Identification and characterization of a novel microsomal enzyme involved in cardiolipin synthesis and remodeling

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored, see: Statement of Contributions in this thesis. This is a true copy of the thesis, including any required final revisions, as requested by my examiners.

I understand that this thesis may be made electronically available to the public.

Statement of Contributions

Chapter 2 of this thesis contains material that has been previously published in the journal Molecular Nutrition and Food Research. The contributions of the authors are listed below:

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Abstract

The Harvey-Ras-like tumor suppressor (HRASLS) enzymes are a homologous group of proteins belonging to the *H-rev107* gene family that are part of a larger papain-like N1pC/P60 superfamily of vertebrate thiol proteases. Five HRASLS enzymes have been identified in humans, with three (*Hrasls1,3,5*) conserved in rodents. Some HRASLS enzymes have been characterized as class II tumor suppressors, but recent studies have focused on their roles in phospholipid metabolism. HRASLS enzymes share sequence homology with lecithin:retinol acyltransferases (LRATs), including a conserved NCEHFV motif in the C-terminal region that is critical for acylation and de-acylation reactions. All members of this enzyme family display varying levels of O-transacylase, N-transacylase, and phospholipase A1/2 activity *in vitro*. However, the biochemical function of these enzymes has not been fully explored. I tested the effect of overexpressing *Hrasls1* in HEK-293 cells, and found a specific increase in total cardiolipin content that had not previously been reported. Thus, the aim of this thesis was to investigate a novel role for HRASLS1 in phospholipid and, more specifically, cardiolipin metabolism. The first objective of this thesis was to define the biochemical function of HRASLS1 in the context of cellular phospholipid metabolism. Endogenous HRASLS1 was found to localize to the endoplasmic reticulum. Evidence from crude lysates from HEK-293 overexpressing cells, and reticulocyte lysates, suggested that HRASLS1 functions as a phosphatidylcholine (PC): monolysocardiolipin (MLCL) transacylase in the synthesis of CL. The second objective of this thesis was to investigate the effects of *Hrasls1* overexpression on cellular phospholipid content and composition *in vivo*, and related effects on mitochondrial physiology. HEK-293 cells overexpressing HRASLS1 had an ~62% higher content of cardiolipin as compared to controls, but no significant increases in any other major phospholipid classes. Interestingly, mitochondrial respiration was impaired in these cells, although mitochondrial DNA content was not different, indicating that total mitochondria had not decreased. Overexpression of HRASLS1 in HEK-293 cells significantly up-regulated the mRNA expression of multiple genes involved in phospholipid biosynthesis, suggesting that *Hrasls1* overexpression may also indirectly alter cardiolipin metabolism through cell signaling that could target transcriptional regulation. HRASLS1 has been demonstrated previously to synthesize N-acylphosphatidylethanolamines (NAPEs) *in vitro*, which increases the down-stream synthesis of bioactive N-acylethanolamines (NAEs). However, treatment of cells with oleoylethanolamine (OEA), stearoylethanolamine (SEA), or palmitoylethanolamine (PEA) did not

significantly increase cardiolipin content in HEK-293 cells. This suggested either that NAEs do not play a significant role in modulating the cardiolipin biosynthetic effects of HRASLS1, or that an NAE other than OEA, SEA, or PEA may be involved in mediating the induction of phospholipid metabolizing genes in *Hrasls1*-overexpressing HEK-293 cells. The final objective of this thesis was to assess the ability of OEA, one of the most abundant cellular NAEs, to modulate cardiolipin content and composition in a model of cellular cardiolipin deficiency. This work was performed in parallel with the studies on OEA, SEA, and PEA in HEK-293 cells, and therefore the negative results of that work did not inform the choice of NAE species. Cultured B-lymphocytes from an individual with Barth Syndrome (BTHS) were utilized as a study model. BTHS is a rare, X-linked genetic disorder that stems from mutations in Tafazzin, a cardiolipin fatty acid remodeling enzyme. Due to their inability to remodel the fatty acyl chains on cardiolipin following *de novo* synthesis, patients with BTHS have decreased cardiolipin content, which leads to a disease state, including heart failure, growth delay, and cyclic neutropenia. BTHS B-lymphocytes treated with 1 μM OEA exhibited a partial rescue of characteristic growth and cardiolipin deficiencies. This was surprising, given that: 1) OEA did not increase cardiolipin in HEK-293 cells, and 2) that a final collaborative characterization study found that HEK-293 cells overexpressing *Hrasls1* had significantly *decreased* OEA and PEA levels relative to control. Of interest, that study also found that *Hrasls1* overexpression may raise levels of linoleoylethanolamine (LEA), strongly suggesting that future studies should examine this target NAE in the context of both cardiolipin synthesis and BTHS treatment. Thus, HRASLS1 is a microsomal PC:MLCL transacylase that increases cardiolipin in cells. Study of this enzyme and related pathways may provide novel therapeutic targets for disorders of cardiolipin metabolism.

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Dedication

This thesis is dedicated to my family. To my parents Paul and Ronit Bradley, to my sister Meghan, and to my nana Anne.

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List of Abbreviations

1, 2-DAG – 1, 2-diacylglcyerol
1-AG – 1-arachidonylglycerol
2-AG – 2-arachidonylglycerol
αTFP – α-trifunctional protein
ACSL4 – acyl-CoA synthase long chain family member 4
AEA – anandamide
AGPAT – acylglycerophosphate acyltransferase
ALA – α-linolenic acid
ALCAT1 – acyl-CoA:lysocardiolipin acyltransferase 1
AdPLA – adipose-specific phospholipase A2
BD – basal diet
BTHS – Barth Syndrome
CB1 – cannabinoid receptor of the central type
CB2 – cannabinoid receptor of the peripheral type
CCCP – carbonyl cyanide *m*-chlorophenyl hydrazone
CDP-DAG – cytidine diphosphate diacylglycerol
CDS – phosphatidate cytidylyltransferase
CL – cardiolipin
CL-ND – cardiolipin nanodisk
CLS – cardiolipin synthase
Ca-NAT – calcium-dependent N-acyltransferase
Co-IP – co-immunoprecipitation
DAG – diacylglycerol
DGAT – diacylglycerol acyltransferase
DHA – docosahexaenoic acid
DHEA – docosahexaenoyl ethanolamine
DLCL – dilysocardiolipin
DTT – dithiothreitol
ER – endoplasmic reticulum
ERT – enzyme replacement therapy
ETC – electron transport chain
FAAH – fatty acid amide hydrolase
FBS – fetal bovine serum
FISH – fluorescent in situ hybridization
GC – gas chromatography
GDE1 – glycerophosphodiester phosphodiesterase 1
GFP – green fluorescent protein
GP-NAE – glycerophospho-N-palmitoyl ethanolamine
GPAT – glycerol-3-phosphate acyltransferase
GPR119 – G-protein coupled receptor 119
GPR55 – G-protein coupled receptor 55
HCO – hydrogenated coconut oil
HEK-293 – human embryonic kidney 293 cells
HRASLS – Harvey-ras like suppressor

HUFA – highly unsaturated fatty acid
IFU – infectious units
iNAT – calcium-independent N-acyltransferase
LEA – linoleoylethanolamine
LPA – lysophosphatidic acid
LPAAT – lysophosphatidic acid acyltransferase
LPC – lysophosphatidylcholine
LPE – lysophosphatidylethanolamine
LPG – lysophosphatidylglycerol
LPI – lysophosphatidylinositol
LPS – lysophosphatidylserine
LRAT – lecithin:retinol acyltransferase
LRT – lipid replacement therapy
LS-MS/MS – liquid chromatography tandem mass spectrometry
MAM – mitochondrial-associated ER membranes
MHC – major histocompatibility complex
MLCL – monolysocardiolipin
MLCL-AT1 – monolysocardiolipin acyltransferase
MOI – multiplicity of infection
MT-ND1 – NADH-ubiquinone oxidoreductase chain 1
MT-TL1- mitochondrially encoded tRNA leucine 1
MUFA – monounsaturated fatty acid
MudPIT – multidimensional protein identification technology
NAAA – N-acylethanolamine acid amidase
NAE – N-acylethanolamine
NAPE – N-acylphosphatidylethanolamine
NAPE-PLD – N-acylphosphatidylethanolamine phospholipase D
NPPE – N-palmitoyl phosphatidylethanolamine
OEA – oleoylethanolamine
P/G/M – pyruvate/glutamate/malate
PA – phosphatidic acid
PAP – phosphatidate phosphatase
PC – phosphatidylcholine
PDH – pyruvate dehydrogenase
PE – phosphatidylethanolamine
PEA – palmitoylethanolamine
PG – phosphatidylglycerol
PGP – phosphatidylglycerol phosphate
PI – phosphatidylinositol
PIP –phosphatidate phosphatase
PLA/AT – phospholipases/acyltransferases
PLA2GXVI – phospholipase A2 group XVI
PLC – phospholipase C
PLS3 – phospholipid scramblase 3
PPARα - peroxisome proliferator-activated receptor alpha
PS – phosphatidylserine

PSS1- phosphatidylserine synthase 1
PSS2 – phosphatidylserine synthase 2
PTPN22 – protein tyrosine phosphatase, non-receptor type 22
PUFA – polyunsaturated fatty acid
rAAV – recombinant adeno-associated virus
RARRES3 – retinoic acid receptor responder protein 3
RIG1 – retinoic acid-inducible gene 1
RLP1 – rat lecithin:retinol acyltransferase-like protein 1
ROS – reactive oxygen species
ROX – residual oxygen consumption
RPMI – Roswell Park Memorial Institute medium
RT-qPCR – real-time reverse transcriptase PCR
SCD1 – stearoyl-CoA desaturase 1
SEA – stearoylethanolamine
SFA – saturated fatty acid
SHIP1 - src homology 2 domain-containing inositol-5-phosphatase 1
sPLA2 - group Ib secretory phospholipase A2
sn – stereospecifically numbered
SUIT – substrate uncoupled inhibitor titration
TAG – triacylglycerol
TAZ – tafazzin
TAZ-KD – tafazzin knock-down mice
TAZ-KO – tafazzin knockout mice
TFP – trifunctional protein
TIG3 - tazarotene-induced gene 3
TLC – thin layer chromatography
TMPD - *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine
TRPV1 – transient vanilloid receptor type 1
UFA – unsaturated fatty acid
WAT – white adipose tissue

Chapter One
General Introduction

The maintenance of cellular homeostasis is dependent on the unique composition of biological membranes. The principal framework of all eukaryotic membranes is the lipid bilayer, a dynamic, semi-fluid membrane that is composed primarily of phospholipids. Phospholipids are amphipathic molecules comprised of both polar sections, *i.e.* a glycerol 'backbone', and a phosphate 'head group', and non-polar sections, *i.e.* fatty acyl 'tails' (see Figure 1 for a general schematic of phospholipid structure). The initial fatty acyl composition of *de novo* synthesized phospholipids is regulated by the specific glycerophosphate-acyltransferase and acyl-glycerophosphate acyltransferase enzymes involved, which each have their own substrate specificities. Typically, however, phospholipids are remodeled to contain saturated fatty acids (SFA) esterified at the stereospecifically numbered 1st carbon (*sn-1)* position, and unsaturated fatty acids (UFA) esterified at the *sn-2* position [1], although this can be variable depending on the individual phospholipid. The third carbon is esterified to a phosphate with a polar head group (**R**) attached. The type of polar head group will determine the class of phospholipid. For example, a choline head group or ethanolamine head group would result in classification of this phospholipid as phosphatidylcholine (PC), or phosphatidylethanolamine (PE), respectively. If no polar head group is attached (*i.e.* R=H, Figure 1), the phospholipid is a phosphatidic acid (PA). Thus, based on variation in the fatty acyl species, positional specificities, and polar head groups, the array of phospholipids created in cells is staggering.

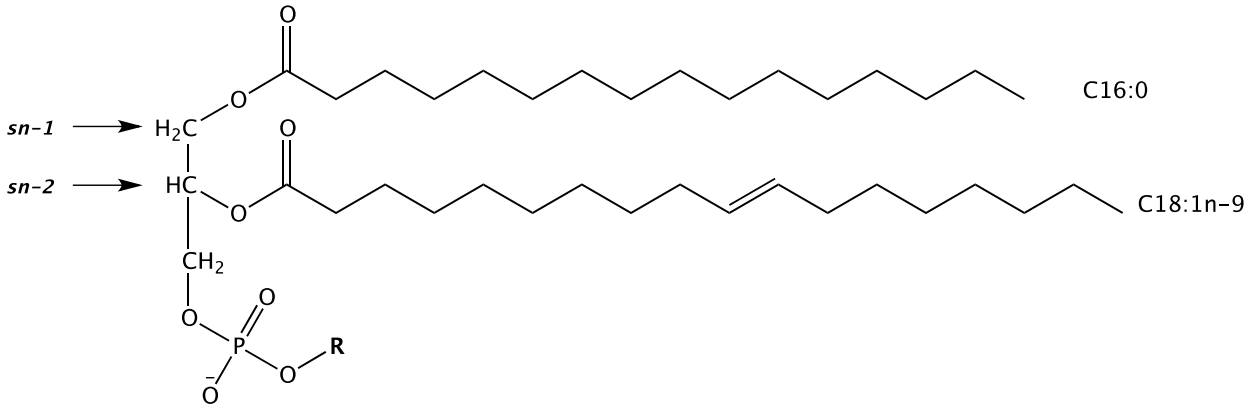

Figure 1: Example schematic of a glycerophospholipid structure.

