0.67	9.91E-03
PPIA	Peptidyl-prolyl cis-trans isomerase A	0.66	1.51E-02

(continued)

Supplementary Table 3 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
PLIN3	Perilipin-3	0.64	1.92E-02
SH3GL1	Endophilin-A2	0.64	1.48E-03
TMOD1	Tropomodulin-1	0.64	8.46E-03
DDAH1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	0.64	2.97E-02
FNBP1L	Formin-binding protein 1-like	0.64	1.02E-03
GRIPAP1	GRIP1-associated protein 1	0.63	2.89E-03
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	0.63	8.70E-06
UQCRC2	Cytochrome b-c1 complex subunit 2, mitochondrial	0.62	1.21E-05
EPHX2	Bifunctional epoxide hydrolase 2	0.62	1.07E-06
GSTO1	Glutathione S-transferase omega-1	0.62	5.27E-05
AK2	Adenylate kinase 2, mitochondrial	0.62	4.11E-03
IRGQ	Immunity-related GTPase family Q protein	0.61	4.08E-02
GPX1	Glutathione peroxidase 1	0.61	2.66E-03
ECE1	Endothelin-converting enzyme 1	0.61	9.44E-03
CLIP2	CAP-Gly domain-containing linker protein 2	0.61	1.35E-03
HOOK3	Protein Hook homolog 3	0.61	1.82E-03
FLNC	Filamin-C	0.59	2.25E-03
VAPB	Vesicle-associated membrane protein-associated protein B/C	0.58	7.06E-03
DBNL	Drebrin-like protein	0.58	1.67E-02
HMOX2	Heme oxygenase 2	0.57	9.57E-03
TXNDC5	Thioredoxin domain-containing protein 5	0.57	4.00E-02
DCTN2	Dynactin subunit 2	0.57	1.56E-02
SERPINB1	Leukocyte elastase inhibitor	0.57	3.88E-04
NCAM1	Neural cell adhesion molecule 1	0.56	4.88E-02
VCL	Vinculin	0.56	2.89E-02
CTTNBP2NL	CTTNBP2 N-terminal-like protein	0.56	1.38E-02
KHSRP	Far upstream element-binding protein 2	0.56	3.26E-02
EMILIN1	EMILIN-1	0.56	1.28E-02
BIN1	Myc box-dependent-interacting protein 1	0.55	3.46E-02
HIBADH	3-hydroxyisobutyrate dehydrogenase, mitochondrial	0.55	2.79E-05
CAPNS1	Calpain small subunit 1	0.54	2.04E-03

(continued)

Supplementary Table 3 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
ALDH1B1	Aldehyde dehydrogenase X, mitochondrial	0.54	1.94E-07
NCOA7	Nuclear receptor coactivator 7	0.54	1.27E-06
APP	Amyloid-beta precursor protein	0.54	2.85E-02
TPI1	Triosephosphate isomerase	0.54	3.91E-02
AKAP12	A-kinase anchor protein 12	0.53	2.64E-02
ENO2	Gamma-enolase	0.53	8.93E-03
LIMCH1	LIM and calponin homology domains-containing protein 1	0.53	2.91E-03
SCP2	Sterol carrier protein 2	0.53	8.17E-03
STIM1	Stromal interaction molecule 1	0.53	6.82E-04
ALDH5A1	Succinate-semialdehyde dehydrogenase, mitochondrial	0.53	1.23E-02
NAGLU	Alpha-N-acetylglucosaminidase	0.53	1.23E-03
VAPA	Vesicle-associated membrane protein-associated protein A	0.52	1.35E-03
LEMD2	LEM domain-containing protein 2	0.51	3.42E-03
HEXA	Beta-hexosaminidase subunit alpha	0.50	1.48E-04

Supplementary Table 4. The list of downregulated proteins from the AK0800-2 proteome analysis, sorted in descending order according to the following criteria: Log₂FC $\geq$ -0.5, Adj.p-val $\leq$ 0.05, and ContrastMaxScore $\geq$ 0.7. Log fold change and adjusted p-values were calculated based on imputed data

<i>Downregulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
HMGCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	-0.53	2.42E-03
ELMO1	Engulfment and cell motility protein 1	-0.54	9.81E-03
CHD7	Chromodomain-helicase-DNA-binding protein 7	-0.55	1.96E-03
DKC1	H/ACA ribonucleoprotein complex subunit DKC1	-0.55	2.14E-06
PNP	Purine nucleoside phosphorylase	-0.58	3.27E-04
DBH	Dopamine beta-hydroxylase	-0.62	2.86E-02
DNA2	DNA replication ATP-dependent helicase/nuclease DNA2	-0.62	6.86E-04
ATAD2	ATPase family AAA domain-containing protein 2	-0.62	1.19E-05
SPTLC2	Serine palmitoyltransferase 2	-0.89	5.45E-09

7.2.3 AK0800-1

Supplementary Table 5. The list of upregulated proteins from the AK0800-1 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \geq 0.35$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Log fold change and adjusted p-values were calculated based on imputed data

<i>Upregulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
AHNAK	Neuroblast differentiation-associated protein	1.91	1.39E-02
CRABP1	Cellular retinoic acid-binding protein 1	1.72	8.13E-04
STMN1	Stathmin	1.01	1.13E-02
TAGLN2	Transgelin-2	0.99	2.53E-03
HN1	Jupiter microtubule associated homolog 1	0.87	4.81E-02
GAP43	Neuromodulin	0.84	1.63E-02
LGALS1	Galectin-1	0.76	1.23E-03
CARHSP1	Calcium-regulated heat-stable protein 1 OS=Homo sapiens OX=9606 GN=CARHSP1 PE=1 SV=2	0.74	2.72E-02
PLEKHA6	Pleckstrin homology domain-containing family A member 6	0.72	7.01E-04
CPT1A	Carnitine O-palmitoyltransferase 1	0.72	1.44E-03
PLIN3	Perilipin-3	0.70	1.90E-02
ACADVL	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial	0.69	6.89E-05
DNPH1	2-deoxynucleoside 5-phosphate N-hydrolase 1	0.67	4.62E-03
STMN2	Stathmin-2	0.67	8.34E-03
ITGB1	Integrin beta-1	0.66	1.50E-03
ATP5H	ATP synthase subunit d, mitochondrial	0.65	9.02E-03
KDEL2	Protein O-glucosyltransferase 3	0.64	4.03E-02
TXNDC5	Thioredoxin domain-containing protein 5	0.64	4.47E-03
PSME2	Proteasome activator complex subunit 2	0.64	8.55E-04
DBNL	Drebrin-like protein	0.63	7.63E-03
SPTAN1	Spectrin alpha chain, non-erythrocytic 1	0.63	4.48E-03

(continued)

Supplementary Table 6 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
TMOD1	Tropomodulin-1	0.63	3.35E-03
PSME1	Proteasome activator complex subunit 1	0.63	1.83E-02
TXN	Thioredoxin	0.62	1.65E-03
MRPL53	39S ribosomal protein L53, mitochondrial	0.61	7.63E-03
CYCS	Cytochrome c	0.60	6.48E-04
DDAH1	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	0.59	1.58E-02
NCAM1	Neural cell adhesion molecule 1	0.59	2.82E-02
EPN1	Epsin-1	0.59	5.12E-03
RANBP1	Ran-specific GTPase-activating protein	0.59	3.01E-02
SAMHD1	Deoxynucleoside triphosphate triphosphohydrolase	0.58	6.85E-03
RBP1	Retinol-binding protein 1	0.58	9.13E-03
TBCA	Tubulin-specific chaperone A	0.58	7.63E-03
CD2BP2	CD2 antigen cytoplasmic tail-binding protein 2	0.57	4.00E-03
DDAH2	N(G),N(G)-dimethylarginine dimethylaminohydrolase 2	0.57	1.50E-03
ALDH1B1	Aldehyde dehydrogenase X, mitochondrial	0.57	4.57E-07
ARPC5	Actin-related protein 2/3 complex subunit 5	0.56	1.88E-02
HIBADH	3-hydroxyisobutyrate dehydrogenase, mitochondrial	0.56	1.26E-04
NDUFA8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	0.56	1.15E-02
AK2	Adenylate kinase 2, mitochondrial	0.55	2.81E-03
ENO2	Gamma-enolase	0.55	1.67E-03
NCOA7	Nuclear receptor coactivator 7	0.54	2.60E-05
PGRMC2	Membrane-associated progesterone receptor component 2	0.53	1.36E-02
HCCS	Holocytochrome c-type synthase	0.52	3.43E-02
UQCRC1	Cytochrome b-c1 complex subunit 1, mitochondrial	0.52	5.44E-06
CSRP2	Cysteine and glycine-rich protein 2	0.52	3.60E-02

(continued)

Supplementary Table 5 (continued)

Gene names	Protein names	Log₂FC	Adj p-val
SH3GL1	Endophilin-A2	0.52	2.97E-03
IRGQ	Immunity-related GTPase family Q	0.51	4.10E-02
KHSRP	Far upstream element-binding protein 2	0.51	1.39E-02
CAPNS1	Calpain small subunit 1	0.50	3.74E-04
GSTO1	Glutathione S-transferase omega-1	0.50	5.85E-06
GAMT	Guanidinoacetate N-methyltransferase	0.49	1.14E-02
UCHL1	Ubiquitin carboxyl-terminal hydrolase isozyme L1	0.49	3.63E-03
ERP29	Endoplasmic reticulum resident protein 29	0.49	3.24E-03
SLC9A3R1	NHRF1_HUMAN Na(+)/H(+) exchange regulatory cofactor	0.48	4.86E-02
UQCRC2	Cytochrome b-c1 complex subunit 2	0.48	7.39E-07
PGRMC1	Membrane-associated progesterone receptor component 1	0.48	3.90E-03
DCTN2	Dynactin subunit 2	0.48	1.59E-02
CTSB	Cathepsin B	0.48	3.90E-02
GPX1	Glutathione peroxidase 1	0.48	6.08E-03
FNBP1L	Formin-binding protein 1-like	0.48	6.99E-03
VCL	Vinculin	0.47	4.62E-02
ANXA4	Annexin A4	0.46	4.56E-02
NDUFA5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	0.46	2.16E-02
AK1	Adenylate kinase isoenzyme 1	0.46	4.89E-03
LIMCH1	LIM and calponin homology domains-containing protein 1	0.45	5.85E-03
CKAP4	Cytoskeleton-associated protein 4	0.45	6.23E-03
PPIA	Peptidyl-prolyl cis-trans isomerase A	0.45	5.51E-03
SLIRP	SRA stem-loop-interacting RNA-binding protein, mitochondrial	0.43	3.23E-02

(continued)

Supplementary Table 5 (continued)

Gene names	Protein names	Log ₂ FC	Adj p-val
NIPSNAP3A	NipSnap homolog 3A	0.43	1.43E-02
KCTD5	BTB/POZ domain-containing protein	0.43	2.31E-02
ST13;ST13P5;ST13P4	Hsc70-interacting protein	0.42	3.90E-02
UBE2L3	Ubiquitin-conjugating enzyme E2 L3	0.42	2.94E-02
VAPA	Vesicle-associated membrane protein-associated protein A	0.42	5.60E-03
ENAH	Protein enabled homolog	0.42	4.53E-02
VAPB	Vesicle-associated membrane protein-associated protein B/C	0.41	4.62E-02
ACTN4	Alpha-actinin-4	0.41	2.50E-03
RNPEP	Aminopeptidase B	0.41	1.29E-04
TPI1	Triosephosphate isomerase	0.41	1.21E-02
DUT	Deoxyuridine 5-triphosphate nucleotidohydrolase, mitochondrial	0.41	9.02E-03
CBX1	Chromobox protein homolog 1	0.41	3.57E-02
FDXR	NADPH:adrenodoxin oxidoreductase, mitochondrial	0.40	4.14E-04
MYO6	Unconventional myosin-VI	0.39	6.58E-05
ADD3	Gamma-adducin	0.39	4.74E-02
NDRG3	NDRG family member 3	0.39	2.68E-02
GOLIM4	Golgi integral membrane protein 4	0.39	4.47E-03
GAA	Lysosomal alpha-glucosidase	0.38	1.29E-03
GRSF1	G-rich sequence factor 1	0.38	4.53E-02
EMD	Emerin	0.37	1.61E-02
HSPE1	10 kDa heat shock protein, mitochondrial	0.37	4.51E-02
SCCPDH	Saccharopine dehydrogenase-like oxidoreductase	0.37	2.60E-05
SRSF1	Serine/arginine-rich splicing factor 1	0.36	4.72E-02
CAT	Catalase	0.35	3.98E-02