Glycerophospholipids contain a three-carbon glycerol backbone, with fatty acids present at the stereospecifically numbered 1st carbon position (*sn-1*), and *sn-2* (2nd carbon) positions. In this example, the *sn-1* position is esterified with C16:0, and the *sn-2* position is esterified with C18:1n-9. The third carbon is esterified to a phosphate with a polar head group (**R**) attached. As no polar head group is attached (*i.e.* R=H), the phospholipid is phosphatidic acid.

Phospholipid composition in cellular membranes varies not only between different cell types [2-4], but also within different regions of a single cell membrane, and within different organelles from the same cell, and this diversity is critical for life [5, 6]. As the primary structural component of the lipid bilayer, phospholipids play important roles in cellular processes mediated by cell membranes, including the regulation of integral membrane protein function [7], cell-to-cell communication [8], and signal transduction cascades [9-11]. Plasma and organellar membranes have unique phospholipid compositions, which ultimately affect function. For example, the plasma membrane of most mammalian cells is primarily composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE) [5, 12], the two most abundant classes of phospholipids in mammalian cells. The inner membrane of mitochondria, however, contains cardiolipin (CL), a phospholipid not present in the plasma membrane of mammalian cells [5]. The presence of CL in the inner mitochondrial membrane is critical for unique mitochondrial processes including oxidative phosphorylation [13], and loss of this phospholipid can lead to impaired mitochondrial respiration, mitochondrial degeneration, and ultimately, cell death [14].

Phospholipids are thought to be synthesized primarily in the endoplasmic reticulum (ER), where most known enzymes involved in the *de novo* biosynthesis of phospholipids reside [15]. However, growing evidence in the literature has now identified less-traditional sites of phospholipid synthesis and fatty acyl remodeling, including, but not limited to, the mitochondria

[3], the Golgi apparatus [16], and the mitochondria-associated ER membranes (MAM) [17]. The discovery of new enzymes involved in phospholipid metabolism is therefore anticipated in association with these findings. Additionally, the relatively recent molecular and biochemical re-examination of several enzyme families has begun redefining the known function of enzymes that were not previously believed to be involved in phospholipid metabolism. This work has been facilitated by data from the human genome project, which allowed for the identification of proteins with sequence homology to known phospholipid metabolizing enzymes. Reporting of these roles is sporadic, and thus our understanding of phospholipid metabolism is currently incomplete. As such, the physiological and biochemical significance of many of these enzymes are unknown and warrant investigation.

In the context of these regards, the overall aim of this thesis was to investigate a novel role for Harvey-Ras-like suppressor 1 (HRASLS1) and its downstream bio-derivatives in phospholipid metabolism. Thus, the first objective of this thesis was to define the cellular and biochemical functions of HRASLS1 in the context of phospholipid metabolism, and expand upon the current literature. Analysis of tissue and cellular localization, as well as detailed *in vitro* enzymological experiments were utilized to complete this objective. Through these experiments, a novel function for HRASLS1 in cardiolipin metabolism was discovered. Specifically, HRASLS1 can function as a phosphatidylcholine (PC): monolysocardiolipin (MLCL) transacylase in the synthesis of CL. The second objective of this thesis was to assess the ability of HRASLS1 to modulate cellular phospholipid content and composition *in vivo*. For this objective, I investigated alterations in gene expression, cellular lipid profiles, and mitochondrial function stemming from HRASLS1 overexpression in cultured HEK-293 cells. The results of this work confirmed an *in vivo* role for HRASLS1 in cardiolipin metabolism. The final objective of this thesis was to assess the ability of N-acylethanolamines (NAE) to modulate cardiolipin content and composition in a model of cardiolipin deficiency. In addition to transacylase activity, HRASLS1 also has N-acyltransferase activity, which contributes to the production of downstream NAE bio-derivatives. Cultured B-lymphocytes taken from an individual with Barth Syndrome (BTHS) were utilized as a study model. BTHS is a rare, X-linked genetic disorder that stems from mutations in Tafazzin, a cardiolipin fatty acid remodeling enzyme. Due to their inability to remodel the fatty acyl chains on cardiolipin following *de novo* synthesis, patients with BTHS have decreased cardiolipin content, which leads to a disease state. BTHS B-lymphocytes treated with 1 μM of oleoylethanolamine

(OEA), a species of NAE, exhibited a partial rescue of characteristic growth and cardiolipin deficiencies. Thus, HRASLS1 and its downstream bioactive products, NAEs, represent a new potential therapy for BTHS patients, and potentially other diseases that present with cardiolipin dysfunction.

Chapter Two
Biochemical Foundations

Harvey-Ras-like suppressor (HRASLS) enzymes

The Harvey-Ras-like tumor suppressor (HRASLS) enzymes are a homologous group of proteins that are a part of a larger papain-like N1pC/P60 superfamily of vertebrate thiol proteases [18, 19]. Belonging to the *H-rev107* gene family, some HRASLS enzymes have been characterized as class II tumor suppressors [20], a class of enzymes that can reversibly down-regulate cellular growth, while themselves remaining unchanged or un-mutated during cancer [19-26]. To date, five HRASLS family members have been found in humans (HRASLS1-5) [24, 27-31], with three of these enzymes (HRASLS1, 3, 5) conserved in rodents as well [32-36]. The HRASLS gene family has been mapped to the human chromosomal region between 11q12.3 and 13.1 [28], with the exception of HRASLS1, which is localized to 3q28 → q29 [27]. In mice, the HRASLS gene family is mapped to chromosome 16 (*Hrasls1*) [37] and chromosome 19 (*Hrasls3* and *Hrasls5*) [38, 39].

Besides their described function as class II tumor suppressors, HRASLS enzymes are reported to play roles in myriad biological processes, yet, their physiological significance is not fully understood. A recent study has implicated HRASLS enzymes in peroxisome metabolism [40]. Uyama *et al*. (2017) observed that overexpression of HRASLS3 in HEK-293 cells was associated with peroxisomal dysfunction by either direct phospholipase activity of peroxisome membrane lipids, or by inhibiting the formation of nascent peroxisomes [40]. Alteration or loss of HRASLS enzymes has also been implicated in genetic disorders including Poland Syndrome, a rare disorder characterized by hypoplasia/aplasia of the pectoralis major muscle [41]. In monozygotic twins presenting with Poland Syndrome, loss of HRASLS2-5 due to a heterozygous deletion event was observed, likely signifying a role for these enzymes in the pathogenesis of this disorder.

HRASLS enzymes share sequence homology with lecithin:retinol acyltransferases (LRATs) [18, 42], including a conserved NCEHFV motif in the C-terminal region that is critical for acylation and de-acylation reactions [36] (see Figure 2 for amino acid alignment of HRASLS and LRAT sequences).

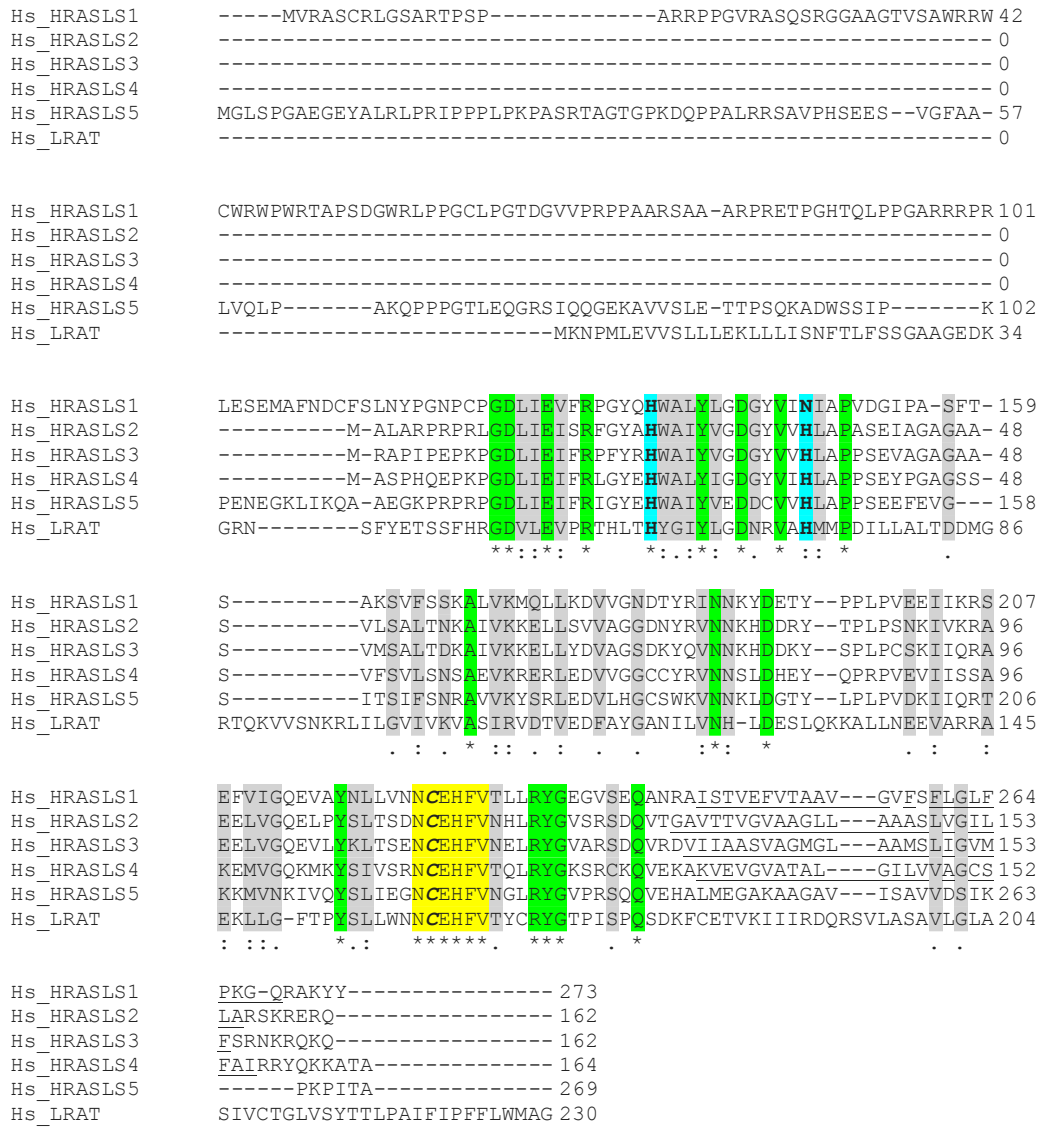

Figure 2: Sequence alignment of human HRASLS and LRAT amino acid sequences.

Figure adapted from Mardian et al. (2015) [19]. Sequence alignment was performed using ClustalOmega [43]. The NCEHFV motif (highlighted in yellow) is critical for acylation/de-acylation activity, and the additional two sites in the catalytic triad are highlighted in blue and bolded. Conserved residues are highlighted in green, and homologous residues are highlighted in grey. The C-terminal hydrophobic transmembrane domain is underlined.

The conserved cysteine residue found within the NCEHFV motif serves as an anchoring point for both acyltransferase and hydrolase reactions, and is part of a catalytic triad along with two histidine residues [18]. In this reaction, the first histidine residue acts to transiently acylate the cysteine residue by causing a nucleophilic attack by the anionic sulfur of the deprotonated cysteine

residue on the *sn-1* acyl chain of the phospholipid. This results in the formation of a thioester intermediate, while the second histidine acts to stabilize the correct imidazole ring of the first histidine [18, 36]. The intermediate is then cleaved into a lysophospholipid and an enzyme-fatty acyl intermediate. The enzyme-fatty acyl intermediate can transfer the fatty acyl to phosphatidylethanolamine, making N-acylphosphatidylethanolamines (NAPEs), or to a lysophospholipid, making phospholipid, or can hydrolyze the enzyme-acyl thioester bond to release a free fatty acid [19]. An illustration of this catalytic mechanism is found in Figure 3.

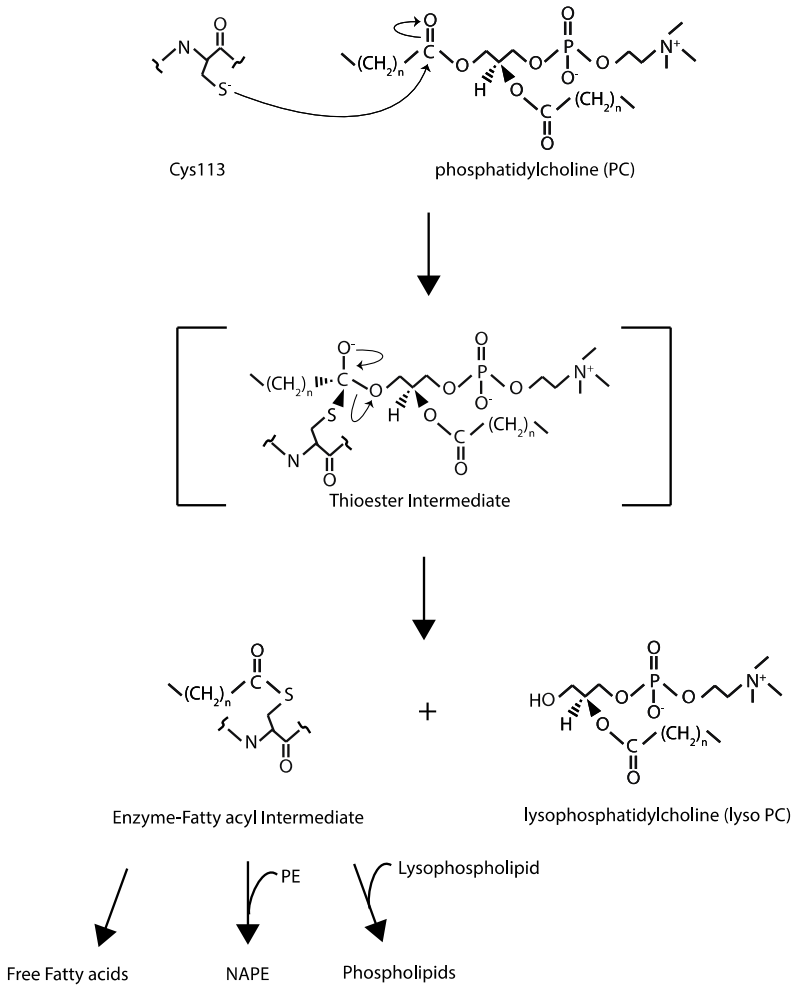

Figure 3: The catalytic mechanism of HRASLS enzymes.

Figure adapted from Mardian, Bradley and Duncan (2015) [19]. The conserved cysteine of HRASLS enzymes is acylated by the first histidine residue, resulting in the formation of a thioester intermediate. The thioester intermediate is hydrolyzed into an enzyme-fatty acyl intermediate, and a lysophospholipid. From here, the enzyme-fatty acyl intermediate can be further hydrolyzed into free fatty acids, NAPE, or phospholipids.

Similar to the mechanism employed by LRAT in de-acylating PC at the *sn-1* position to esterify the fatty acyl species onto the hydroxyl group of all-*trans* retinol [40, 44] to form retinyl ester, mass spectrometry analysis has demonstrated that HRASLS family members predominantly utilize a fatty acyl from the *sn-1* position of PC in the generation of glycerophospholipids [18]. Thus, in this context, HRASLS enzymes have been reported to play important roles in phospholipid metabolism [45], specifically due to their demonstrated *in vitro* abilities to act as both O- and N-acyltransferases, and as phospholipases [45]. While the HRASLS enzyme family is known by many names, their enzymatic functions in lipid metabolism have garnered a new name: PLA/ATs (phospholipases A1/2/acyltransferases) [26, 36, 46].

Since HRASLS enzymes can encode multiple complex enzymatic functions, an initial summary of each enzyme's discovery and characterization is summarized below. Following these summaries, a comprehensive review of the major enzymatic functions of HRASLS enzymes including their activities as O-acyltransferases, N-acyltransferases, and phospholipases A1/2 is discussed.

HRASLS1

HRASLS1, originally known as A-C1, and also known as PLA/AT1, was initially cloned and characterized in mice in 1999 by Akiyama and colleagues, and in humans in 2001 by Ito *et al.* [27]. HRASLS1 was discovered through comparative display between two murine cell lines: embryonic fibroblast C3H10T1/2 and chondrogenic ATDC5 [33]. The murine *Hrasls1* gene encodes for a protein that is 167 amino acids in length and shows 46% homology with a rat gene known as H-rev107, now known as HRASLS3 [33]. Akiyama *et al.* determined that murine *Hrasls1* is ubiquitously expressed, albeit most highly in skeletal muscle, heart, brain, and bone marrow [33], a finding that has been corroborated by the work of Hussain *et al.* (2016) [47]. However, this expression profile varies slightly in humans, as the content of HRASLS1 is relatively low in most tissues other than the testes, skeletal muscle, and heart [27, 45]. Chromosomal mapping by fluorescent *in situ* hybridization (FISH) analysis found that the human *HRASLS1* gene localizes to 3q28 → q29 [33]. Subcellular analysis indicated that HRASLS1 localizes to the cytoplasm, and the peri-nuclear region of transfected COS-7 cells, with no detectable expression in the nucleus [33].

HRASLS2

HRASLS2 was initially cloned from SW40 human colon cancer cells and characterized in 2008 [28]. Shyu *et al*. demonstrated that HRASLS2, also known as PLA/AT2, is highly expressed in the gastrointestinal system, specifically in the small intestine, but also shows expression in the kidney and trachea as well [28]. The coding region of *HRASLS2* encodes for an 18 kDa protein that is comprised of 162 amino acids, and shares 47 and 69% protein sequence homology with *HRASLS1* and *HRASLS3*, respectively [28]. Wildtype HRASLS2 protein transfected into HtTA cells preferentially localizes in a perinuclear pattern with some expression in the cytoplasm as well, whereas both C- and N-truncated HRASLS2 protein are found equally between the cytoplasm and the nucleus [28]. Like other HRASLS enzymes, HRASLS2 has been observed to exhibit anti-Ras activity, as well as growth inhibitory activity in cancer cells [28]. In 2012, the crystal structure of HRASLS2 was solved by Golczak and colleagues, demonstrating that this enzyme contains three α-helices, and four antiparallel β-sheets [18].

HRASLS3

HRASLS3 was the first member of the HRASLS enzyme family to be cloned and characterized [32]. Also known as H-rev107, and adipose-specific phospholipase A2 (AdPLA) [48], *Hrasls3* was identified through subtractive hybridization in H-ras transformed rat fibroblasts [32]. Human HRASLS3 shares 46% protein sequence homology with human HRASLS1. This enzyme, like other members of the HRASLS family, has been reported to suppress growth in cancer cell lines [20, 25, 32], and is thus identified as a class II tumour suppressor. Further analysis of HRASLS3 demonstrated that although it is found in all tissues examined thus far, it is expressed at highest levels in adipocytes [48]. The crystal structure of HRASLS3 was published at the same time as HRASLS2 by the same group, and indicated that like HRASLS2, HRASLS3 contains three α-helices, and four antiparallel β-sheets [18].

HRASLS4

HRASLS4 was identified on three separate occasions and classified under three alternative names: tazarotene-induced gene 3 (TIG3) [29], retinoid-inducible gene 1 (RIG1) [30], and retinoic acid receptor responder 3 (RARRES3) [24]. HRASLS4 shares high sequence homology with HRASLS3/AdPLA, and inhibits H-Ras-mediated signaling [28, 49, 50], and also shares 48% protein sequence homology with HRASLS1. Interestingly, while HRASLS4 is anchored to both

the endoplasmic reticulum and the Golgi apparatus, the anti-cancer effects of this protein are limited to effects mediated within the Golgi, as only Golgi-targeted HRASLS4 induces apoptosis in cancer cells [49]. The NMR structure of HRASLS4 was published in 2015 by Wei *et al.*, indicating that the overall structure of the N-terminal domain of HRASLS4 is very similar to that of HRASLS3 [51]. However, HRASLS4 has less flexible C- and N-terminal domains than that of HRASLS3, which may result in functional differences between the two enzymes [51].

HRASLS5

Originally named rat LRAT-like protein (RLP1) for similarities in its sequence homology to LRAT, HRASLS5 was discovered in 2007 as part of an effort to identify enzymes involved in the generation of NAPEs [31]. HRASLS5 shares 45% protein sequence homology with HRASLS1. Studied in rats, this enzyme shows highest expression in testes [31, 34] and is proposed to play an important role in spermatogenesis in addition to its proposed role as a tumor suppressor [34]. However, the physiological significance of HRASLS5, and whether it does indeed possess anti-cancer activities similar to the other members of the HRASLS family, is still unknown [34]. Sequence homology between the human HRASLS enzymes is shown in Table 1.

Table 1: Sequence homology of human HRASLS enzymes

HRASLS Protein	Seq. homology to HRASLS1	Seq. homology to HRASLS2	Seq. homology to HRASLS3	Seq. homology to HRASLS4	Seq. homology to HRASLS5
HRASLS1 (NP_065119.2)	-	47%	46%	48%	45%
HRASLS2 (NP_060348.1)	47%	-	69%	57%	51%
HRASLS3 (NP_001121675.1)	46%	69%	-	51%	53%
HRASLS4 (NP_004576.2)	48%	57%	51%	-	56%
HRASLS5 (NP_001140200.1)	45%	51%	53%	56%	-

O-Transacylase activity

In the context of phospholipid metabolism, O-transacylase activity involves the transfer of an acyl group from a phospholipid to glycerol-3-phosphate or a lysophospholipid, in a process that is also referred to as O-acyltransferase activity [35, 45] (see Figure 4 for an illustration of transacylase/O-acyltransferase activity). The term O-transacylase is preferred over O-acyltransferase, because it distinguishes the CoA-independent phospholipid-to-phospholipid transfer of a fatty acid from the Co-A-dependent activity of acyltransferase enzymes such as the lysophosphatidic acid acyltransferase enzymes (LPAATs). The HRASLS enzymes have all been found to demonstrate O-transacylase activity *in vitro* [45, 46].

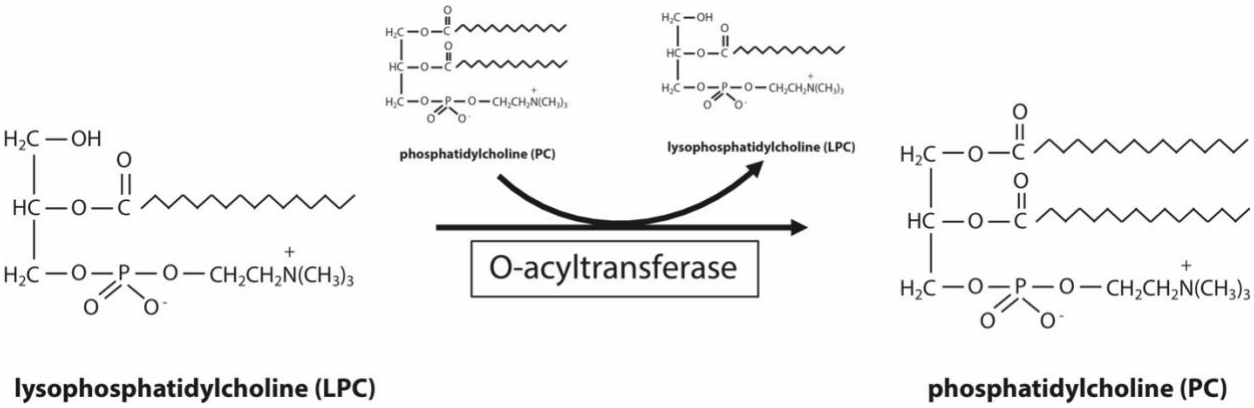

Figure 4: PC O-transacylase/O-acyltransferase activity of HRASLS enzymes.

HRASLS enzymes have the ability to produce phospholipids by functioning as lysophospholipid O-acyltransferases. In this example, HRASLS enzymes will derive a select fatty acyl group from the *sn-1* or *sn-2* position of PC for the incorporation into the *sn-1* position of LPC, to produce PC. This activity is typically employed by HRASLS enzymes to form phospholipids with specific acyl compositions.

The specific O-transacylase activity of HRASLS1 was not described until 2011 when Shinohara *et al*. reported that human HRASLS1 has *in vitro* O-transacylation activity utilizing PC as an acyl donor and lysophosphatidylcholine (LPC) as an acyl acceptor [45]. Consistent with the enzymatic mechanisms proposed by Golczak *et al.* (2012) [18] in solving the structures of HRASLS2 and HRASLS3, HRASLS1 prefers esterolysis at the *sn-1* position of PC, and esterification at the *sn-1* position of LPC [45]. This role for HRASLS1 in acyl remodeling of PC operates in a CoA-independent manner, and requires dithiothreitol (DTT) and Nonidet P-40 for full enzymatic activity [45]. The O-transacylase activity of HRASLS1 is approximately 8-fold

higher than its N-transacylase activity, and approximately 5-fold higher than its PLA1/2 activity, indicating that *in vitro*, HRASLS1 predominately acts as an O-transacylase [45].

The *in vitro* O-transacylase activities of human HRASLS2, HRASLS3, and HRASLS4 were first reported in 2009 by Uyama *et al.* [46], and later also reported in the Shinohara study [45]. Like HRASLS1, HRASLS2 prefers to esterify fatty acyls at the *sn-1* rather than the *sn-2* position. HRASLS2 used 1-hydroxy-2-[^{14}C] palmitoyl-LPC as an acyl acceptor, although it also utilized 1-[^{14}C] palmitoyl-2-hydroxy-LPC to a lesser extent, demonstrating 2.4 – 4.7-fold lower activity with the latter [46]. Similarly, both HRASLS3 and HRASLS4 show higher *in vitro* LPC O-transacylase activity when using 1-hydroxy-2-[^{14}C] palmitoyl LPC, although these enzymes show higher phospholipase A1/2 activity than O-transacylase activity [46]. *In vitro* O-transacylase activity of HRASLS5 was reported in the Shinohara study [45]. In line with the other HRASLS family members, HRASLS5 prefers 1-hydroxy-2-[^{14}C] palmitoyl LPC to 1-[^{14}C] palmitoyl-2-hydroxy- LPC (~2.5-fold higher activity), although the authors found that HRASLS5 also has higher *in vitro* phospholipase activity than O-transacylase activity [45].