Supplementary Table 7. The list of downregulated proteins from the AK0800-1 proteome analysis, sorted in descending order according to the following criteria: $\text{Log}_2\text{FC} \leq -0.35$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$. Log fold change and adjusted p-values were calculated based on imputed data

<i>Downregulated proteins in SPTSSA KO compared to control clones</i>			
Gene names	Protein names	Log ₂ FC	Adj p-val
DLD	Dihydrolipoyl dehydrogenase, mitochondrial	-0.35	1.99E-02
AKR1B1	Aldo-keto reductase family 1 member B1	-0.35	5.23E-03
RPL32	60S ribosomal protein L32	-0.35	1.11E-03
DDX56	Probable ATP-dependent RNA helicase DDX56	-0.35	2.55E-03
RPS6	40S ribosomal protein S6	-0.36	3.01E-04
UTP3	Something about silencing protein 10	-0.36	2.02E-04
RPL26;RPL26L1	60S ribosomal protein L26	-0.36	3.94E-03
RPL24	60S ribosomal protein L24	-0.37	4.74E-04
HTATSF1	HIV Tat-specific factor 1	-0.37	2.68E-03
RPL36	60S ribosomal protein L36	-0.37	4.62E-02
SPTLC1	Serine palmitoyltransferase 1	-0.37	7.85E-03
NPM3	Nucleoplasmin-3	-0.37	1.91E-02
RPL10	60S ribosomal protein L10	-0.38	3.83E-02
PDCD11	Protein RRP5 homolog	-0.38	6.82E-06
DDX24	ATP-dependent RNA helicase DDX24	-0.39	1.90E-03
VDAC1	Voltage-dependent anion-selective channel protein 1	-0.39	3.35E-02
RPL23A	60S ribosomal protein L23a	-0.39	1.44E-03
VDAC2	Voltage-dependent anion-selective channel protein 2	-0.40	1.98E-02
RRM2	Ribonucleoside-diphosphate reductase subunit M2	-0.41	3.04E-04
RPS24	40S ribosomal protein S24	-0.41	3.27E-02
TOP2A	DNA topoisomerase 2-alpha	-0.41	1.27E-05
SSRP1	FACT complex subunit	-0.42	2.24E-03
SUPT16H	FACT complex subunit	-0.42	4.18E-03
RPL13	60S ribosomal protein L13	-0.43	6.66E-04

(continued)

Supplementary Table 6 (continued)

Gene names	Protein names	Log₂FC	Adj p-val
RPL37A	60S ribosomal protein L37a	-0.44	2.33E-03
DNAJA1	DnaJ homolog subfamily A member 1	-0.44	2.21E-03
VDAC3	Voltage-dependent anion-selective channel protein 3	-0.45	3.32E-02
SDAD1	Protein SDA1 homolog	-0.45	2.19E-03
MSI1	RNA-binding protein Musashi homolog 1	-0.46	2.52E-03
NOLC1	Nucleolar and coiled-body phosphoprotein 1	-0.47	2.67E-04
RPL17	60S ribosomal protein L17	-0.50	5.76E-04
KIAA0101	PCNA-associated factor	-0.51	2.80E-02
DKC1	H/ACA ribonucleoprotein complex subunit DKC1	-0.53	3.24E-05
METAP2	Methionine aminopeptidase 2	-0.54	3.74E-04
HK2	Hexokinase-2	-0.55	1.13E-02
RPL22L1	60S ribosomal protein L22-like 1	-0.56	1.23E-02
PNP	Purine nucleoside phosphorylase	-0.57	5.76E-04
ABCF2	ATP-binding cassette sub-family F member 2	-0.57	1.36E-05
DBH	Dopamine beta-hydroxylase	-0.61	2.94E-02
HMGCS1	Hydroxymethylglutaryl-CoA synthase, cytoplasmic	-0.65	2.17E-03
MT-CO2	Cytochrome c oxidase subunit 2	-5.00	4.62E-08

Network Stats

number of nodes: 131
 number of edges: 393
 average node degree: 6
 avg. local clustering coefficient: 0.505

expected number of edges: 218
 PPI enrichment p-value: < 1.0e-16
your network has significantly more interactions than expected (what does that mean?)

Functional enrichments in your network

[explain columns](#)

Biological Process (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0006122	Mitochondrial electron transport, ubiquinol to cytochrome c	3 of 13	1.54	0.0363
GO:0046031	ADP metabolic process	5 of 55	1.14	0.0178
GO:0002181	Cytoplasmic translation	11 of 123	1.13	2.21e-05
GO:0019646	Aerobic electron transport chain	7 of 87	1.08	0.0024
GO:0032272	Negative regulation of protein polymerization	6 of 77	1.07	0.0084

(more ...)

Molecular Function (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0015288	Porin activity	3 of 5	1.96	0.0095
GO:0003735	Structural constituent of ribosome	11 of 169	0.99	3.86e-05
GO:0045296	Cadherin binding	15 of 334	0.83	2.65e-05
GO:0050839	Cell adhesion molecule binding	17 of 560	0.66	0.00026
GO:0003779	Actin binding	12 of 448	0.61	0.0259

(more ...)

Cellular Component (Gene Ontology)				
GO-term	description	count in network	strength	false discovery rate
GO:0035101	FACT complex	2 of 2	2.18	0.0115
GO:0008537	Proteasome activator complex	2 of 3	2.0	0.0172
GO:0031429	Box H/ACA snoRNP complex	2 of 5	1.78	0.0303
GO:0022625	Cytosolic large ribosomal subunit	8 of 56	1.33	1.49e-06
GO:0046930	Pore complex	3 of 27	1.22	0.0326

(more ...)

Supplementary Figure 12. GO terms (Biological Process, Molecular Function, Cellular Component) associated with data from supplementary table 5 and supplementary table 6 based on STRING analysis. Each ontology ranked using strength in descendent order.

7.3 Phosphoproteome Analysis

Supplementary Table 8. Differentially regulated proteins and their associated phosphosites from AK0800-1 phosphoproteomic analysis. The list is sorted in alphabetical order according to the following criteria: $\text{Log}_2\text{FC} \geq 1$ or $\text{Log}_2\text{FC} \leq -1$, $\text{Adj.p-val} \leq 0.05$, and $\text{ContrastMaxScore} \geq 0.7$

Gene names	Phospho Position	Amino acid	Log ₂ FC	Adj p-val
AHNAK	135	S	1.2	9.58E-04
AHNAK	216	S	1.7	2.36E-03
AHNAK	2397	S	1.5	1.32E-04
AHNAK	5841	S	1.5	7.94E-03
AHNAK	93	S	1.6	6.59E-03
ATL1	23	S	2.6	1.06E-11
BCLAF1	257	T	1.2	6.29E-03
BIRC6	480	S	2.2	1.20E-08
BOD1L1	2986	S	-2.6	1.75E-11
CARHSP1	30	S	1.1	1.14E-02
CARHSP1	30	S	1.5	2.34E-02
CARHSP1	32	S	1.5	2.34E-02
CARHSP1	41	S	1.5	2.34E-02
CDC42BPB	1690	S	1.0	9.72E-04
CHGB	263	S	1.2	2.62E-02
CPSF1	756	S	-2.6	1.16E-08
CTNNA1	654	T	-1.2	3.94E-03
CTNNA2	653	T	-1.2	3.94E-03
CTTN	401	T	1.1	2.38E-02
CTTN	405	S	1.1	2.38E-02
CWC22	51	S	-1.1	5.98E-06
DNTTIP2	145	S	-1.1	6.02E-05
DPYSL3	522	S	-1.0	1.03E-03
DST	7510	S	1.1	2.85E-04
EDF1	4	S	-1.1	1.94E-04
EIF2B4	191	S	-1.0	2.95E-04
EPB41	85	S	-1.1	3.22E-02
EPN1	435	S	1.1	5.25E-04
ESYT2	758	S	2.2	8.96E-10

(continued)

Supplementary Table 7 (continued)

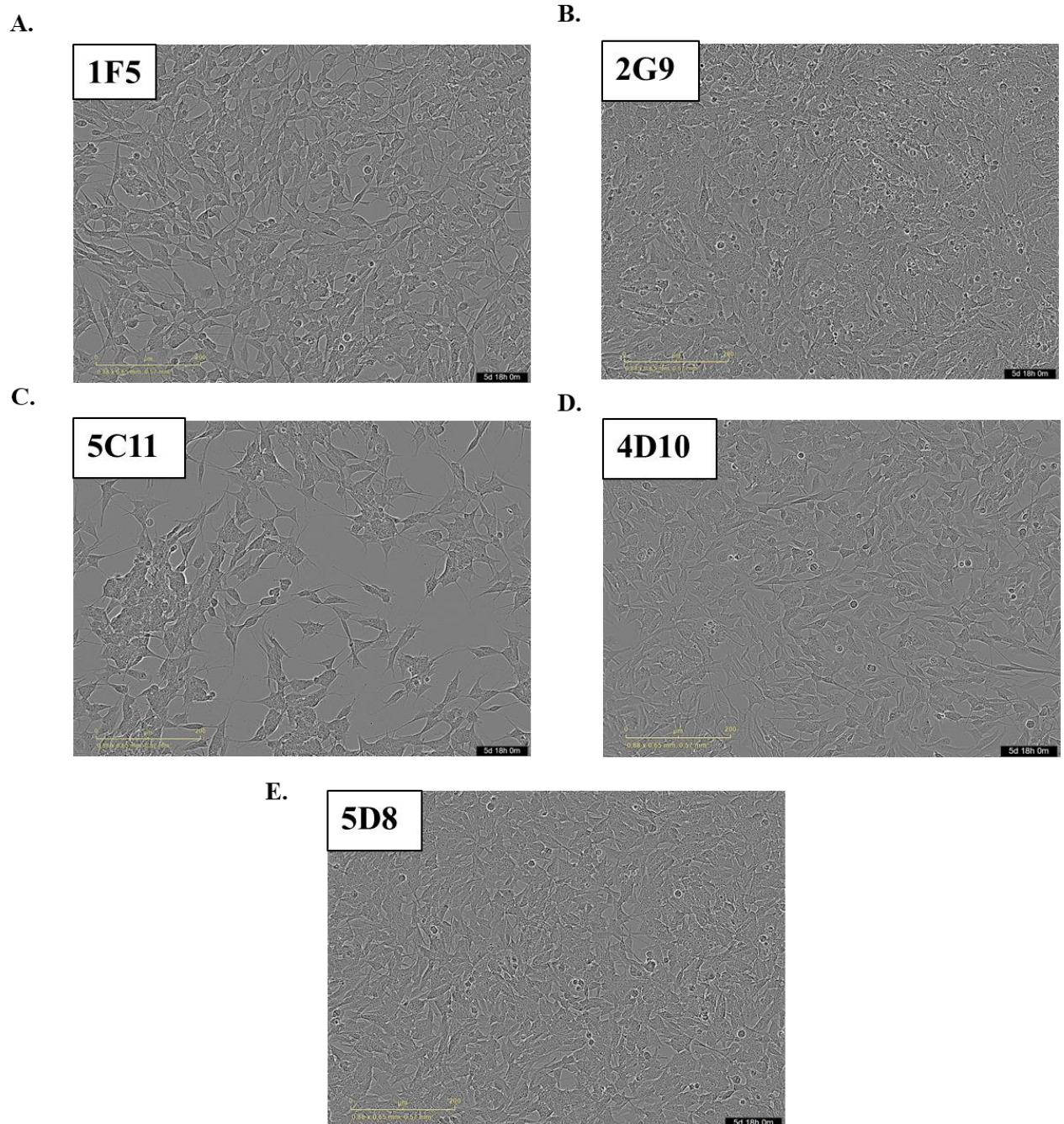
Gene names	Phospho Position	Amino acid	Log ₂ FC	Adj p-val
FAM21A;FAM21C	802	S	-1.1	5.17E-04
FARP1	24	T	-1.1	2.57E-02
FXR2	601	S	-3.1	7.47E-10
FXR2	603	S	-2.9	6.45E-11
HTATSF1	409	S	-1.1	7.12E-05
HTATSF1	624	S	-3.2	1.20E-09
INTS3	972	S	-1.2	6.53E-04
IRS2	388	S	-1.0	9.84E-06
IRS2	391	S	-1.0	9.84E-06
JUN	63	S	-2.2	5.21E-09
LEO1	629	T	4.3	3.12E-15
MAP1B	1252	S	2.3	1.06E-11
MCM2	41	S	-1.6	2.73E-07
MPRIP	542	T	-1.1	7.42E-03
NFIC	333	S	-2.5	7.47E-10
NFIC	339	S	-2.6	2.52E-12
NSUN2	751	S	1.2	1.42E-06
PACS1	534	S	1.0	1.86E-06
PHF6	155	S	-1.1	1.24E-04
PHYHIPL	15	S	1.1	2.04E-03
PPP1R11	77	S	-2.0	5.27E-10
PRRC2C	1263	S	1.0	7.12E-04
PSME1	38	S	3.5	2.93E-08
RBM10	60	S	2.6	2.41E-10
RRM2	20	S	-1.2	1.36E-06
SEPT6	418	T	-1.4	2.68E-03
SEPT7	426	T	-1.3	2.08E-03
SH3BP5L	343	S	1.0	7.38E-03
SH3BP5L	350	S	1.0	7.38E-03
SLC38A1	52	S	-1.5	8.83E-11
SMARCA4	1570	S	2.4	3.63E-08
SMARCA4	1575	S	2.6	1.34E-10