Phospholipase activity

All of the HRASLS enzymes have been reported to possess varying degrees of Ca^{2+}–independent phospholipase A1/2 activity [31, 45, 46]. The catalytic activity of HRASLS enzymes (Figure 3) results in cleavage of a fatty acyl chain from a phospholipid at either the *sn-1* position or at the *sn-2* position that is coupled with the formation of a fatty acyl-enzyme intermediate complex, and the release of a lysophospholipid. If the fatty acyl group is released from the catalytic cleft, rather than transferred to a new phospholipid through the O- or N-transacylase activities of the enzyme, then the enzyme is displaying phospholipase activity. This is characterized as phospholipase A1 activity if the fatty acyl released was generated from the *sn-1* position, and phospholipase A2 activity if the fatty acyl released from the *sn-2* position. An example of phospholipase A1/2 activity is shown in Figure 5.

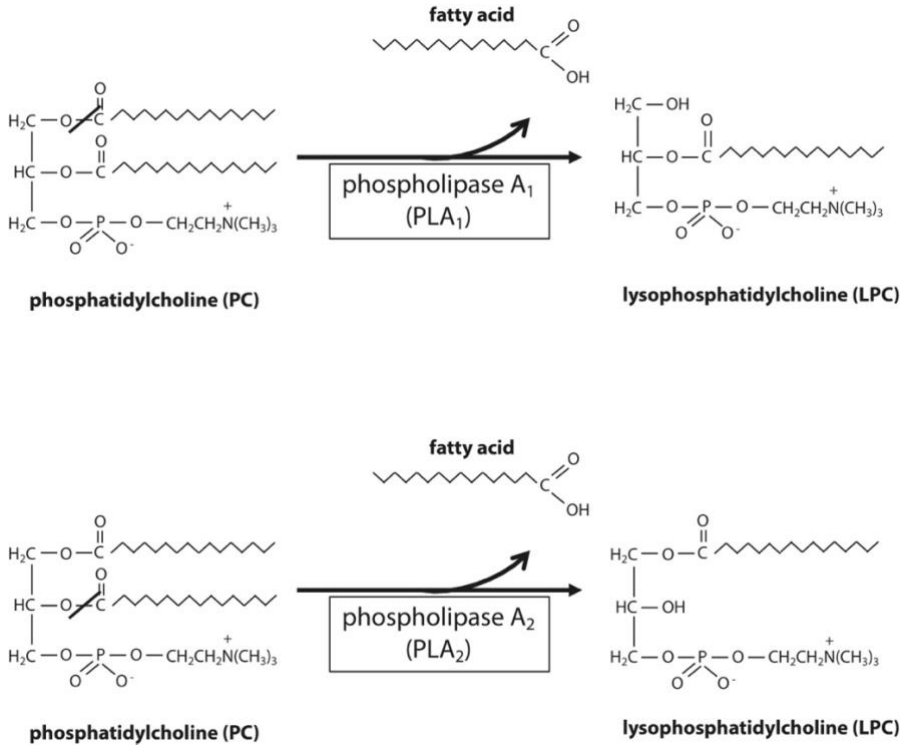

Figure 5: Schematic of phospholipase A1/2 activity

HRASLS enzymes have phospholipase activity. In this example, HRASLS enzymes are able to hydrolyze fatty acids from either the *sn-1* (top figure), or *sn-2* (bottom figure) positions of phospholipids. These fatty acids are released, resulting in the production of a lysophospholipid.

The phospholipase A1/2 activity of HRASLS1 was first described in 2011, through a study identifying a role for this enzyme in phospholipid metabolism [45]. Optimal PLA1/2 activity of HRASLS is dependent on several factors including optimal pH (around 8-9), the presence of EDTA (approximately 1 mM), and the absence of Ca^{2+} [45]. In addition, catalytic activity of HRASLS1 as a phospholipase requires the presence of DTT and Nonidet-P40, similar to the requirement for these compounds for O- or N- transacylase activities [45]. Although HRASLS1 can catalyze the hydrolysis of acyl groups from both the *sn-1* and *sn-2* positions of PC, it much prefers to act as a PLA1, showing ~56% higher activity when acting as a PLA1 versus acting as a PLA2 [45]. HRASLS1 shows phospholipase activity with both PC and PE. However, it does not show phospholipase activity with either 1-[^{14}C] palmitoyl-2-hydroxy-LPC or 1-hydroxy-2-[^{14}C] palmitoyl-LPC, indicating activity as a PLA1/2 but not a PLB [45]. When compared to other members of the HRASLS family, HRASLS1 has modest PLA1/2 activity, and instead prefers to act as a PC O-transacylase *in vitro* [45].

HRASLS2 has also been reported to have Ca^{2+}–independent PLA1/2 activity. Like HRASLS1, HRASLS2 requires the presence of DTT and Nonidet P-40 for optimal PLA1/2 activity, and functions best at pH 8-9 [46]. HRASLS2 shows a striking preference for PLA1 activity to PLA2 activity, as PLA1 activity for this enzyme was 4.4 – 27.6-fold higher than PLA2 activity depending on the substrate [46]. However, much like HRASLS1, HRASLS2 prefers O-transacylase activity *in vitro* over PLA1/2 or N-transacylase activity [45].

HRASLS3 was initially described as an *in vitro* N-acyltransferase by Jin *et al*. (2007) [31]. However, a thorough characterization of this enzyme *in vitro* and in adipose tissue found that phospholipase activity is the predominant function of this enzyme, which is responsible for 80% of adipocyte phospholipase activity [52]. As a result, HRASLS3 was renamed adipose-specific phospholipase A2 (AdPLA), and given the gene name PLA2 Group XVI (PLA2GXVI) [48, 52]. Later studies by others confirmed these findings [35, 45]. HRASLS3 has been shown to be critical for the development of obesity, and to be a regulator of lipolysis in mice [48, 52]. However, the PLA1/2 preference of HRASLS3/AdPLA is debated. While it is agreed that the major enzymatic activity of HRASLS3 is as a phospholipase, a study performed by Uyama *et al*. (2009) [46] suggests that HRASLS3 has predominant PLA1 activity, whereas the Duncan study (2008) indicates that PLA2 is slightly dominates as the major phospholipase activity of this enzyme [48]. As well, the Uyama study showed PLA activity with PC and PE as substrates [26], which was furthered by the Duncan study, which demonstrated that HRASLS3 has PLA activity with phosphatidylserine (PS) and phosphatidylinositol (PI), but not phosphatidic acid (PA) [48]. Relative to the other HRASLS enzymes, HRASLS3 has the greatest *in vitro* PLA1/2 activity [45].

Like HRASLS3, HRASLS4 acts primarily as a Ca^{2+}–independent phospholipase A1/2 *in vitro*, although it has only weak O- and N-transacylase activity [46]. HRASLS4 preferentially hydrolyses fatty acids from the *sn-1* position of PC and PE, and similar to HRASLS1, 2, and 5, it functions optimally between pH 8-9, and requires DTT and Nonidet P-40 for maximal catalytic activity [46]. Although HRASLS5 primarily functions as an N-transacylase, it does also have phospholipase A1/2 activity [36]. Whereas most HRASLS enzymes prefer *sn-1* hydrolysis, as indicated by preferential PLA1 activity in phospholipase assays, initial investigation indicated that HRASLS5 does not show a clear preference for use of fatty acyl groups from either the *sn-1* or *sn-2* position of PC during N-transacylation [19, 36].

N-transacylase activity

N-transacylase activity describes the transfer of a fatty acyl group from the *sn-1* or *sn-2* position of a phospholipid, to the amino group of the PE polar head group (see Figure 6 for an illustration of N-transacylase activity) [35]. Most studies utilize PC as an acyl donor [26, 35, 40, 45, 46, 53], but other phospholipids and lysophospholipids can also be utilized as acyl donors including 1-acyl-LPC, PE, and CL [54]. These reactions are catalyzed by three groups of enzymes: Ca^{2+}–dependent N-transacylases (Ca-NATs), Ca^{2+}–independent N-transacylases (iNATs), and HRASLS enzymes, which are also largely Ca^{2+}–independent, but constitute a separate group [55].

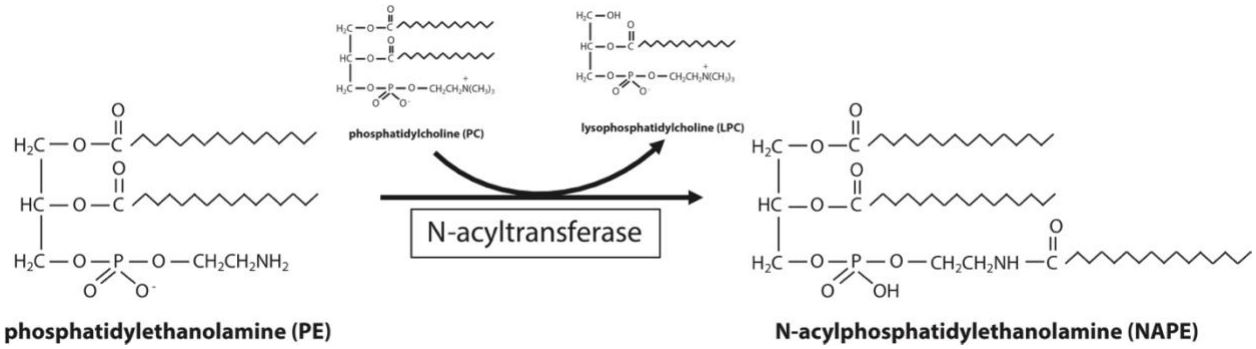

Figure 6: Illustration of N-transacylase activity

HRASLS enzymes can function as N-transacylases in the synthesis of NAPE. In this example, the HRASLS enzyme cleaves a fatty acid from the *sn-1* position of PC for selective esterification onto the nitrogen of the ethanolamine polar head group of PE. During this reaction, lysophosphatidylcholine is released, and a molecule of NAPE is produced. The species of NAPE will depend on which fatty acid is esterified onto the ethanolamine head group. NAPE serve as precursors for the synthesis of NAE.

The result of HRASLS-mediated N-transacylase activity is the production of a molecule of NAPE. Many different species of NAPE can be produced, with the molecular species of N-acylethanolamine depending on the fatty acyl species extracted from the donor glycerophospholipid [54]. Unlike O-acyltransferase activity, where fatty acyl-CoA can be utilized, the synthesis of NAPE is dependent on acyl species derived from existing glycerophospholipids [54]. Since glycerophospholipids tend to have a saturated fatty acyl in the *sn*-1 position, and an unsaturated fatty acyl in *sn*-2 position, the N-acylethanolamine group produced by transacylation will, to some extent, be influenced by the positional-preference of the N-transacylase enzyme. The

species of NAPE produced in cells can affect cellular function, since NAPE are important precursors to biologically active signaling molecules known as N-acylethanolamines (NAEs).

The observation that HRASLS1 exhibits PE N-acylation activity has been determined by *in vitro* PE N-transacylation activity assays, as well as metabolic labeling experiments in intact cells [35, 36, 45, 53]. In the 2011 study by Shinohara and colleagues, purified human HRASLS1 was found to have N-transacylase activity when utilizing PE as an acyl acceptor and [^{14}C] PC as an acyl donor [45]. While this activity was relatively modest (~0.08 μmol [^{14}C] NPPE/min/mg protein) as compared to the *sn-1* O-transacylase activity of this enzyme using 1-hydroxy-2-[^{14}C]16:0-LPC as an acyl acceptor (~1.2 μmol PC/min/mg protein), it is comparable to the *in vitro* PC phospholipase (~0.15 μmol 16:0/min/mg protein) and *sn-2* O-transacylase activities (~0.10 μmol [^{14}C] PC/min/mg protein using 1-[^{14}C16:0]-2-hydroxy-LPC as an acyl acceptor) [45]. In experiments where [^{14}C] palmitate was used to metabolically label COS-7 cells, stable overexpression of HRASLS1 caused an ~100-fold increase in the formation of [^{14}C] N-palmitoyl-PE (NPPE), as compared to controls, indicating the ability of HRASLS1 to synthesize NAPE *in vivo* as well [45]. Further studies investigating the *in vitro* N-transacylase ability of HRASLS1 found that it has approximately 1.6-fold higher activity *in vitro* as an N-transacylase than as a phospholipase [35], and that overexpression of this enzyme in COS-7 cells increases levels of total endogenous NAPE [35].

In comparison to HRASLS3 and HRASLS4, Uyama et al. (2009) found that only HRASLS2 had detectable *in vitro* N-acyltransferase activity resulting in the formation of [^{14}C] NPPE (~0.10 μmol [^{14}C] NPPE/min/mg protein) when utilizing 1,2-[^{14}C] dipalmitoyl-PC as an acyl donor and PE as an acyl acceptor, and found virtually no activity with HRASLS3 or HRASLS4 [46]. The subsequent Shinohara study found comparable levels of N-acyltransferase activity for HRASLS2 (~0.10 μmol [^{14}C] NPPE/min/mg protein) by utilizing the same conditions [45]. A more recent study performed by Uyama and colleagues characterized HRASLS2 activity *in vitro* as predominately N-acyltransferase, as compared to its ability to act as a phospholipase (~5.6-fold higher activity) [35]. In addition, transient expression of HRASLS2 in COS-7 cells resulted in an accumulation in total NAPE species, while knockdown of HRASLS2 caused a significant drop in N-transacylase activity and NAPE levels [35], indicating a role for HRASLS2 in NAPE production *in vivo*.

The N-transacylase activity of HRASLS3 was first reported in 2007 by Jin *et al.*, who identified that this enzyme exhibits *in vitro* N-transacylase activity [31]. However, the N-transacylase abilities of HRASLS3 are the lowest amongst the other HRASLS family members [35, 45, 46]. In comparison to its ability to act as a phospholipase, HRASLS3 has exceedingly low *in vitro* N-transacylase activity [35]. When assayed *in vitro*, the ratio of N-transacylase activity to phospholipase A1/2 activity of HRASLS3 is a trivial 0.1 [35].

Although HRASLS4 displays limited N-acyltransferase activity *in vitro* [46], overexpression of HRASLS4 in COS-7 cells increases cellular NAPE content [35]. HRASLS4 has higher activity as an N-acyltransferase than as a phospholipase *in vitro* [35], but this is opposite *in vivo* [35, 45, 46] . HRASLS5 was originally identified as a Ca^{2+}–dependent N-acyltransferase that synthesizes anandamide [31]. HRASLS5 possesses *in vitro* N-acyltransferase activity that predominates over both O-transacylase and phospholipase A1/2 activity [31]. This observation was supported by experiments performed *in vivo*, where overexpression of HRASLS5 in cultured cells was found to enrich cellular content of both NAPE and NAEs [31, 35, 36, 45]. HRASLS5 has been suggested to be of importance in the production of anandamide due to its lack of preference in utilizing acyl chains from either the *sn-1* or *sn-2* positions of glycerophospholipids to produce NAPE and NAE [31]. While other HRASLS family members show preference for the *sn-1* position, HRASLS5 will utilize fatty acyl chains from the *sn*-2 position equally well, and this site is more likely to be enriched in arachidonic acid required for N-arachidonoyl ethanolamine and downstream anandamide production.

N-acylethanolamines (NAEs) and their synthesis from NAPEs

The generation of NAPE by Ca^{2+}–dependent N-acyltransferases, along with HRASLS enzymes, is part of a larger pathway in the synthesis of NAEs, known as the phosphodiesterase-transacylation pathway [35], illustrated in Figure 7.

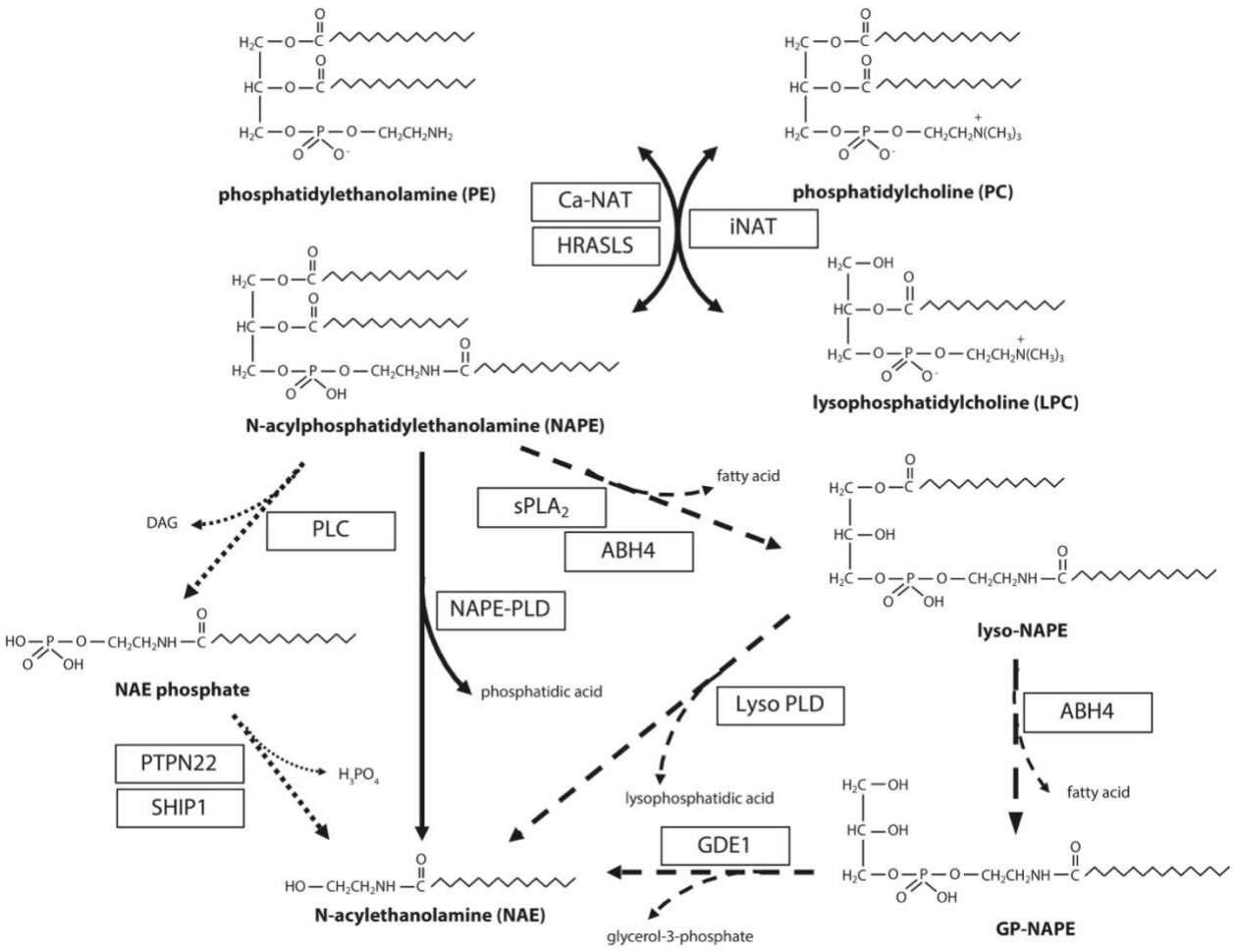

Figure 7: Illustration of the phosphodiesterase-transacylation pathway of NAE synthesis.

Figure adapted from Ueda *et al.* (2010) [54]. NAE are generated through the phosphodiesterase-transacylation pathway. The majority of NAE are produced from NAPE-PLD-mediated hydrolysis of NAPE, which also results in the release of a molecule of PA. Alternatively, NAPE can be hydrolyzed by PLC into NAE-phosphate, which is then de-phosphorylated by either PTPN22 or SHIP1 to catalyze the formation of NAE. NAE can also be produced in a less direct, but also utilized pathway. In this alternative pathway, NAE can be produced by the hydrolysis of the *sn-2* acyl group in NAPE by the enzyme ABH4 or $sPLA_2$, which results in the production of a molecule of lyso-NAPE. Lyso-NAPE can then be directly hydrolyzed into NAE by the enzyme lyso-PLD, or be converted into GP-NAPE by the hydrolysis of the *sn-1* fatty acid on lyso-NAPE, which will generate a molecule of GP-NAPE. Once GP-NAPE is produced, NAE can be formed by the hydrolytic activity of the enzyme GDE1.

Along with NAEs, NAPE molecules are themselves bioactive, accumulating in sites of inflammation following injurious events such as myocardial infarction [56, 57] and neural ischemia [58, 59]. However, NAPEs have been better characterized as important precursors to the

synthesis of NAEs. NAEs play diverse roles in a multitude of biological processes including, but not limited to nocioception [60], tumor growth suppression [61], apoptosis [62], and PPAR agonism [62]. Quantitatively, the most abundant NAEs are N-palmitoylethalomine (PEA), N-oleoylethanolamine (OEA), N-stearoylethanolamine (SEA), and N-linoleoylethanolamine (LEA) [54]. Perhaps the most well-known NAE, however, is N-arachidonylethanolamine, better known as anandamide (AEA) [60]. NAEs including AEA signal through a host of receptors, including the cannabinoid receptor of the central and peripheral types (CB1 and CB2, respectively) [63], the transient vanilloid receptor type 1 (TRPV1) [64], G-protein-coupled receptors 55 [65] and 119 [66] (GPR55 and GPR119), and peroxisome proliferator-activated receptor α (PPARα) [67, 68]. NAEs form a relatively minor component of membranes, and are synthesized on-demand, rather than stored in cells [54]. As such, their regulation is highly dependent on the enzymes involved in their synthesis and degradation [54].

The majority of NAEs are generated from NAPEs through the enzyme NAPE-phospholipase D (NAPE-PLD) in a one-step reaction [69]. NAPE-PLD is a 46 kDa integral membrane protein, which acts as a phosphodiesterase of the phospholipase D type [70, 71]. As such, NAPE-PLD releases a molecule of NAPE and a molecule of phosphatidic acid following NAPE degradation, and is the only mammalian enzyme to directly hydrolyze NAE from NAPE [54]. Unlike HRASLS enzymes, with N-acyltransferase activity that is activated by Ca^{2+}, NAPE-PLD largely operates in a Ca^{2+}–independent manner, as its activity is only minimally stimulated upon the introduction of Ca^{2+} [55]. Interestingly, although NAPE-PLD is the only known mammalian enzyme to directly hydrolyze NAPE into NAE, studies from *NAPE-PLD*$^{-/-}$ mice still show the formation of NAE, which suggested the existence of alternative NAPE degradation pathways [72]. As such, two multi-step pathways independent of NAPE-PLD have been discovered in the formation of NAEs from NAPE [73-76].

In one pathway independent of NAPE-PLD, a group Ib secretory PLA2 (sPLA2) hydrolyzes the fatty acyl group from the *sn-2* position of a NAPE molecule, resulting in lyso-NAPE [77]. Following this, lyso-NAPE can be either be further hydrolyzed into NAE by an enzyme known as lyso-PLD [77], or it can be converted into glycerophospho-N-acylethanolamide (GP-NAE) by the hydrolysis of the *sn-1* acyl group by the enzyme ABH4 [73]. The molecule of GP-NAE can then be hydrolyzed by the enzyme glycerophosphodiester phosphodiesterase 1 (GDE1) [78], resulting in the release of glycerol-3-phosphate and a molecule of NAE.

In a separate pathway that is independent of NAPE-PLD-mediated NAE synthesis, NAPE can be hydrolyzed into diacylglycerol (DAG) and NAE-phosphate by phospholipase C (PLC) activity [79]. However, the enzyme responsible for the PLC activity observed in this pathway has not yet been characterized [55]. Following the generation of NAE-phosphate, two separate phosphatases can mediate the hydrolysis of NAE-phosphate into NAE and phosphoric acid [55]. The first phosphatase identified is known as protein tyrosine phosphatase, non-receptor type 22 (PTPN22) [80]. PTPN22 had been previously cloned and characterized in cultured RAW264.7 murine macrophages, whereby its stable overexpression caused doubling in endogenous anandamide content [80]. As well, investigations on PTPN22-deficient mice have shown that PTPN22 is at least partially responsible for the cleavage of anandamide-phosphate into anandamide [55, 75]. Thus, a role for PTPN22 in the formation of NAE from NAE-phosphate has been demonstrated, and studies are ongoing to investigate possible inhibitors of this enzyme [55]. The other known phosphatase is an enzyme known as src homology 2 domain-containing inositol-5-phosphatase 1 (SHIP1), which was identified in 2005 [81]. SHIP1 activity is stimulated by lipopolysaccharide, and has been shown to cleave anandamide-phosphate into anandamide [75]. Interestingly, studies have indicated that the sPLA2/ABH4-mediated pathway seemingly occurs at a much faster rate (i.e. 6x faster) than the PLC/PTPN22/SHIP1-mediated pathway in brain homogenates, indicating that it may be favoured *in vivo* [55]. However, analysis of RAW264.7 cells treated with lipopolysaccharide showed anandamide formation through only the PLC/PTPN22/SHIP1-mediated pathway [55]. Further characterization of NAE synthesis pathways independent of NAPE-PLD are warranted to appreciate their full physiological significance.