(continued)

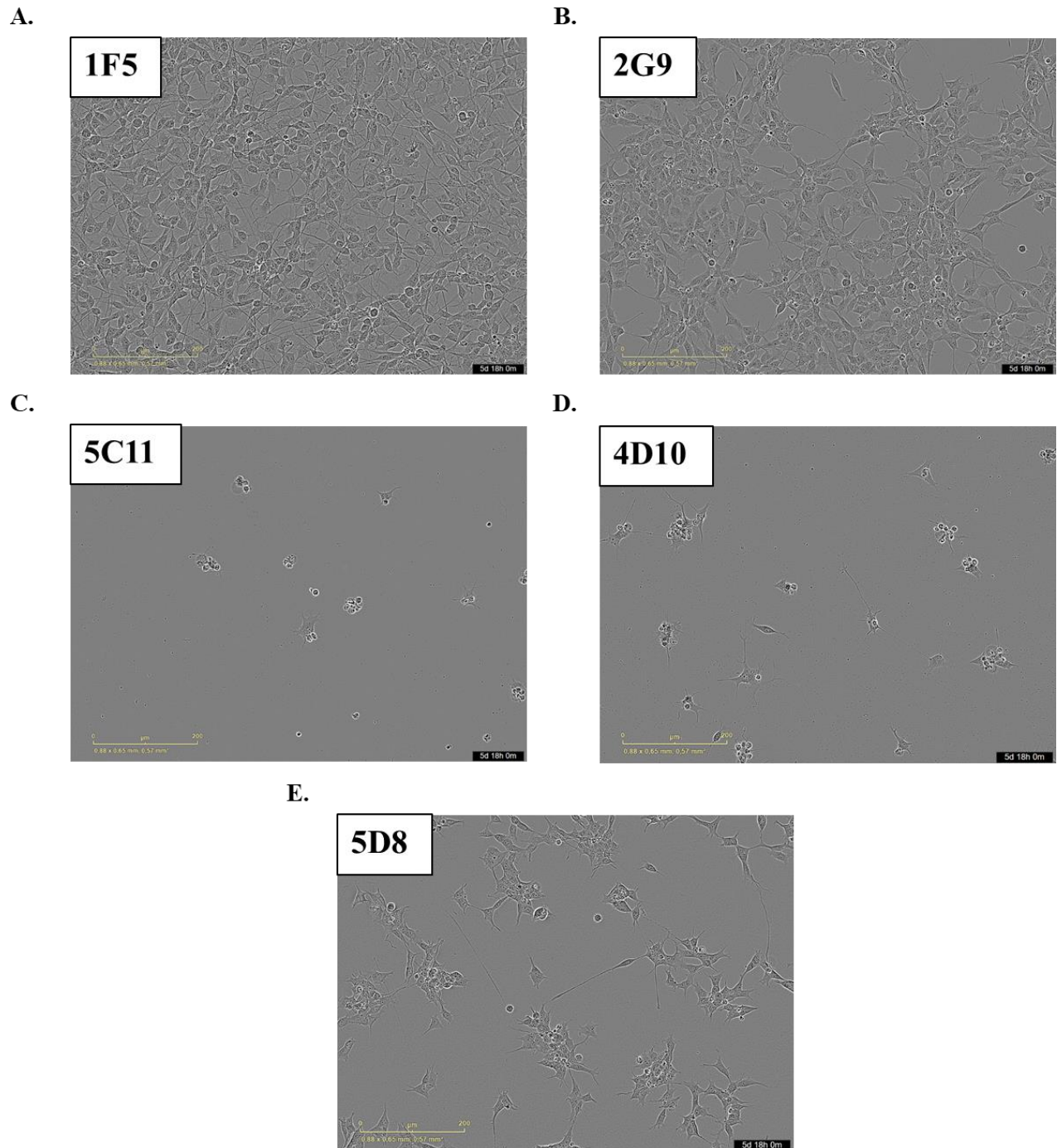
Supplementary Table 7 (continued)

Gene names	Phospho Position	Amino acid	Log₂FC	Adj p-val
SRRM2	435	S	-2.3	1.16E-08
STMN1	16	S	1.2	1.64E-03
STMN2	50	S	1.0	7.99E-03
TAOK3	324	S	3.7	5.06E-14
TOP1	10	S	-1.2	1.60E-05
TOP2B	1581	S	-1.6	3.30E-04
TOP2B	1588	S	-1.6	4.72E-04
TOR1AIP1	220	T	1.1	2.82E-06
TPD52L2	166	S	1.2	2.40E-02
VGF	420	S	1.9	1.93E-04
ZC3H13	318	S	-2.0	3.02E-02
ZC3H18	74	S	-1.2	2.73E-02
ZC3H4	908	S	-2.9	8.84E-14
ZFP36L2	57	S	3.9	2.02E-13

7.4 All-Trans Retinoic Acid Induced Differentiation of SPTSSA KO and Control Clones

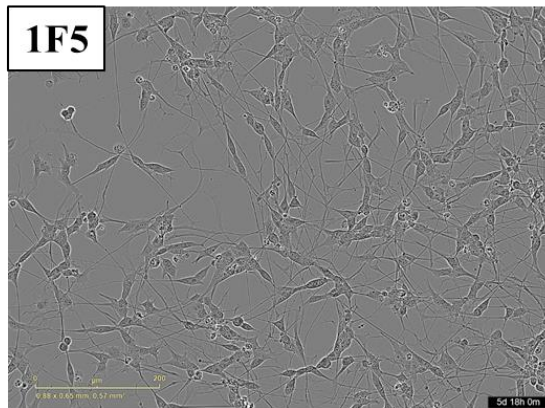


Supplementary Figure 13. The phase-contrast images of SPTSSA KO and controls cells on 96-well plate in DMEM/F12 supplemented with 10% FBS and %1 P/S (DMEM/F12). Images were taken by IncuCyte® S3-Live Cell Images System at the end of the experiment (Day 5).

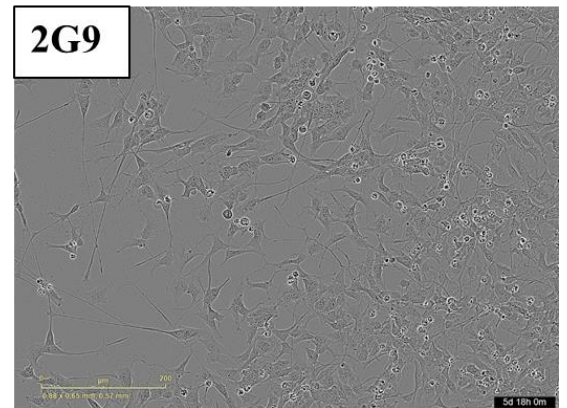


Supplementary Figure 14. The phase-contrast images of SPTSSA KO and controls cells on 96-well plate in Neurobasal media supplemented with 1X B27 and 1X Glutamax (B27 only). Images were taken by IncuCyte® S3-Live Cell Images System at the end of the experiment (Day 5).

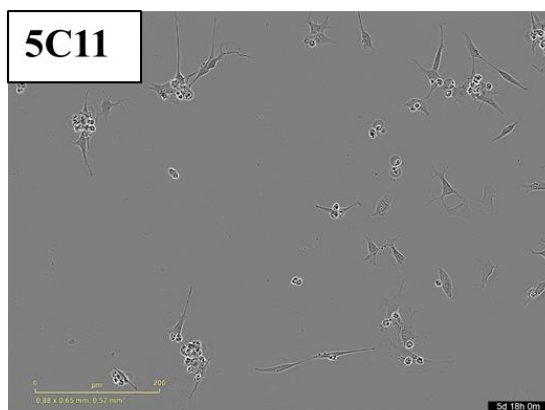
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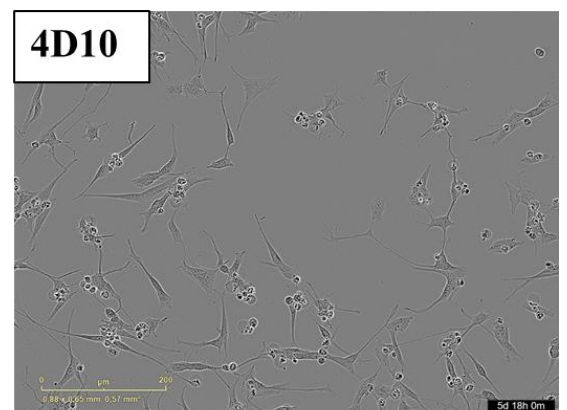
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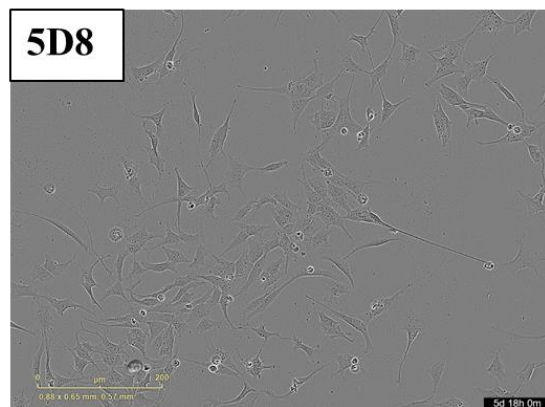
C.



D.

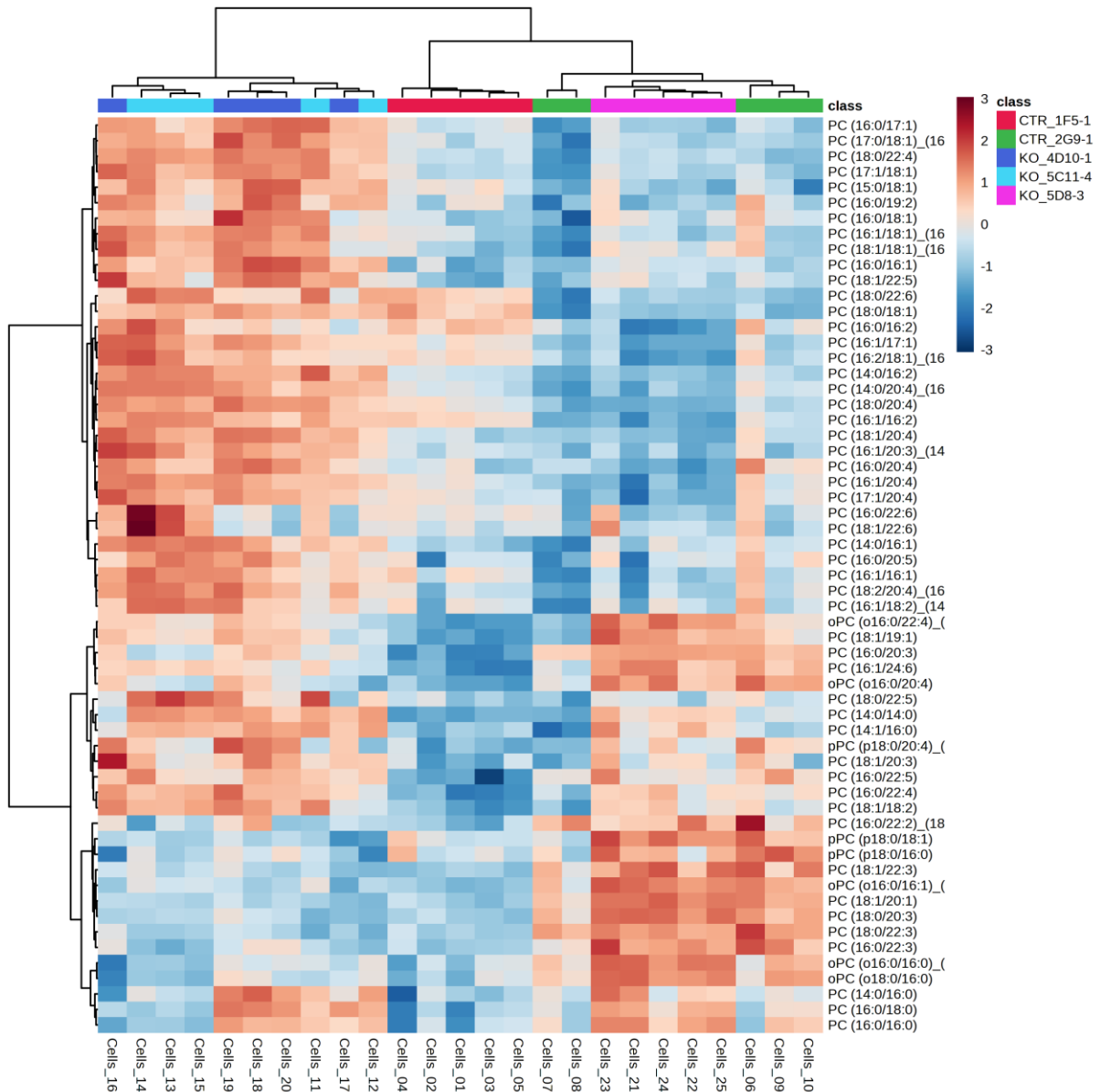


E.

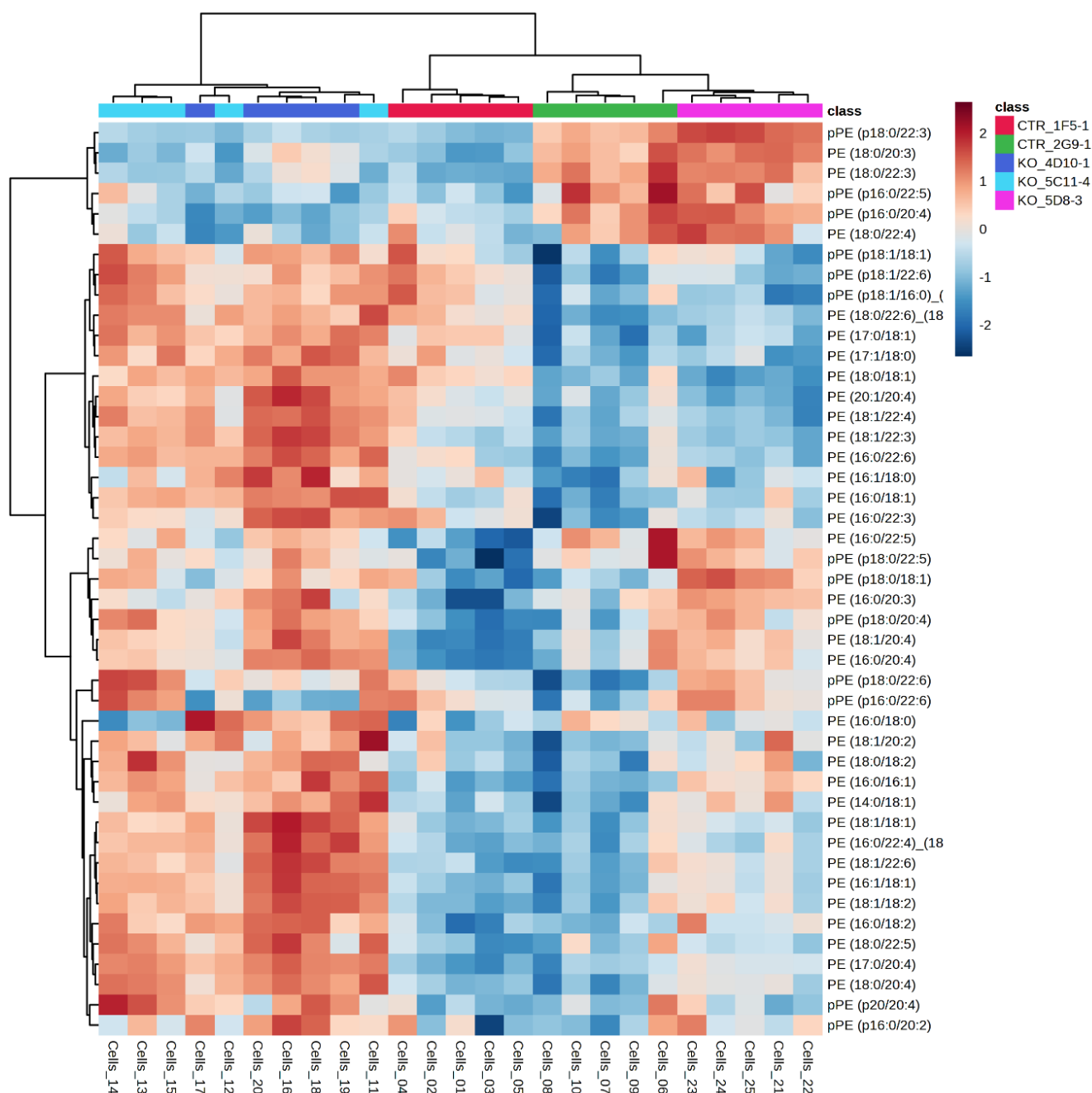


Supplementary Figure 15. The phase-contrast images of SPTSSA KO and controls cells on 96-well plate in Neurobasal media supplemented with 1X B27, 10 μ M ATRA and 1X Glutamax (B27+ATRA). Images were taken by IncuCyte® S3-Live Cell Images System at the end of the experiment (Day 5).

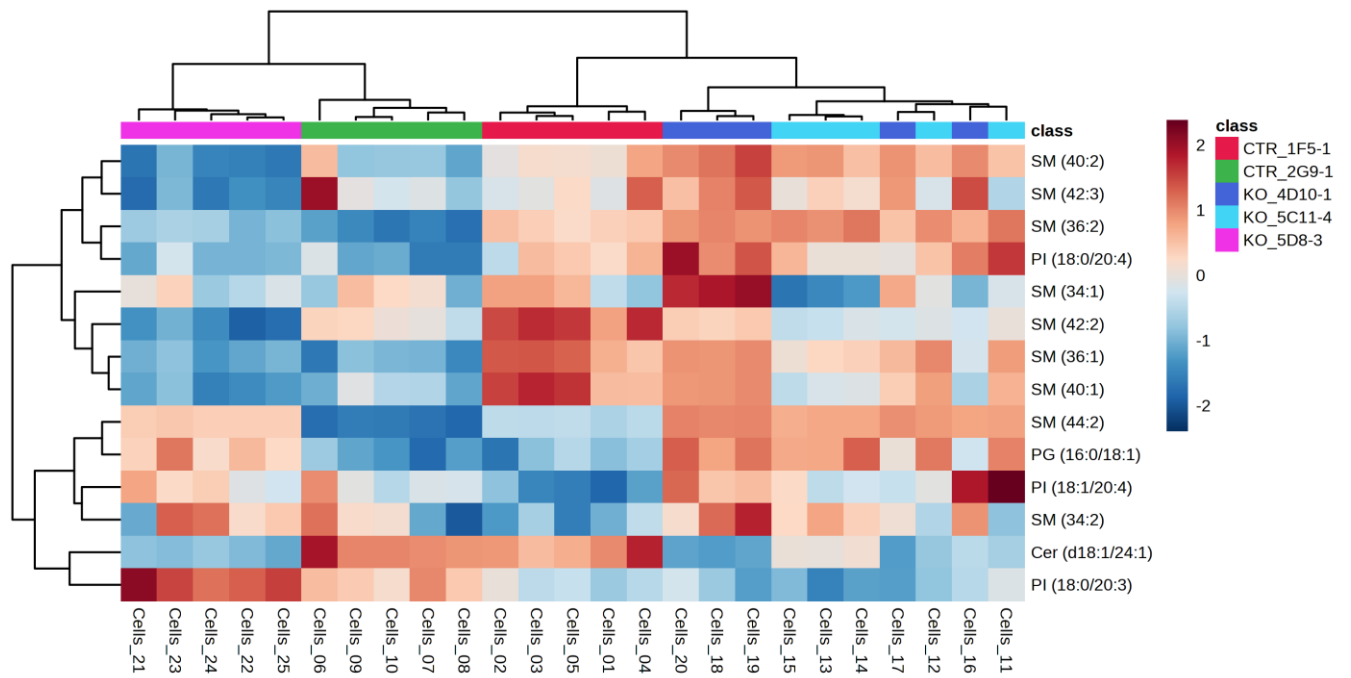
7.5 Lipidome Analysis



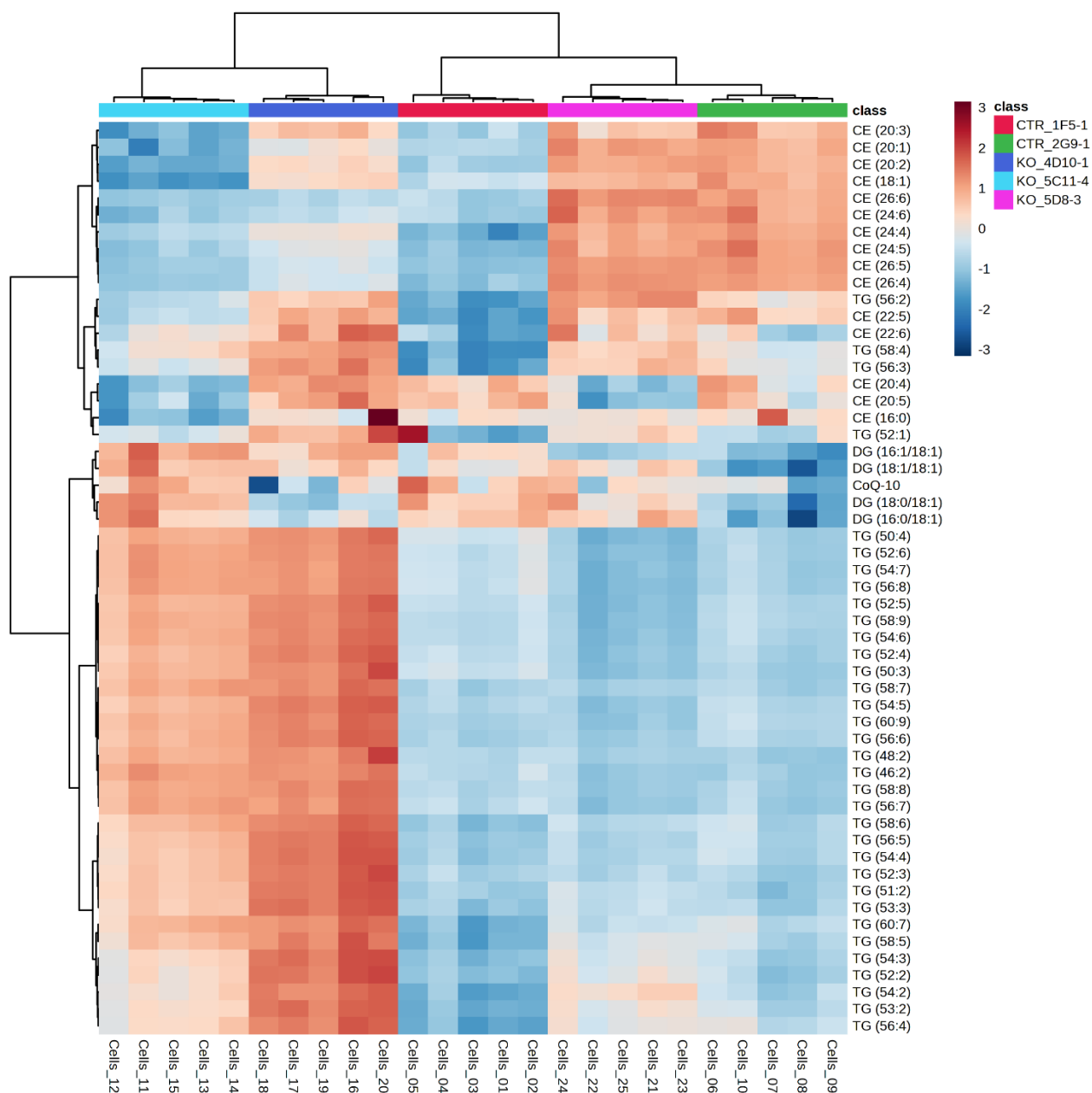
Supplementary Figure 16. A heatmap analysis was generated to display significantly altered phosphatidylcholine (PC), plasanyl-phosphatidylcholine (oPC) and plasmeyl-phosphatidylcholine (pPC) in SPTSSA KO and control clones, as determined by lipidome analysis. Out of 60 species, a total of 59 were altered. Significance was assessed by performing one-way ANOVA with a p-value threshold set to ≤ 0.05 . Representing z-scores, upregulated lipid in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.