Finally, there is recent evidence to suggest that the formation of NAE from NAPE can occur spontaneously within the cell [55] [82-84]. McCue and colleagues observed at high (150 mM) concentrations of ethanolamine, anandamide was generated through the condensation of ethanolamine and arachidonyl-CoA [85]. The spontaneous formation of NAE through this method has been confirmed as the reverse reaction of the degradation of NAE through the enzyme fatty acid amide hydrolase (FAAH) [86-88], described below. However, due to the supra-physiological concentration of ethanolamine required for the spontaneous synthesis of anandamide, the physiological relevance of this reaction is unknown [55]

Pathways of NAE degradation

Perhaps the best characterized pathway of NAE degradation is mediated through FAAH, which hydrolyses NAE into ethanolamine and free fatty acid [89]. Although FAAH has highest affinity for anandamide, it is able to mediate the degradation of a number of NAE species including OEA, PEA, and LEA [82]. In humans, an isozyme of FAAH, termed FAAH-2 has also been shown to mediate the degradation of NAE [90]. However, FAAH-2 only shares 20% protein sequence homology with FAAH, and also differs in its cellular localization and physiological role [91].

Another enzyme responsible for the degradation of NAE is the protein N-acylethanolamine-hydrolyzing acid amidase (NAAA), which was discovered in 1999 [92]. NAAA is a 31 kDa NAE-hydrolyzing enzyme that was first purified in rat lung [93], and then later cloned and characterized in humans [94]. NAAA shares no sequence homology with FAAH [94], and prefers the hydrolysis of PEA to anandamide [93]. NAAA functions similarly to a ceramide hydrolase by cleaving the amide bond of NAE to release ethanolamine and a free fatty acid [54, 94].

Statement of the problem

The aim of this thesis was to investigate a novel role for HRASLS1 and its downstream bio-derivatives in phospholipid metabolism. Although Shinohara and colleagues have previously shown PC O-transacylase, PLA1/2, and N-transacylase activity [45], a full characterization of the ability of HRASLS1 to directly or indirectly synthesize additional phospholipids had not been tested. Specifically, a role for HRASLS1 in the metabolism of the mitochondrial phospholipid cardiolipin had not yet been investigated. Initial investigations indicated that overexpression of HRASLS1 in cells specifically increased the content of cardiolipin. The O-transacylase (i.e. PC transacylase) activity of HRASLS enzymes suggests that HRASLS1 could participate directly in cardiolipin remodeling, by transferring a fatty acyl chain from the *sn-1* position of PC to MLCL or DLCL in a reaction similar to that catalyzed by the transacylase *Tafazzin* (TAZ). HRASLS1 may also indirectly affect cardiolipin synthesis, through its role as an N-acyltransferase functioning in the synthesis of NAPEs and resultant NAEs [35]. Thus, a review of the phospholipid biosynthetic pathway and mechanisms of acyl remodeling follows. In addition, special attention is given to the biological importance and tissue- and enzymatic regulation of cardiolipin.

Kennedy Pathway of phospholipid and TAG synthesis

The *de novo* synthesis of phospholipids and triacylglycerol occurs in the Kennedy Pathway, initially described by Eugene Kennedy in 1956 [15]. Phospholipid and triacylglycerol (TAG) synthesis largely takes place in the endoplasmic reticulum, MAM, and the mitochondria [5]. The membranes of these organelles contain enzymes involved in glycerophospholipid synthesis including glycerol-3-phosphate acyltransferases (GPATs), and AGPATs/lysophosphatidic acid acyltransferases (LPAATs).

In the initial step of this pathway, a fatty acyl-CoA is esterified to a molecule of glycerol-3-phosphate by a member of the GPAT family of enzymes, producing a molecule of lysophosphatidic acid (LPA). To date, there have been four GPAT enzymes identified, the first two of which are mitochondrial [95, 96], and the latter two of which are microsomal [97, 98]. Following the production of a molecule of LPA, an AGPAT/LPAAT enzyme will esterify it with another fatty acyl chain, catalyzing the formation of phosphatidic acid (PA). Phosphatidic acid represents a unique branching point in the Kennedy Pathway. In one branch, PA can be dephosphorylated by a phosphatidate phosphatase (PAP) enzyme to yield 1, 2-diacylglycerol (1, 2 – DAG), which can be used as a substrate to produce a molecule of TAG by one of two diacylglycerol acyltransferases (DGATs) [99, 100]. Alternatively, 1,2-DAG can be used as a substrate in either the CDP-choline pathway to produce the glycerophospholipid phosphatidylcholine (PC), or in the CDP-ethanolamine pathway to produce phosphatidylethanolamine (PE) [15]. Together, PC and PE jointly constitute 60-80% of glycerophospholipids found in eukaryotic cell membranes [5]. Among these, PC is the most abundant, representing anywhere from 45 – 55% of phospholipids in plasma membranes [5], while PE, which is typically enriched within the inner leaflet of membranes comprises 15-25% [5]. The synthesis of the glycerophospholipid phosphatidylserine (PS) can be achieved using either PC or PE as substrates, which are metabolized by either phosphatidylserine synthase 1 (PSS1) [101] or phosphatidylserine synthase 2 (PSS2) [102].

If PA is used in the alternative branch of the Kennedy Pathway, glycerophospholipids that are typically enriched within the mitochondria can be synthesized. Once produced, PA can be converted into cytidine diphosphate diacylglycerol (CDP-DAG) by CDP-diacylglycerol synthase (CDS) [103]. CDP-DAG can then be combined with a molecule of inositol to produce the glycerophospholipid phosphatidylinositol. PI is an important signaling molecule that is

particularly enriched in the brain as compared to most other tissues [104-106]. Importantly, PI acts as a source of polyphosphorylated phosphoinositides (PIPs) that function in a multitude of brain signaling pathways including neurotransmission [107] and neuronal exocytosis [108], and have been implicated in regulating functional and structural plasticity [109-111]. Alternatively, CDP-DAG can be converted into phosphatidylglycerol phosphate (PGP), which is dephosphorylated by a phosphatase to produce the glycerophospholipid phosphatidylglycerol (PG). Phosphatidylglycerol typically constitutes less than 1% of membrane glycerophospholipids, but importantly can be used as a precursor to the mitochondrial phospholipid cardiolipin. Cardiolipin is a dimeric phospholipid found almost exclusively in the inner mitochondrial membrane in eukaryotic cells, and is formed through the condensation of PG and CDP-DAG in a reaction catalyzed by the enzyme cardiolipin synthase (CLS) [112]. The mammalian synthesis of cardiolipin, known as the CDP-DG pathway, was initially discovered by Karl Hostetler in the laboratory of Laurens van Deenen [113]. A schematic of the Kennedy Pathway is shown in Figure 8.

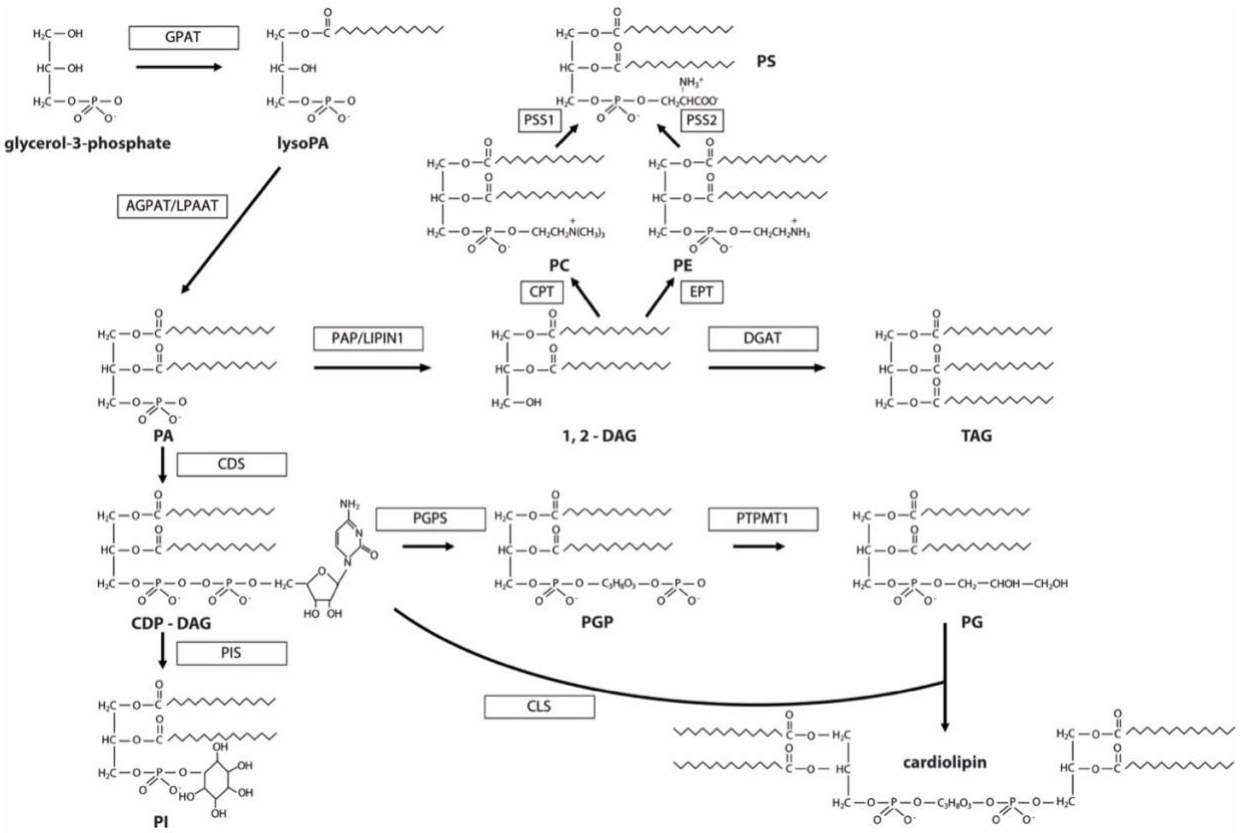

Figure 8: The Kennedy Pathway of glycerophospholipid and triacylglycerol synthesis

Phospholipids and triacylglycerol are produced *de novo* in the Kennedy Pathway. In this pathway, a molecule of glycerol-3-phosphosphate is acylated at the *sn-1* position by a member of the GPAT family to form lysoPA. Next, a member of the AGPAT/LPAAT family will acylate lysoPA at the *sn-2* position to produce PA. PA represents a branching point in the pathway, where it can be dephosphorylated, and acylated to form 1,2-DAG that can serve as precursor to the synthesis of PC, PS, or PE. Alternatively, 1-2 DAG can be further acylated into TAG. In the other branch of the pathway, PA is converted into CDP-DAG by CDS enzymes. CDP-DAG then serves as a precursor to form either phosphatidylglycerol phosphate (PGP), or the phospholipid PI. Once PGP is formed, it can be de-phosphorylated into the phospholipid PG by PTPMT1. Finally, cardiolipin synthase is able to produce the mitochondrial phospholipid cardiolipin through the condensation of CDP-DAG and PG.

Cardiolipin

Cardiolipin is structurally unique from other glycerophospholipids since it contains four, rather that two, fatty acyl side chains. These can vary in composition, and the molecular species of cardiolipin present in any particular tissue is highly specific [114, 115] and often conserved across related species [114, 116, 117]. The substrate specificities of known, and likely also of 'as-of-yet uncharacterized' cardiolipin-remodeling enzymes are key in determining cardiolipin acyl moieties in any given tissue [118-122].

Tissue-specific molecular cardiolipin composition

In eukaryotes, cardiolipin is found predominately in the inner mitochondrial membrane where it is required for oxidative phosphorylation [13], and is critical in stabilizing electron transport chain (ETC) complexes [123]. A mitochondrial insult event, such as a loss of membrane potential, causes cardiolipin translocation to the outer mitochondrial membrane, catalyzed by the protein NDPK-D [124] and/or phospholipid scramblase 3 (PLS3) [125]. This externalization can serve as a target for mitochondrial-mediated apoptosis [126] and mitochondrial-specific autophagy (a process deemed mitophagy) [127, 128]. However, under normal conditions, the tissue content of cardiolipin in eukaryotes is specifically found in mitochondrial membranes. In contrast, prokaryotes, lack mitochondria as well as any other membrane-bound organelle, and cardiolipin is only found in the plasma membrane [129]. Interestingly, the presence of cardiolipin in the plasma membrane of prokaryotes, coupled with the absence of cardiolipin in any other eukaryotic structure besides mitochondria, helped give rise to the serial endosymbiont theory. This idea states that protoeukaryotic organisms took up an alpha-protobacterium, in an event that led to the evolution of eukaryotic organisms, which now contain a descendant of that protobacterium mitochondria [130].

The total cardiolipin content of tissues varies in proportion with oxidative demand, reflecting the higher mitochondrial content. A number of studies have characterized the cardiolipin content of mammalian organs, and these are outlined below. In the heart, cardiolipin constitutes approximately 12-15% of the total phospholipid present [131], and is the third most abundant glycerophospholipid, following PC and PE [114, 132, 133]. In the kidneys, cardiolipin amounts to 6-7% of total phospholipid [131]. The brain has a normalized energetic demand that is close to half that of cardiac muscle or kidney per unit mass, and similarly has a significantly lower

proportion of cardiolipin relative to total phospholipid content, accounting for only 1-2% [105]. Relative to total phospholipid, cardiolipin accounts for 2-3% by weight in lung, 5-6% in liver, and 2-3% in testes [105]. The unique tissue-specific distribution of cardiolipin side chain composition is expanded below, and is summarized in Table 2.