Supplementary Figure 17. A heatmap analysis was generated to display significantly altered phosphatidylethanolamine (PE) and plasmalogen phosphatidylethanolamine (pPE) in SPTSSA KO and control clones, as determined by lipidome analysis. Out of 49 species, a total of 45 were altered. Significance was assessed by performing one-way ANOVA with a p-value threshold set to ≤ 0.05 . Representing z-scores, upregulated lipid in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.

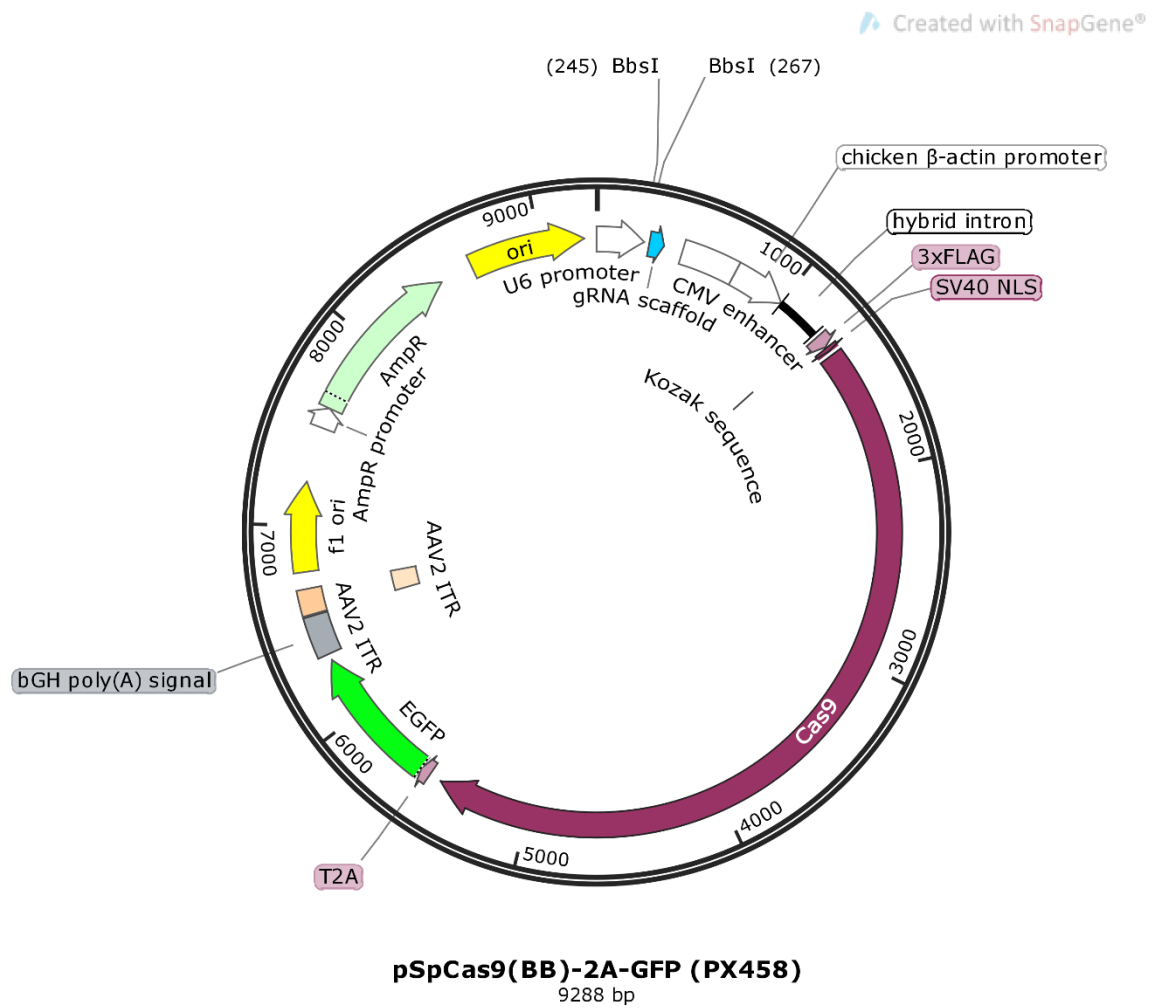


Supplementary Figure 18. A heatmap analysis was generated to display significantly altered phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylserine (PS), sphingomyelin (SM) and ceramide (Cer) in SPTSSA KO and control clones, as determined by lipidome analysis. Out of 16 species, a total of 14 were altered. Significance was assessed by performing one-way ANOVA with a p-value threshold set to ≤ 0.05 . Representing z-scores, upregulated in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.



Supplementary Figure 19. A heatmap analysis was generated to display significantly altered cholesteryl ester (CE), diacylglycerol (DG), triacylglycerol (TG) and coenzyme Q (CoQ)bin SPTSSA KO and control clones, as determined by lipidome analysis. Out of 55 species, a total of 54 were altered. Significance was assessed by performing one-way ANOVA with a p-value threshold set to ≤ 0.05 . Representing z-scores, upregulated lipid in SPTSSA KO clones compared to control are depicted in red, while downregulated ones in blue.

7.6 Additional Information for Material and Methods



Supplementary Figure 20. The map of pSpCas9(BB)-2A-GFP (PX458) used in Crisp-Cas9 mediated gene editing.

Compound Description (Isotopic Label, Enrichment)	Neat Material Chemical Purity	Target Concentration (mmol/L)	Concentration by Gravimetry (mmol/L)	Analyzed Concentration (mmol/L)
L-Alanine (¹³ C ₃ , 99%; ¹⁵ N, 99%)	100%	2.50	2.50	2.52 ± 0.08
L-Arginine:HCl (¹³ C ₆ , 99%; ¹⁵ N ₄ , 99%)	99.9%	2.50	2.50	2.50 ± 0.04
L-Asparagine:H ₂ O (¹³ C ₄ , 99%; ¹⁵ N ₂ , 99%)	98.6%	2.50	2.50	2.46 ± 0.09
L-Aspartic Acid (¹³ C ₄ , 99%; ¹⁵ N, 99%)	99.5%	2.50	2.50	2.22 ± 0.13
L-Cystine (¹³ C ₆ , 99%; ¹⁵ N ₂ , 99%)	98.3%	1.25	1.25	1.48 ± 0.09
L-Glutamine (¹³ C ₅ , 99%; ¹⁵ N ₂ , 99%)	98.2%	2.50	2.50	2.52 ± 0.08
L-Glutamic Acid (¹³ C ₅ , 99%; ¹⁵ N, 99%)	99.9%	2.50	2.50	2.53 ± 0.05
Glycine (¹³ C ₂ , 99%; ¹⁵ N, 99%)	99.1%	2.50	2.50	2.58 ± 0.14
L-Histidine:HCl:H ₂ O (¹³ C ₆ , 97-99%; ¹⁵ N ₃ , 97-99%)	>98%	2.50	2.50	2.53 ± 0.21
L-Isoleucine (¹³ C ₆ , 99%; ¹⁵ N, 99%)	99.8%	2.50	2.50	2.59 ± 0.15
L-Leucine (¹³ C ₆ , 99%; ¹⁵ N, 99%)	99.9%	2.50	2.50	2.48 ± 0.12
L-Lysine:2HCl (¹³ C ₆ , 99%; ¹⁵ N ₂ , 99%)	99.4%	2.50	2.50	2.70 ± 0.10
L-Methionine (¹³ C ₅ , 99%; ¹⁵ N, 99%)	99.7%	2.50	2.50	2.55 ± 0.05
L-Phenylalanine (¹³ C ₉ , 99%; ¹⁵ N, 99%)	97.9%	2.50	2.50	2.43 ± 0.18
L-Proline (¹³ C ₅ , 99%; ¹⁵ N, 99%)	99.2%	2.50	2.50	2.57 ± 0.11
L-Serine (¹³ C ₃ , 99%; ¹⁵ N, 99%)	100%	2.50	2.50	2.56 ± 0.04
L-Threonine (¹³ C ₄ , 97-99%; ¹⁵ N, 97-99%)	99.6%	2.50	2.50	2.49 ± 0.10
L-Tryptophan (¹³ C ₁₁ , 99%; ¹⁵ N ₂ , 99%)	100%	2.50	2.50	2.44 ± 0.06
L-Tyrosine (¹³ C ₉ , 99%; ¹⁵ N, 99%)	99.0%	2.50	2.50	2.49 ± 0.09
L-Valine (¹³ C ₅ , 99%; ¹⁵ N, 99%)	98.3%	2.50	2.50	2.41 ± 0.22

Supplementary Figure 21. List of amino acids used in the analysis of water soluble metabolites (Table 19)

8. REFERENCES

- Aaltonen, M. J., Alecu, I., König, T., Bennett, S. A. L., & Shoubbridge, E. A. (2022). Serine palmitoyltransferase assembles at ER-mitochondria contact sites. *Life Science Alliance*, 5(2). https://doi.org/ARTN_e202101278
10.26508/lsa.202101278
- Al Sazzad, M. A., Yasuda, T., Murata, M., & Slotte, J. P. (2017). The Long-Chain Sphingoid Base of Ceramides Determines Their Propensity for Lateral Segregation. *Biophys J*, 112(5), 976-983. <https://doi.org/10.1016/j.bpj.2017.01.016>
- Allen, J. A., Halverson-Tamboli, R. A., & Rasenick, M. M. (2007). Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci*, 8(2), 128-140. <https://doi.org/10.1038/nrn2059>
- Antony, B., Vanni, S., Shindou, H., & Ferreira, T. (2015). From zero to six double bonds: phospholipid unsaturation and organelle function. *Trends in Cell Biology*, 25(7), 427-436. <https://doi.org/10.1016/j.tcb.2015.03.004>
- Arkhipov, A., Shan, Y., Das, R., Endres, N. F., Eastwood, M. P., Wemmer, D. E., Kuriyan, J., & Shaw, D. E. (2013). Architecture and membrane interactions of the EGF receptor. *Cell*, 152(3), 557-569. <https://doi.org/10.1016/j.cell.2012.12.030>
- Babiychuk, E. B., & Draeger, A. (2000). Annexins in cell membrane dynamics:: Ca-regulated association of lipid microdomains. *Journal of Cell Biology*, 150(5), 1113-1123. https://doi.org/DOI_10.1083/jcb.150.5.1113
- Barba-Bon, A., Nilam, M., & Hennig, A. (2020). Supramolecular Chemistry in the Biomembrane. *Chembiochem*, 21(7), 886-910. <https://doi.org/10.1002/cbic.201900646>
- Bardou, P., Mariette, J., Escudie, F., Djemiel, C., & Klopp, C. (2014). jvenn: an interactive Venn diagram viewer. *BMC Bioinformatics*, 15(1), 293. <https://doi.org/10.1186/1471-2105-15-293>
- Benaud, C., Gentil, B. J., Assard, N., Court, M., Garin, J., Delphin, C., & Baudier, J. (2004). AHNAK interaction with the annexin 2/S100A10 complex regulates cell membrane cytoarchitecture. *Journal of Cell Biology*, 164(1), 133-144. https://doi.org/DOI_10.1083/jcb.200307098
- Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society Series*