Heart

Cardiolipin has been best described in heart, where it was first characterized, and from whence it derives its name [134]. Cardiac cardiolipin has a signature fatty acyl profile that is predominated by linoleic acid (18:2n-6). Approximately ~80% of cardiolipin molecules in heart are fully esterified with this acyl species at all four positions, forming tetra-linoleoyl cardiolipin [114]. This molecular specificity is highly conserved across mammals, with similar compositions observed in bovine, rodent, and human hearts [114, 116-118, 132, 133, 135-140]. Although this composition is relatively well preserved throughout many changing cellular conditions, alterations are reported. Lee *et al*. found an effect of aging on the composition of cardiolipin in rat hearts [116]. In that work, the authors reported that the linoleate content decreased approximately 34% between 12 and 24 months of age [116]. However, linoleate remained the major fatty acyl species in rat cardiac cardiolipin at all time points measured (4, 12, and 24 months of age). Furthermore, even at 24 months, 18:2n-6 was present at levels more than 10-fold higher than the next most abundant fatty acids, oleic acid (18:1n-9) and docosahexaenoic acid [116]. The predominance of linoleate in heart cardiolipin is also conserved during certain drug interventions that cause profound changes in other phospholipid species. Injection of rats with norepinephrine has been found to reduce the cardiac content of linoleate in phosphatidylcholine and phosphatidylethanolamine approximately in half, without affecting the amount of linoleate in cardiolipin [135]. While this treatment did lead to a small increase in the cardiolipin content of docosahexaenoic acid (DHA), changes in other fatty acyl species were negligible, especially when compared to the total amount of linoleate present [135].

Oleic acid is typically the next most abundant fatty acyl found in heart cardiolipin [114, 116, 117]. Cardiolipin esterified with one oleic acid in place of a linoleic acid (18:2-18:2)-(18:2-18:1) is the second most common molecular cardiolipin species detected in bovine, rodent, and human hearts [114, 116, 117, 135]. The third most abundant form of heart cardiolipin differs depending on the animal examined, although this may be reflective of differences in diet-induced cardiolipin remodeling rather than differences in host species, *per se*. In humans and rodents,

stearic acid (18:0) is commonly the third most abundant fatty acyl species esterified in cardiolipin [114, 116], whereas bovine cardiolipin is typically more enriched with α-linolenic acid (18:3n-3) [117].

Brain

Brain has an entirely different cardiolipin fatty acyl profile compared to heart, and there are species-specific differences in these profiles. In bovine [141] and human [115] brains, oleate is the most abundant fatty acyl within cardiolipin, accounting for ~54% and 32% of the total fatty acyl species, respectively. In rats, however, stearate is the most abundant, accounting for close to half of fatty acyl species in cardiolipin [142]. There is also species variation in which fatty acyl is the second most abundant within brain cardiolipin. In humans, stearate is often found in relative abundance, whereas in rodents, oleate predominates [115]. In contrast, linoleate typically accounts for <10% of total brain cardiolipin fatty acyl species [115].

The highly distinct profile of brain cardiolipin, and degree of difference compared to that of heart cardiolipin, has garnered speculation regarding biological significance. One hypothesis is that brain cardiolipin is composed of less unsaturated fatty acids than heart cardiolipin in order to help mitigate the effects of aging on lipid peroxidation [143]. Because of its proximity to molecular oxygen in mitochondria, cardiolipin is readily oxidized by reactive oxygen species (ROS) [143-145]. Evidence has shown that the oxygenation of brain cardiolipin generates neuronal death signals [146, 147]. Thus, adopting a less unsaturated fatty acyl profile may render brain cardiolipin less likely to undergo peroxidation, and may promote the conservation of neurons that are non-replicating and functionally irreplaceable. Conversely, a higher degree of cardiolipin unsaturation in heart may be necessary to achieve the very high rate of oxidative metabolism characteristic of that tissue [148-150].

Alterations in the cardiolipin content or composition of brain may have pathological consequences [106, 136, 143, 151, 152]. Decreases in the total cardiolipin content of brain mitochondria have been reported in Parkinson's Disease [127, 153], Alzheimer's Disease [154], and in age-related dementia [154]. Changes in the fatty acyl composition of brain cardiolipin are also reported. Analysis of brains taken after death from individuals with Alzheimer's Disease reveals an increased content of palmitate (16:0) in cardiolipin isolated from the frontal cortex, and a lower content of docosahexaenoate (22:6n-3) in cardiolipin isolated from the temporal cortex,

compared to brains from healthy controls [115]. Altered mitochondrial phospholipid composition has been shown to affect the capacity of mitochondria to perform oxidative phosphorylation. A study by Divakaran and Venkataraman (1977) examined the cardiolipin fatty acyl composition and oxidative phosphorylation capacity of mitochondria of Wistar rats fed a hydrogenated coconut oil (HCO)-supplemented diet or Wistar rats fed a safflower-oil supplemented diet [155]. Rats fed the HCO-diet had increases in cardiolipin oleic and palmitoleic acid content, which was associated with a decrease in the oxidative phosphorylative capacity of their liver mitochondria when using glutamate and malate as substrates, as compared to rats fed the safflower oil-supplemented diet, which had greater content of cardiolipin containing oleic and arachidonic acids, and had a greater capacity for oxidative phosphorylation. The observed decreases in capacity for oxidative phosphorylation in HCO-supplemented rats was hypothesized to be attributed to structural abnormalities in mitochondrial ultrastructure, due to an altered cardiolipin fatty acyl profile which may provide an unfavourable environment for the synthesis of high energy intermediates [155]. The specific composition of cardiolipin acyl chains has been studied to a degree, and a number of studies have built on the premise that mitochondria must contain a high degree of unsaturated 18 carbon-rich cardiolipin needed for oxidative phosphorylation. It has been shown that mitochondria deficient in unsaturated 18 carbon-rich cardiolipin have impairments in the activity of ETC complexes including, but not limited to, complex IV (cytochrome c oxidase) [156], and complex II + III [157].

Mitochondrial cardiolipin content has been studied in detail in neurons, and found to be proportionately higher in non-synaptic compared to synaptic mitochondria [158]. Interestingly, however, cardiolipin in non-synaptic mitochondria appears to be less resistant to aging-mediated changes, undergoing a decrease in total content with advancing age, while the cardiolipin content of synaptic mitochondria remains well preserved [158]. Conversely, in at least one model of Alzheimer's Disease, synaptic mitochondria are shown to undergo a decline in most cardiolipin species, while it is the non-synaptic mitochondria that are well maintained [154].

Other tissues

The composition of liver cardiolipin is well conserved across species, with a high degree of similarity between rodents and bovines in fatty acyl profiles. Similar to heart, linoleate comprises more than two-thirds of the fatty acyl species in hepatic cardiolipin [142]. The next most abundant fatty acyl species in hepatic cardiolipin is oleate, which comprises ~19%, followed

by palmitate, which comprises ~6% [142, 159]. Markedly fewer studies have examined cardiolipin in lung, kidney, skeletal muscle, testes, and spleen, compared to heart, and brain. Linoleic acid is reported to be the predominant fatty acyl species in cardiolipin isolated from kidney (~61%) [142] and spleen (~49%) [142]. This profile is not seen in other tissues, which are often found with a more saturated cardiolipin profile. Rodent lung and skeletal muscle are reported to have a greater content of saturated fatty acyl species, primarily stearate (~44% and ~40%, respectively) [142], whereas cardiolipin found in testes is predominated by palmitate (~55%) [142]. Additional studies on the cardiolipin content and composition of these and other tissues would fill an important gap in the primary literature. A summary of cardiolipin fatty acyl species by abundance in each tissue is shown in Table 2.

Table 2: Summary of the most abundant fatty acyl species found in cardiolipin in mammalian tissues

Organ	Tissue source	Order of abundance	Reference
Heart	Human	18:2n-6 >> 18:1n-9/20:4n-6 > 18.0	[114]
	Rodent	18:2n-6 >> 18:1n-9 > 18:0/22:6n-3	[116]
	Bovine	18:2n-6 >> 18:1n-9 > 18:3n-3	[117]
Brain	Human	18:1n-9 >> 18:0 > 22:6n-3	[115]
	Rodent	18:0 >> 18:1n-9 > 16:0	[142]
	Bovine	18:1n-9 >> 18:2n-6 > 16:1n-9	[141]
Liver	Rodent	18:2n-6 >> 18:1n-9 > 16:0	[142, 159]
	Bovine	18:2n-6 >> 18:1n-9 > 16:0	[159]
Lung	Rodent	18:0 >> 16:0 > 18:1n-9/18:2n-6	[142]
Kidney	Rodent	18:2n-6 >> 18:1n-9 > 18:0	[142]
Skeletal muscle	Rodent	18:0 >> 16:0 > 18:2n-6	[142]
	Bovine	18:2n-6 >> 18:1n-9 > 18:3n-3	[160]
Spleen	Rodent	18:2n-6 >> 18:0 > 18:1n-9	[142]
Testes	Rodent	16:0 >> 18:0 > 18:1n-9	[142]

Enzymes involved in cardiolipin acyl-chain remodeling

To date, four enzymes have been identified that can re-synthesize cardiolipin by acylating MLCL or DLCL. These enzymes play crucial roles in the remodeling of 'immature' or nascent

cardiolipin produced *de novo* in the Kennedy pathway to 'mature' cardiolipin that contains a profile of fatty acyl chains specific to, and functionally appropriate for, the tissue in which it is located. During the initial synthesis of cardiolipin, the enzyme CLS demonstrates a similar level of activity with substrates regardless of fatty acyl composition [161-163]. Cardiolipin synthesized *de novo* therefore tends to have the same essential composition as its major PG and CDP-diacylglycerol precursors [161, 162, 164]. The biochemical characterization of human CLS found that *de novo* synthesis of nascent cardiolipin occurs at a high level with a number of PG species including dilinoleoyl (18:2n-6/18:2n-6)-PG and dioleoyl (18:1n-9/18:1n-9)-PG. CLS also had activity with 16:0/18:2n-6-PG, 16:0/18:1n-9-PG, dipalmitate (16:0/16:0)-PG, albeit to a lesser extent, where CLS activity was approximately halved with these PG species. In contrast, CLS had little-to-no activity relative to dilinoleoyl- and dioleoyl-PG with dimyristoyl-PG (14:0/14:0) [161]. Interestingly, CLS had similar activity with dimyristoyl-CDP-DAG, egg phosphatidylcholine, and dioleoyl-CDP-DAG, but substantially much less activity (~30%) with dipalmitoyl-CDP-DAG [161]. These glycerolipids are produced in earlier steps of the Kennedy Pathway using phosphatidic acid as the ultimate precursor. Since phosphatidic acid is acylated by glycerophosphate acyltransferases (GPATs) and acyl-glycerophosphate acyltransferases (AGPATs) that tend to prefer saturated and monounsaturated fatty acids [165], the remodeling process is critical to achieve the high degree of cardiolipin acyl chain specificity that is apparent in different tissues.

Cardiolipin remodeling largely occurs via the Lands' remodeling pathway [166, 167]. Remodeling of 'immature' cardiolipin is achieved through the joint action of phospholipases A1 and A2, CoA-dependent acyltransferases, and O-transacylases. There are dozens of different known mammalian PLAs, and they vary widely in substrate specificity, with preferences for substrates based on glycerophospholipid species, fatty acyl side chain moieties, or both [168-171]. There are also over a dozen known acyl-CoA-dependent mammalian acyltransferases and acyl-CoA-independent transacylases. These tend to show significant acyl donor and acyl acceptor substrate specificities [168, 170], recognizing only a narrow class of glycerophospholipids. A PLA2 will either cleave a fatty acid from one of the two *sn-2* positions, resulting in the intermediate species MLCL, or it can cleave both fatty acids from the *sn-2* positions, resulting in DLCL. Similarly, a PLA1 will act on either one or both of the *sn-1* positions of cardiolipin. Re-acylation at one or both of these sites may be performed by an acyltransferase that utilizes fatty acyl-CoAs

as donor substrates, or a transacylase that cleaves and transfers a fatty acyl chain from another phospholipid such as phosphatidylcholine, generating reformed (*i.e.* fully acylated) cardiolipin and a lysophospholipid such as LPC (see Fig. 9 for an overview of cardiolipin remodeling).

Tafazzin

The enzyme Tafazzin (encoded by the *TAZ* gene in humans) was first identified in 1996 [172]. Tafazzin is a mitochondrial enzyme, found both on the outer- and inner-mitochondrial membranes [173]. It is most highly expressed in cardiac and skeletal muscle, with varying levels in other tissues [151], and has been shown to act predominantly as a linoleoyl transacylase in the remodeling of MLCL [118]. Although Tafazzin also exhibits some lesser activity with palmitoleoyl residues, it does not utilize oleoyl residues [118]. Tafazzin functions by deacylating linoleoyl residues from PC, which it then esterifies onto MLCL to produce linoleate-enriched cardiolipin [118]. Cardiolipin produced by Tafazzin usually contains linoleate at all *sn-1* and *sn-2* positions [174]. Tafazzin can also catalyze the reverse reaction, esterifying LPC with acyl groups it derives from cardiolipin, PE, or PC, forming mature PC. However, the forward reaction is typically favoured *in vivo* [118]. This is supported by findings that knockdown of Tafazzin results in an overall decrease in the synthesis of tetra-linoleoyl cardiolipin, with accumulation of MLCL and linoleate-deficient cardiolipin species in cells [151]. Mutations in the *TAZ* gene also cause aberrant cardiolipin remodeling in humans [175-177], resulting in Barth Syndrome. This X-linked genetic disorder is characterized by a decrease in total cardiolipin and tetra-linoleoyl cardiolipin levels, and an accumulation of MLCL, resulting in skeletal- and cardiomyopathies, neutropenia, and frequently some degree of cognitive impairment [172, 177-180].