- B-Statistical Methodology*, 57(1), 289-300. <https://doi.org/DOI> 10.1111/j.2517-6161.1995.tb02031.x
- Bonadonna, M., Altamura, S., Tybl, E., Palais, G., Qatato, M., Polycarpou-Schwarz, M., Schneider, M., Kalk, C., Ruediger, W., Ertl, A., Anstee, N., Bogeska, R., Helm, D., Milsom, M. D., & Galy, B. (2022). Iron regulatory protein (IRP)-mediated iron homeostasis is critical for neutrophil development and differentiation in the bone marrow. *Science Advances*, 8(40). <https://doi.org/ARTN> eabq4469 10.1126/sciadv.abq4469
- Chakraborty, M., & Jiang, X. C. (2013). Sphingomyelin and Its Role in Cellular Signaling. *Lipid-Mediated Protein Signaling*, 991, 1-14. https://doi.org/10.1007/978-94-007-6331-9_1
- Chaves, A. B., Pinto, I. F. D., Dantas, L. S., Xavier, A. M., Inague, A., Faria, R. L., Medeiros, M. H. G., Glezer, I., Yoshinaga, M. Y., & Miyamoto, S. (2019). Alterations in lipid metabolism of spinal cord linked to amyotrophic lateral sclerosis. *Scientific Reports*, 9. <https://doi.org/ARTN> 11642 10.1038/s41598-019-48059-7
- Chen, Y., Yang, B., & Jacobson, K. (2004). Transient confinement zones: a type of lipid raft? *Lipids*, 39(11), 1115-1119. <https://doi.org/10.1007/s11745-004-1337-9>
- Concordet, J. P., & Haeussler, M. (2018). CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res*, 46(W1), W242-W245. <https://doi.org/10.1093/nar/gky354>
- Coskun, U., Grzybek, M., Drechsel, D., & Simons, K. (2011). Regulation of human EGF receptor by lipids. *Proc Natl Acad Sci U S A*, 108(22), 9044-9048. <https://doi.org/10.1073/pnas.1105666108>
- Cox, J., Hein, M. Y., Lubner, C. A., Paron, I., Nagaraj, N., & Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. *Mol Cell Proteomics*, 13(9), 2513-2526. <https://doi.org/10.1074/mcp.M113.031591>
- D'Aprile, C., Prioni, S., Mauri, L., Prinetti, A., & Grassi, S. (2021). Lipid rafts as platforms for sphingosine 1-phosphate metabolism and signalling. *Cellular Signalling*, 80. <https://doi.org/10.1016/j.cellsig.2021.109929>

- De Luca, A., Carotenuto, A., Rachiglio, A., Gallo, M., Maiello, M. R., Aldinucci, D., Pinto, A., & Normanno, N. (2008). The role of the EGFR signaling in tumor microenvironment. *J Cell Physiol*, 214(3), 559-567. <https://doi.org/10.1002/jcp.21260>
- Dranka, B. P., Hill, B. G., & Darley-USmar, V. M. (2010). Mitochondrial reserve capacity in endothelial cells: The impact of nitric oxide and reactive oxygen species. *Free Radic Biol Med*, 48(7), 905-914. <https://doi.org/10.1016/j.freeradbiomed.2010.01.015>
- Erwied, P., Gu, Y., Simon, L., Schneider, M., Helm, D., Michel, M. S., Nuhn, P., Nitschke, K., & Worst, T. S. (2024). Optimized workflow of EV enrichment from human plasma samples for downstream mass spectrometry analysis [preprint]. *Discover Oncology*. <https://doi.org/10.21203/rs.3.rs-3914589/v1>
- Feles, S., Overath, C., Reichardt, S., Diegeler, S., Schmitz, C., Kronenberg, J., Baumstark-Khan, C., Hemmersbach, R., Hellweg, C. E., & Liemersdorf, C. (2022). Streamlining Culture Conditions for the Neuroblastoma Cell Line SH-SY5Y: A Prerequisite for Functional Studies. *Methods Protoc*, 5(4). <https://doi.org/10.3390/mps5040058>
- Fessler, M. B., & Parks, J. S. (2011). Intracellular Lipid Flux and Membrane Microdomains as Organizing Principles in Inflammatory Cell Signaling. *Journal of Immunology*, 187(4), 1529-1535. <https://doi.org/10.4049/jimmunol.1100253>
- Gable, K., Han, G., Monaghan, E., Bacikova, D., Natarajan, M., Williams, R., & Dunn, T. M. (2002). Mutations in the yeast and genes, including those corresponding to the hereditary sensory neuropathy type I mutations, dominantly inactivate serine palmitoyltransferase. *Journal of Biological Chemistry*, 277(12), 10194-10200. <https://doi.org/10.1074/jbc.M107873200>
- Gerritsen, J. S., Faraguna, J. S., Bonavia, R., Furnari, F. B., & White, F. M. (2023). Predictive data-driven modeling of C-terminal tyrosine function in the EGFR signaling network. *Life Sci Alliance*, 6(8). <https://doi.org/10.26508/lsa.202201466>
- Goh, M. W. S., & Tero, R. (2021). Cholesterol-induced microdomain formation in lipid bilayer membranes consisting of completely miscible lipids. *Biochimica Et Biophysica Acta-Biomembranes*, 1863(8). <https://doi.org/ARTN183626> 10.1016/j.bbamem.2021.183626
- Golub, T., Wacha, S., & Caroni, P. (2004). Spatial and temporal control of signaling through lipid rafts. *Current Opinion in Neurobiology*, 14(5), 542-550. <https://doi.org/https://doi.org/10.1016/j.conb.2004.08.003>

- Grasmann, G., Mondal, A., & Leithner, K. (2021). Flexibility and Adaptation of Cancer Cells in a Heterogenous Metabolic Microenvironment. *International Journal of Molecular Sciences*, 22(3). https://doi.org/ARTN_1476_10.3390/ijms22031476
- Gupta, G., & Surolia, A. (2010). Glycosphingolipids in microdomain formation and their spatial organization. *Febs Letters*, 584(9), 1634-1641. <https://doi.org/10.1016/j.febslet.2009.11.070>
- Han, G., Gupta, S. D., Gable, K., Niranjanakumari, S., Moitra, P., Eichler, F., Brown, R. H., Jr., Harmon, J. M., & Dunn, T. M. (2009). Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proc Natl Acad Sci U S A*, 106(20), 8186-8191. <https://doi.org/10.1073/pnas.0811269106>
- Han, W.-Q., Xia, M., Xu, M., Boini, K. M., Ritter, J. K., Li, N.-J., & Li, P.-L. (2012). Lysosome fusion to the cell membrane is mediated by the dysferlin C2A domain in coronary arterial endothelial cells. *Journal of Cell Science*, 125(5), 1225-1234. <https://doi.org/10.1242/jcs.094565>
- Hanada, K. (2003). Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta*, 1632(1-3), 16-30. [https://doi.org/10.1016/s1388-1981\(03\)00059-3](https://doi.org/10.1016/s1388-1981(03)00059-3)
- Hannun, Y. A., & Obeid, L. M. (2018). Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol*, 19(3), 175-191. <https://doi.org/10.1038/nrm.2017.107>
- Harayama, T., & Riezman, H. (2018). Understanding the diversity of membrane lipid composition. *Nat Rev Mol Cell Biol*, 19(5), 281-296. <https://doi.org/10.1038/nrm.2017.138>
- Harmon, J. M., Bacikova, D., Gable, K., Gupta, S. D., Han, G., Sengupta, N., Somashekarappa, N., & Dunn, T. M. (2013). Topological and functional characterization of the ssSPTs, small activating subunits of serine palmitoyltransferase. *J Biol Chem*, 288(14), 10144-10153. <https://doi.org/10.1074/jbc.M113.451526>
- Hartmann, D., Lucks, J., Fuchs, S., Schiffmann, S., Schreiber, Y., Ferreirós, N., Merkens, J., Marschalek, R., Geisslinger, G., & Grösch, S. (2012). Long chain ceramides and very long chain ceramides have opposite effects on human breast and colon cancer cell growth. *International Journal of Biochemistry & Cell Biology*, 44(4), 620-628. <https://doi.org/10.1016/j.biocel.2011.12.019>

- Hofman, E. G., Ruonala, M. O., Bader, A. N., van den Heuvel, D., Voortman, J., Roovers, R. C., Verkleij, A. J., Gerritsen, H. C., & Henegouwen, P. M. P. V. (2008). EGF induces coalescence of different lipid rafts. *Journal of Cell Science*, 121(15), 2519-2528. <https://doi.org/10.1242/jcs.028753>
- Holopainen, J. M., Subramanian, M., & Kinnunen, P. K. (1998). Sphingomyelinase induces lipid microdomain formation in a fluid phosphatidylcholine/sphingomyelin membrane. *Biochemistry*, 37(50), 17562-17570. <https://doi.org/10.1021/bi980915e>
- Hornemann, T., Penno, A., Rutti, M. F., Ernst, D., Kivrak-Pfiffner, F., Rohrer, L., & von Eckardstein, A. (2009). The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. *J Biol Chem*, 284(39), 26322-26330. <https://doi.org/10.1074/jbc.M109.023192>
- Hsu, P. D., Scott, D. A., Weinstein, J. A., Ran, F. A., Konermann, S., Agarwala, V., Li, Y., Fine, E. J., Wu, X., Shalem, O., Cradick, T. J., Marraffini, L. A., Bao, G., & Zhang, F. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*, 31(9), 827-832. <https://doi.org/10.1038/nbt.2647>
- Huang, J., & Feigenson, G. W. (1999). A microscopic interaction model of maximum solubility of cholesterol in lipid bilayers. *Biophys J*, 76(4), 2142-2157. [https://doi.org/10.1016/S0006-3495\(99\)77369-8](https://doi.org/10.1016/S0006-3495(99)77369-8)
- Huber, W., von Heydebreck, A., Sultmann, H., Poustka, A., & Vingron, M. (2002). Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*, 18 Suppl 1, S96-104. https://doi.org/10.1093/bioinformatics/18.suppl_1.s96
- Hur, J. Y., Teranishi, Y., Kihara, T., Yamamoto, N. G., Inoue, M., Hosia, W., Hashimoto, M., Winblad, B., Frykman, S., & Tjernberg, L. O. (2012). Identification of Novel γ -Secretase-associated Proteins in Detergent-resistant Membranes from Brain. *Journal of Biological Chemistry*, 287(15), 11991-12005. <https://doi.org/10.1074/jbc.M111.246074>
- Isik, O. A., & Cizmecioglu, O. (2023). Rafting on the Plasma Membrane: Lipid Rafts in Signaling and Disease. *Adv Exp Med Biol*, 1436, 87-108. https://doi.org/10.1007/5584_2022_759
- Jennemann, R., Sandhoff, R., Langbein, L., Kaden, S., Rothermel, U., Gallala, H., Sandhoff, K., Wiegandt, H., & Grone, H. J. (2007). Integrity and barrier function of the epidermis

- critically depend on glucosylceramide synthesis. *J Biol Chem*, 282(5), 3083-3094. <https://doi.org/10.1074/jbc.M610304200>
- Johnson, J. L., Yaron, T. M., Huntsman, E. M., Kerelsky, A., Song, J., Regev, A., Lin, T. Y., Liberatore, K., Cizin, D. M., Cohen, B. M., Vasan, N., Ma, Y., Krismer, K., Robles, J. T., van de Kooij, B., van Vlimmeren, A. E., Andree-Busch, N., Kaufer, N. F., Dorovkov, M. V., . . . Cantley, L. C. (2023). An atlas of substrate specificities for the human serine/threonine kinome. *Nature*, 613(7945), 759-766. <https://doi.org/10.1038/s41586-022-05575-3>
- Jorissen, R. N., Walker, F., Pouliot, N., Garrett, T. P., Ward, C. W., & Burgess, A. W. (2003). Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res*, 284(1), 31-53. [https://doi.org/10.1016/s0014-4827\(02\)00098-8](https://doi.org/10.1016/s0014-4827(02)00098-8)
- Julien, S., Bobowski, M., Steenackers, A., Le Bourhis, X., & Delannoy, P. (2013). How Do Gangliosides Regulate RTKs Signaling? *Cells*, 2(4), 751-767. <https://www.mdpi.com/2073-4409/2/4/751>
- Kim, D. H., Triet, H. M., & Ryu, S. H. (2021). Regulation of EGFR activation and signaling by lipids on the plasma membrane. *Prog Lipid Res*, 83, 101115. <https://doi.org/10.1016/j.plipres.2021.101115>
- Kitatani, K., Idkowiak-Baldys, J., & Hannun, Y. A. (2008). The sphingolipid salvage pathway in ceramide metabolism and signaling. *Cell Signal*, 20(6), 1010-1018. <https://doi.org/10.1016/j.cellsig.2007.12.006>
- Kovacs, T., Zakany, F., & Nagy, P. (2022). It Takes More than Two to Tango: Complex, Hierarchical, and Membrane-Modulated Interactions in the Regulation of Receptor Tyrosine Kinases. *Cancers*, 14(4). https://doi.org/ARTN_94410.3390/cancers14040944
- Kovalevich, J., & Langford, D. (2013). Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods Mol Biol*, 1078, 9-21. https://doi.org/10.1007/978-1-62703-640-5_2
- Kurzchalia, T. V., & Parton, R. G. (1999). Membrane microdomains and caveolae. *Curr Opin Cell Biol*, 11(4), 424-431. [https://doi.org/10.1016/s0955-0674\(99\)80061-1](https://doi.org/10.1016/s0955-0674(99)80061-1)
- Lam, B. W. S., Yam, T. Y. A., Chen, C. P., Lai, M. K. P., Ong, W. Y., & Herr, D. R. (2021). The noncanonical chronicles: Emerging roles of sphingolipid structural variants. *Cell Signal*, 79, 109890. <https://doi.org/10.1016/j.cellsig.2020.109890>
- Laviad, E. L., Albee, L., Pankova-Kholmyansky, I., Epstein, S., Park, H., Merrill, A. H., Jr., & Futerman, A. H. (2008). Characterization of ceramide synthase 2: tissue distribution,

- substrate specificity, and inhibition by sphingosine 1-phosphate. *J Biol Chem*, 283(9), 5677-5684. <https://doi.org/10.1074/jbc.M707386200>
- Levental, I., Lingwood, D., Grzybek, M., Coskun, U., & Simons, K. (2010). Palmitoylation regulates raft affinity for the majority of integral raft proteins. *Proc Natl Acad Sci U S A*, 107(51), 22050-22054. <https://doi.org/10.1073/pnas.1016184107>
- Li, B., Qin, Y., Yu, X., Xu, X., & Yu, W. (2022). Lipid raft involvement in signal transduction in cancer cell survival, cell death and metastasis. *Cell Prolif*, 55(1), e13167. <https://doi.org/10.1111/cpr.13167>
- Li, S., Xie, T., Liu, P., Wang, L., & Gong, X. (2021). Structural insights into the assembly and substrate selectivity of human SPT-ORMDL3 complex. *Nat Struct Mol Biol*, 28(3), 249-257. <https://doi.org/10.1038/s41594-020-00553-7>
- Lietha, D., & Izard, T. (2020). Roles of Membrane Domains in Integrin-Mediated Cell Adhesion. *International Journal of Molecular Sciences*, 21(15). <https://doi.org/10.3390/ijms21155531>
- Lim, H. J., Kang, D. H., Lim, J. M., Kang, D. M., Seong, J. K., Kang, S. W., & Bae, Y. S. (2013). Function of Ahnak protein in aortic smooth muscle cell migration through Rac activation. *Cardiovascular Research*, 97(2), 302-310. <https://doi.org/10.1093/cvr/cvs311>
- Lopes, F. M., Schröder, R., da Frota, M. L. C., Zanotto, A., Müller, C. B., Pires, A. S., Meurer, R. T., Colpo, G. D., Gelain, D. P., Kapczinski, F., Moreira, J. C. F., Fernandes, M. D., & Klamt, F. (2010). Comparison between proliferative and neuron-like SH-SY5Y cells as an model for Parkinson disease studies. *Brain Research*, 1337, 85-94. <https://doi.org/10.1016/j.brainres.2010.03.102>
- Massey, J. B. (2001). Interaction of ceramides with phosphatidylcholine, sphingomyelin and sphingomyelin/cholesterol bilayers. *Biochim Biophys Acta*, 1510(1-2), 167-184. [https://doi.org/10.1016/s0005-2736\(00\)00344-8](https://doi.org/10.1016/s0005-2736(00)00344-8)
- Matrella, M. L., Valletti, A., Gigante, I., De Rasmio, D., Signorile, A., Russo, S., Lobasso, S., Lobraico, D., Dibattista, M., Pacelli, C., & Cocco, T. (2024). High OXPHOS efficiency in RA-FUdr-differentiated SH-SY5Y cells: involvement of cAMP signalling and respiratory supercomplexes. *Sci Rep*, 14(1), 7411. <https://doi.org/10.1038/s41598-024-57613-x>

- Maula, T., Artetxe, I., Grandell, P. M., & Slotte, J. P. (2012). Importance of the sphingoid base length for the membrane properties of ceramides. *Biophys J*, 103(9), 1870-1879. <https://doi.org/10.1016/j.bpj.2012.09.018>
- Maxfield, F. R. (2002). Plasma membrane microdomains. *Curr Opin Cell Biol*, 14(4), 483-487. [https://doi.org/10.1016/s0955-0674\(02\)00351-4](https://doi.org/10.1016/s0955-0674(02)00351-4)
- Mayor, S., & Maxfield, F. R. (1995). Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. *Mol Biol Cell*, 6(7), 929-944. <https://doi.org/10.1091/mbc.6.7.929>
- McCampbell, A., Truong, D., Broom, D. C., Allchorne, A., Gable, K., Cutler, R. G., Mattson, M. P., Woolf, C. J., Frosch, M. P., Harmon, J. M., Dunn, T. M., & Brown, R. H. (2005). Mutant SPTLC1 dominantly inhibits serine palmitoyltransferase activity and confers an age-dependent neuropathy. *Human Molecular Genetics*, 14(22), 3507-3521. <https://doi.org/10.1093/hmg/ddi380>
- Megha, & London, E. (2004). Ceramide selectively displaces cholesterol from ordered lipid domains (rafts): implications for lipid raft structure and function. *J Biol Chem*, 279(11), 9997-10004. <https://doi.org/10.1074/jbc.M309992200>
- Mihaly, S. R., Ninomiya-Tsuji, J., & Morioka, S. (2014). TAK1 control of cell death. *Cell Death and Differentiation*, 21(11), 1667-1676. <https://doi.org/10.1038/cdd.2014.123>
- Mirkin, B. L., Clark, S. H., & Zhang, C. (2002). Inhibition of human neuroblastoma cell proliferation and EGF receptor phosphorylation by gangliosides GM, GM GD and GT. *Cell Proliferation*, 35(2), 105-115. <https://doi.org/DOI> 10.1046/j.1365-2184.2002.00228.x
- Mizutani, Y., Kihara, A., & Igarashi, Y. (2005). Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. *Biochem J*, 390(Pt 1), 263-271. <https://doi.org/10.1042/BJ20050291>
- Mollinedo, F., & Gajate, C. (2020). Lipid rafts as signaling hubs in cancer cell survival/death and invasion: implications in tumor progression and therapy. *Journal of Lipid Research*, 61(5), 611-635. <https://doi.org/10.1194/jlr.TR119000439>
- Moriki, T., Maruyama, H., & Maruyama, I. N. (2001). Activation of preformed EGF receptor dimers by ligand-induced rotation of the transmembrane domain. *J Mol Biol*, 311(5), 1011-1026. <https://doi.org/10.1006/jmbi.2001.4923>

- Muller, T., Kalxdorf, M., Longuespee, R., Kazdal, D. N., Stenzinger, A., & Krijgsveld, J. (2020). Automated sample preparation with SP3 for low-input clinical proteomics. *Mol Syst Biol*, 16(1), e9111. <https://doi.org/10.15252/msb.20199111>
- Murillo, J. R., Goto-Silva, L., Sanchez, A., Nogueira, F. C. S., Domont, G. B., & Junqueira, M. (2017). Quantitative proteomic analysis identifies proteins and pathways related to neuronal development in differentiated SH-SY5Y neuroblastoma cells. *EuPA Open Proteom*, 16, 1-11. <https://doi.org/10.1016/j.euprot.2017.06.001>
- Nica, A. F., Tsao, C. C., Watt, J. C., Jiffar, T., Kurinna, S., Jurasz, P., Konopleva, M., Andreeff, M., Radomski, M. W., & Ruvolo, P. P. (2008). Ceramide promotes apoptosis in chronic myelogenous leukemia-derived K562 cells by a mechanism involving caspase-8 and JNK. *Cell Cycle*, 7(21), 3362-3370. https://doi.org/DOI_10.4161/cc.7.21.6894
- Nicolau, D. V., Burrage, K., Parton, R. G., & Hancock, J. F. (2006). Identifying optimal lipid raft characteristics required to promote nanoscale protein-protein interactions on the plasma membrane. *Molecular and Cellular Biology*, 26(1), 313-323. <https://doi.org/10.1128/Mcb.26.1.313-323.2006>
- Okonechnikov, K., Golosova, O., Fursov, M., & Team, U. (2012). Unipro UGENE: a unified bioinformatics toolkit. *Bioinformatics*, 28(8), 1166-1167. <https://doi.org/10.1093/bioinformatics/bts091>
- Oliferenko, S., Paiha, K., Harder, T., Gerke, V., Schwarzler, C., Schwarz, H., Beug, H., Gunthert, U., & Huber, L. A. (1999). Analysis of CD44-containing lipid rafts: Recruitment of annexin II and stabilization by the actin cytoskeleton. *J Cell Biol*, 146(4), 843-854. <https://doi.org/10.1083/jcb.146.4.843>
- Oliva, C. R., Nozell, S. E., Diers, A., McClugage, S. G., 3rd, Sarkaria, J. N., Markert, J. M., Darley-USmar, V. M., Bailey, S. M., Gillespie, G. Y., Landar, A., & Griguer, C. E. (2010). Acquisition of temozolomide chemoresistance in gliomas leads to remodeling of mitochondrial electron transport chain. *J Biol Chem*, 285(51), 39759-39767. <https://doi.org/10.1074/jbc.M110.147504>
- Pilz, R., Opalka, L., Majcher, A., Grimm, E., Van Maldergem, L., Mihalceanu, S., Schakel, K., Enk, A., Aubin, F., Bursztejn, A. C., Brischoux-Boucher, E., Fischer, J., & Sandhoff, R. (2022). Formation of keto-type ceramides in palmoplantar keratoderma based on biallelic KDSR mutations in patients. *Human Molecular Genetics*, 31(7), 1105-1114. <https://doi.org/10.1093/hmg/ddab309>

- Pinto, S. N., Silva, L. C., Futerman, A. H., & Prieto, M. (2011). Effect of ceramide structure on membrane biophysical properties: the role of acyl chain length and unsaturation. *Biochim Biophys Acta*, 1808(11), 2753-2760. <https://doi.org/10.1016/j.bbamem.2011.07.023>
- Pons, A., Timmerman, P., Leroy, Y., & Zanetta, J. P. (2002). Gas-chromatography/mass-spectrometry analysis of human skin constituents as heptafluorobutyrate derivatives with special reference to long-chain bases. *Journal of Lipid Research*, 43(5), 794-804. <Go to ISI>:/WOS:000175398800017
- Quinville, B. M., Deschenes, N. M., Ryckman, A. E., & Walia, J. S. (2021). A Comprehensive Review: Sphingolipid Metabolism and Implications of Disruption in Sphingolipid Homeostasis. *Int J Mol Sci*, 22(11). <https://doi.org/10.3390/ijms22115793>
- Rabionet, M., Engel, R., & Sandhoff, R. (2023). Chapter 1 - Structure and function of mammalian sphingolipids in health and disease. In J. M. Ntambi (Ed.), *Cellular Lipid in Health and Disease* (pp. 1-65). Academic Press. <https://doi.org/https://doi.org/10.1016/B978-0-323-95582-9.00016-4>
- Reynolds, C. P., Maurer, B. J., & Kolesnick, R. N. (2004). Ceramide synthesis and metabolism as a target for cancer therapy. *Cancer Letters*, 206(2), 169-180. <https://doi.org/10.1016/j.canlet.2003.08.034>
- Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res*, 43(7), e47. <https://doi.org/10.1093/nar/gkv007>
- Rosenberg, A., & Stern, N. (1966). Changes in Sphingosine and Fatty Acid Components of Gangliosides in Developing Rat and Human Brain. *Journal of Lipid Research*, 7(1), 122-+. <Go to ISI>:/WOS:A19667144400020
- Sabbah, D. A., Hajjo, R., & Sweidan, K. (2020). Review on Epidermal Growth Factor Receptor (EGFR) Structure, Signaling Pathways, Interactions, and Recent Updates of EGFR Inhibitors. *Curr Top Med Chem*, 20(10), 815-834. <https://doi.org/10.2174/1568026620666200303123102>
- Salazar-Cavazos, E., Nitta, C. F., Mitra, E. D., Wilson, B. S., Lidke, K. A., Hlavacek, W. S., & Lidke, D. S. (2020). Multisite EGFR phosphorylation is regulated by adaptor protein abundances and dimer lifetimes. *Molecular Biology of the Cell*, 31(7), 695-708. <https://doi.org/10.1091/mbc.E19-09-0548>

- Saltiel, A. R., & Pessin, J. E. (2003). Insulin signaling in microdomains of the plasma membrane. *Traffic*, 4(11), 711-716. <https://doi.org/DOI> 10.1034/j.1600-0854.2003.00119.x
- Sandhoff, R., & Sandhoff, K. (2023). Neuronal Ganglioside and Glycosphingolipid (GSL) Metabolism and Disease : Cascades of Secondary Metabolic Errors Can Generate Complex Pathologies (in LSDs). *Adv Neurobiol*, 29, 333-390. https://doi.org/10.1007/978-3-031-12390-0_12
- Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc*, 3(6), 1101-1108. <https://doi.org/10.1038/nprot.2008.73>
- Schneider, L., Giordano, S., Zelickson, B. R., M, S. J., G, A. B., Ouyang, X., Fineberg, N., Darley-Usmar, V. M., & Zhang, J. (2011). Differentiation of SH-SY5Y cells to a neuronal phenotype changes cellular bioenergetics and the response to oxidative stress. *Free Radic Biol Med*, 51(11), 2007-2017. <https://doi.org/10.1016/j.freeradbiomed.2011.08.030>
- Schneider, P. B., & Kennedy, E. P. (1967). Sphingomyelinase in normal human spleens and in spleens from subjects with Niemann-Pick disease. *J Lipid Res*, 8(3), 202-209. <https://www.ncbi.nlm.nih.gov/pubmed/4962590>
- Schwanhausser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W., & Selbach, M. (2011). Global quantification of mammalian gene expression control. *Nature*, 473(7347), 337-342. <https://doi.org/10.1038/nature10098>
- Sengupta, P., Baird, B., & Holowka, D. (2007). Lipid rafts, fluid/fluid phase separation, and their relevance to plasma membrane structure and function. *Seminars in Cell & Developmental Biology*, 18(5), 583-590. <https://doi.org/10.1016/j.semcdb.2007.07.010>
- Sezgin, E., Levental, I., Mayor, S., & Eggeling, C. (2017). The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nature Reviews Molecular Cell Biology*, 18(6), 361-374. <https://doi.org/10.1038/nrm.2017.16>
- Shipley, M. M., Mangold, C. A., & Szpara, M. L. (2016). Differentiation of the SH-SY5Y Human Neuroblastoma Cell Line. *J Vis Exp*(108), 53193. <https://doi.org/10.3791/53193>
- Shirakabe, K., Yamaguchi, K., Shibuya, H., Irie, K., Matsuda, S., Moriguchi, T., Gotoh, Y., Matsumoto, K., & Nishida, E. (1997). TAK1 mediates the ceramide signaling to stress-activated protein kinase/c-Jun N-terminal kinase. *J Biol Chem*, 272(13), 8141-8144. <https://doi.org/10.1074/jbc.272.13.8141>

- Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., & Di Fiore, P. P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Developmental Cell*, 15(2), 209-219. <https://doi.org/10.1016/j.devcel.2008.06.012>
- Simons, K., & Ikonen, E. (1997). Functional rafts in cell membranes. *Nature*, 387(6633), 569-572. <https://doi.org/10.1038/42408>
- Sonnino, S., & Chigorno, V. (2000). Ganglioside molecular species containing C18- and C20-sphingosine in mammalian nervous tissues and neuronal cell cultures. *Biochim Biophys Acta*, 1469(2), 63-77. [https://doi.org/10.1016/s0005-2736\(00\)00210-8](https://doi.org/10.1016/s0005-2736(00)00210-8)
- Sonnino, S., & Prinetti, A. (2010). Lipids and membrane lateral organization. *Frontiers in Physiology*, 1. <https://doi.org/ARTN> 153 10.3389/fphys.2010.00153
- Stekhoven, D. J., & Buhlmann, P. (2012). MissForest--non-parametric missing value imputation for mixed-type data. *Bioinformatics*, 28(1), 112-118. <https://doi.org/10.1093/bioinformatics/btr597>
- Stewart, M. E., & Downing, D. T. (1995). Free sphingosines of human skin include 6-hydroxysphingosine and unusually long-chain dihydrosphingosines. *J Invest Dermatol*, 105(4), 613-618. <https://doi.org/10.1111/1523-1747.ep12323736>
- Suzuki, M., Ohno, Y., & Kihara, A. (2022). Whole picture of human stratum corneum ceramides, including the chain-length diversity of long-chain bases. *J Lipid Res*, 63(7), 100235. <https://doi.org/10.1016/j.jlr.2022.100235>
- Takayama, M., Maeda, S., Watanabe, D., Takebayashi, K., Hiroshima, M., & Ueda, M. (2024). Cholesterol suppresses spontaneous activation of EGFR-mediated signal transduction. *Biochem Biophys Res Commun*, 704, 149673. <https://doi.org/10.1016/j.bbrc.2024.149673>
- Teppola, H., Sarkanen, J. R., Jalonen, T. O., & Linne, M. L. (2016). Morphological Differentiation Towards Neuronal Phenotype of SH-SY5Y Neuroblastoma Cells by Estradiol, Retinoic Acid and Cholesterol. *Neurochem Res*, 41(4), 731-747. <https://doi.org/10.1007/s11064-015-1743-6>
- Thankamony, S. P., & Knudson, W. (2006). Acylation of CD44 and its association with lipid rafts are required for receptor and hyaluronan endocytosis. *Journal of Biological Chemistry*, 281(45), 34601-34609. <https://doi.org/10.1074/jbc.M601530200>

- Tidhar, R., & Futerman, A. H. (2013). The complexity of sphingolipid biosynthesis in the endoplasmic reticulum. *Biochim Biophys Acta*, 1833(11), 2511-2518. <https://doi.org/10.1016/j.bbamcr.2013.04.010>
- Tyanova, S., & Cox, J. (2018). Perseus: A Bioinformatics Platform for Integrative Analysis of Proteomics Data in Cancer Research. *Methods Mol Biol*, 1711, 133-148. https://doi.org/10.1007/978-1-4939-7493-1_7
- Tyanova, S., Temu, T., & Cox, J. (2016). The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat Protoc*, 11(12), 2301-2319. <https://doi.org/10.1038/nprot.2016.136>
- van Meer, G., Voelker, D. R., & Feigenson, G. W. (2008). Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*, 9(2), 112-124. <https://doi.org/10.1038/nrm2330>
- Wang, C. W., Sun, M., Shao, C. X., Schlicker, L., Zhuo, Y., Harim, Y., Peng, T. P., Tian, W. L., Stoeffler, N., Schneider, M., Helm, D., Chu, Y. J., Fu, B. B., Jin, X. L., Mallm, J. P., Mall, M., Wu, Y. H., Schulze, A., & Liu, H. K. (2024). A multidimensional atlas of human glioblastoma-like organoids reveals highly coordinated molecular networks and effective drugs. *Npj Precision Oncology*, 8(1). <https://doi.org/ARTN> 19 10.1038/s41698-024-00500-5
- Wang, Y., Niu, Y., Zhang, Z., Gable, K., Gupta, S. D., Somashekarappa, N., Han, G., Zhao, H., Myasnikov, A. G., Kalathur, R. C., Dunn, T. M., & Lee, C. H. (2021). Structural insights into the regulation of human serine palmitoyltransferase complexes. *Nat Struct Mol Biol*, 28(3), 240-248. <https://doi.org/10.1038/s41594-020-00551-9>
- Wattenberg, B. W. (2021). Kicking off sphingolipid biosynthesis: structures of the serine palmitoyltransferase complex. *Nat Struct Mol Biol*, 28(3), 229-231. <https://doi.org/10.1038/s41594-021-00562-0>
- Wee, P., & Wang, Z. (2017). Epidermal Growth Factor Receptor Cell Proliferation Signaling Pathways. *Cancers (Basel)*, 9(5). <https://doi.org/10.3390/cancers9050052>
- Wee, P., & Wang, Z. (2018). Regulation of EGFR Endocytosis by CBL During Mitosis. *Cells*, 7(12). <https://doi.org/10.3390/cells7120257>
- Willaime-Morawek, S., Brami-Cherrier, K., Mariani, J., Caboche, J., & Brugg, B. (2003). c-Jun N-terminal kinases/c-Jun and p38 pathways cooperate in ceramide-induced neuronal apoptosis. *Neuroscience*, 119(2), 387-397. [https://doi.org/10.1016/S0306-4522\(02\)00996-X](https://doi.org/10.1016/S0306-4522(02)00996-X)

- Xu, X., Bittman, R., Duportail, G., Heissler, D., Vilcheze, C., & London, E. (2001). Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide. *J Biol Chem*, 276(36), 33540-33546. <https://doi.org/10.1074/jbc.M104776200>
- Yamaji, T., Sekizuka, T., Tachida, Y., Sakuma, C., Morimoto, K., Kuroda, M., & Hanada, K. (2019). A CRISPR Screen Identifies LAPT4A and TM9SF Proteins as Glycolipid-Regulating Factors. *Science*, 11, 409-+. <https://doi.org/10.1016/j.isci.2018.12.039>
- Yoon, S.-J., Nakayama, K.-i., Hikita, T., Handa, K., & Hakomori, S.-i. (2006). Epidermal growth factor receptor tyrosine kinase is modulated by GM3 interaction with N-linked GlcNAc termini of the receptor. *Proceedings of the National Academy of Sciences*, 103(50), 18987-18991. <https://doi.org/doi:10.1073/pnas.0609281103>
- Yu, C., Alterman, M., & Dobrowsky, R. T. (2005). Ceramide displaces cholesterol from lipid rafts and decreases the association of the cholesterol binding protein caveolin-1. *J Lipid Res*, 46(8), 1678-1691. <https://doi.org/10.1194/jlr.M500060-JLR200>
- Zaman, S. N., Resek, M. E., & Robbins, S. M. (2008). Dual acylation and lipid raft association of Src-family protein tyrosine kinases are required for SDF-1/CXCL12-mediated chemotaxis in the Jurkat human T cell lymphoma cell line. *Journal of Leukocyte Biology*, 84(4), 1082-1091. <https://doi.org/10.1189/jlb.1007698>
- Zhao, L. H., Spassieva, S., Gable, K., Gupta, S. D., Shi, L. Y., Wang, J. P., Bielawski, J., Hicks, W. L., Krebs, M. P., Naggert, J., Hannun, Y. A., Dunn, T. M., & Nishina, P. M. (2015). Elevation of 20-carbon long chain bases due to a mutation in serine palmitoyltransferase small subunit b results in neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America*, 112(42), 12962-12967. <https://doi.org/10.1073/pnas.1516733112>
- Zhuo, D., & Guan, F. (2020). Corrigendum: Ganglioside GM1 promotes contact inhibition of growth by regulating the localization of epidermal growth factor receptor from glycosphingolipid-enriched microdomain to caveolae Article DOI: 10.1111/cpr.12639. *Cell Prolif*, 53(1), e12748. <https://doi.org/10.1111/cpr.12748>

CORRIGENDUM

3. RESULTS

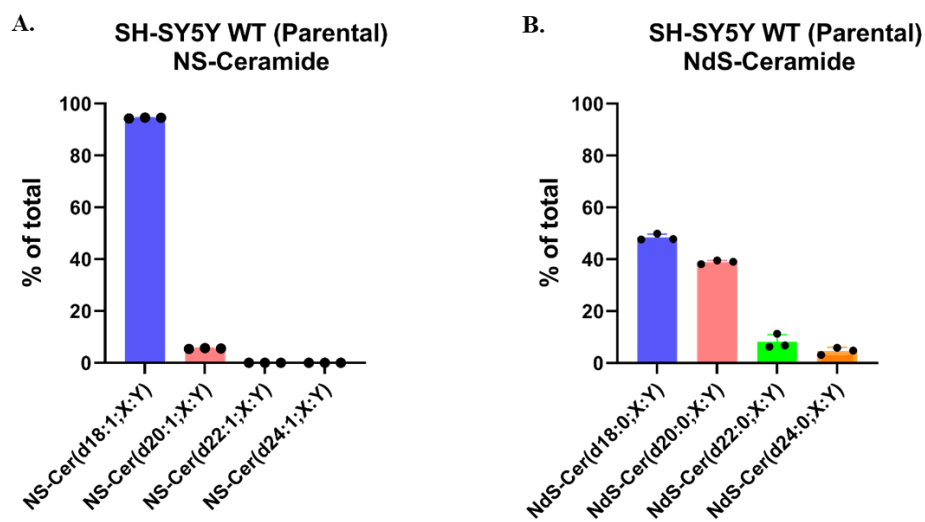


Figure 8. Distribution of NS-ceramides or NdS- ceramides containing 18-, 20-, 22- or 24- carbon sphingoid bases with various fatty acyl chain length in SH-SY5Y wt (Parental) cells. Calculations were performed in LC-MS/MS data of Figure 7.