Monolysocardiolipin-Acyltransferase 1 (MLCL-AT1)

MLCL-AT1 was first characterized by the laboratory of Grant Hatch in 1999 as a mitochondrial monolysocardiolipin acyltransferase enriched in the hearts of rats [181]. In 2003, the Hatch laboratory purified MLCL-AT1 from pig liver mitochondria and identified the protein sequence by mass spectrometry [182]. MLCL-AT1 is a 74 kDa protein that preferentially esterifies linoleoyl-CoA and oleoyl-CoA onto MLCL. Although it also utilizes palmitoyl-CoA, MLCL-AT1 has 10-fold higher activity with unsaturated fatty acids, and no activity with any other lysophospholipid substrates, including DLCL. MLCL-AT1 activity has been found to increase during apoptosis in both cultured cells [183], and in rat hearts during heart failure [157], which

may reflect a compensatory mechanism employed to maintain homeostatic levels of cardiolipin that decline during apoptosis [184].

alpha-Trifunctional Protein (αTFP)

In 2009, the human homolog of MLCL-AT1 was identified [119]. Human MLCL-AT1 is a 59 kDa acyl-CoA dependent enzyme that preferentially incorporates linoleate into MLCL *in vivo* [119]. Sequence analysis indicated that it is identical to the *α*-subunit of human trifunctional protein (TFP), which lacks the initial 227 N-terminal amino acids of the full-length protein. A detailed analysis of the cardiolipin-remodeling activity of αTFP has shown that it has the highest activity with linoleoyl-CoA, but can also utilize oleoyl-CoA and palmitoyl-CoA, similar to MLCL-AT1 [120].

ALCAT1

ALCAT1 is a 44 kDa acyl-CoA:lysocardiolipin acyltransferase that was characterized in 2004 as an acyl-CoA dependent enzyme that can use both MLCL and DLCL as acyl acceptors [121], although it has shown activity with other anionic lysophospholipid acceptors as well, including LPI and LPG [185]. *In vivo*, ALCAT1 preferentially esterifies MLCL and DLCL with very long-chain highly unsaturated fatty acids (HUFA, ≥20 carbons, ≥3 double bonds), at the expense of 16-18 carbon saturated (SFA), monounsaturated (MUFA), and n-6- and n-3 polyunsaturated fatty acids (n-6 PUFA, n-3 PUFA) [143]. This enzyme is predominantly expressed in the heart and liver and, importantly, has been found to localize to the MAM [143], which indicates participation in a cardiolipin-remodeling pathway that is distinct from the other known mitochondrial pathways [161]. Because ALCAT1 prefers HUFA, cardiolipin formed by this enzyme is highly unsaturated, and may be more prone to peroxidation [143]. ALCAT1 is therefore implicated in mitochondrial dysfunction [143]. A schematic of CL remodeling by ALCAT1, MLCL-AT1, Tafazzin, and αTFP is shown in Figure 9.

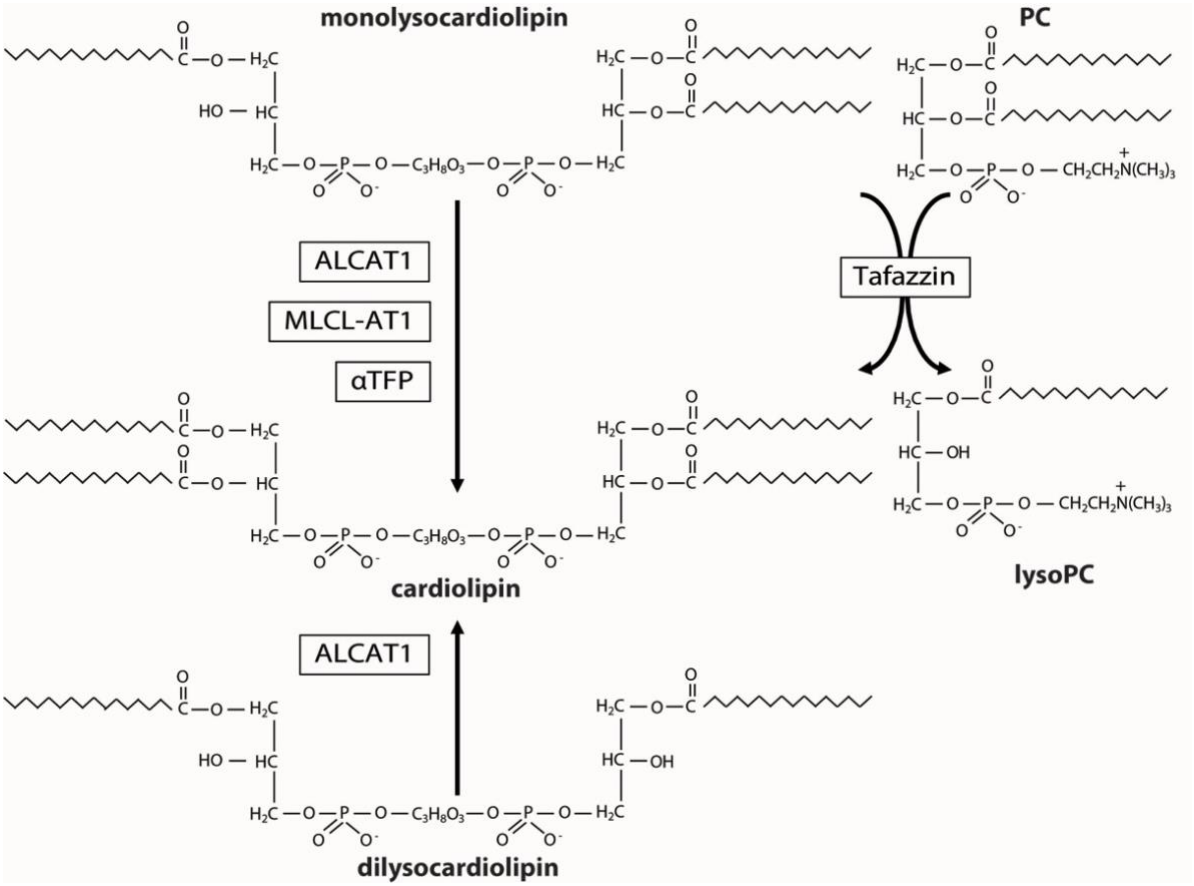

Figure 9: Enzymes involved in cardiolipin remodeling

Cardiolipin produced during *de novo* synthesis must undergo acyl remodeling by one of four known enzymes in order for incorporation into the inner mitochondrial membrane. Tafazzin functions as a PC:MLCL transacylase by hydrolyzing a fatty acid from the *sn-1* position of PC for incorporation into MLCL. This results in the production of a molecule of CL and a molecule of LPC. One enzyme, ALCAT1 can remodel CL from both MLCL and DLCL, and has the ability to incorporate fatty acyl-CoAs into either precursor molecule. Finally, two MLCL acyltransferases have been identified. Both MLCL-AT1 and αTFP can esterify a fatty acid into MLCL for the remodeling of CL.

Dietary regulation of cardiolipin acyl-chain profile

Differences in the dietary composition of fatty acids can influence body phospholipid fatty acyl profiles to the extent that functional consequences become evident, such as alterations in membrane fluidity [186-190]. Several studies have investigated the effects of modulating dietary fatty acid species on cardiolipin fatty acyl profiles, with outcomes examined most often in the heart and liver [133, 137, 138, 191]. Since a high degree of variability is evident between reported studies with regards to the methods used, source of fatty acids, and duration of treatment, the data

discussed here compare different dietary interventions only when performed within the same study. A summary of diet intervention on cardiolipin content and composition is given in Table 3.

Dietary enrichment with saturated fatty acids (SFA) versus polyunsaturated fatty acids (PUFA)

In a study performed by Aoun *et al*. (2012), alterations in hepatic cardiolipin were observed when 6-week old Wistar rats were fed for 12 weeks on diets enriched in lard or fish oils, or fed a basal diet (BD) containing a mixture of olive oil, safflower oil, and lard, at either 5 or 30% total fat contents (w/w) [189]. The level of linoleate rose from ~50% to over 70% of fatty acyl species in hepatic cardiolipin when animals were fed a higher fat diet, regardless of the source. This increase was accounted for primarily by decreases in total monounsaturated fatty acid (MUFA) species (16:1n-7, 18:1n-9, and 18:1n-7) where the largest single change observed was an ~10% decrease in the proportion of palmitoleate (16:1n-7). Interestingly, the proportion of 22:6n-3 in hepatic cardiolipin was similar in rats across all dietary treatments, including the fish oil-enriched diet, when animals were fed fat at a 5% total fat level, indicating little effect of fish oil supplementation at low levels [189]. When the dietary content of fat was increased to 30%, the proportion of hepatic cardiolipin 22:6n-3 decreased in animals from all diet groups except for the fish oil-enriched diet, which stayed essentially the same. In animals fed BD, lard, or fish oil diets at a 5% total fat level, hepatic cardiolipin arachidonate content varied significantly from ~3% to 2% to 1%, respectively. When animals were fed these same diets at a 30% fat level, the content of arachidonate made up a similar proportion of total hepatic cardiolipin fatty acyl species in BD and lard-enriched diets (~1.5 to 2%), whereas it represented a significantly lower proportion when animals were fed fish oil-enriched diets. Interestingly, the total content of saturated fatty acids in hepatic cardiolipin was not different across dietary groups, regardless of the percentage fat in the diet, and comprised ~8-10% of total fatty acyl species.

Another study performed by Chicco *et al.* (2008) examined the effects on heart cardiolipin content and composition of spontaneous hypertensive heart failure prone rats (SHHR) ($Mcc^{facp-/-}$) fed either a 20 kcal% lard diet, or a 20 kcal% high-linoleate safflower oil diet, compared to animals fed a control chow diet [138]. This strain of rats exhibits progressive cardiac hypertrophy leading to a distinct decrease in tetra-linoleoyl cardiolipin with advancing age. Dietary treatments were therefore initiated relatively late in life, at 18 months, and were continued until natural death occurred, with a mean duration of exposure to diets of approximately 5 months. In this study,

changes in cardiolipin composition between dietary groups were substantial. While the lard-enriched diet had no significant effect relative to the low-fat chow diet on the proportion of tetra-linoleoyl cardiolipin in rat hearts, which was close to 40% in both groups at death, the high-18:2n-6 safflower-enriched diet resulted in a significant increase in cardiac tetra-linoleoyl cardiolipin species to ~80% of the total. Given the importance of maintaining tetra-linoleoyl cardiolipin in heart, this work suggests that dietary linoleic acid supplementation may have benefit for the maintenance of cardiac health in later life.

Dietary enrichment with MUFA vs. PUFA

A study by Kramer and colleagues using 3-week old male Sprague-Dawley rats fed diets containing 20% (w/w) corn oil, 20% (w/w) soybean oil, or 20% (w/w) olive oil [137]. The most striking difference between these diets was in the relative contents of 18:2n-6 and 18:1n-9. The corn oil and soybean oil enriched diets contained ~60% and ~50% linoleic acid, respectively, but only ~24-25% oleic acid, whereas the olive oil-enriched diet contained over 75% oleic acid, but only ~7% linoleic acid. Over the course of a 16-week trial, rats fed the corn oil or soybean oil diets displayed significantly higher levels of cardiac cardiolipin linoleate (~85% of fatty acyl species) compared to rats fed the olive oil diet, where cardiac cardiolipin linoleate content was only ~52%. Not surprisingly, the difference in the proportionate content of fatty acyl species in cardiac cardiolipin in olive oil diet-fed rats was largely accounted for by enrichment with oleate. This study is important since it highlights the striking degree of influence that dietary fatty acyl composition can exert on cardiac cardiolipin profile. It also highlights that this change is possible either in animals at either a very young [137] or advanced age [138]. In addition, this study also emphasizes the apparent importance for a specific cardiolipin fatty acyl species in heart. The cardiac mitochondrial membranes of rats fed diets rich in oleic acid (*i.e.* olive oil) or alpha-linolenic acids (*i.e.* soybean oil) over 16 weeks were more easily lysed, and generated a higher content of free fatty acids and lysophosphatidylcholine during lipid extraction than those fed diets high in linoleic acid (*i.e.* corn oil) [137]. These effects were positively correlated with heart lesions in male rats fed oils high in oleic acid and alpha-linolenic acid for an extended period of time [137].

Dietary enrichment with n-3 PUFA vs. n-6 PUFA

Perhaps the most intriguing dietary intervention studies are those that examine the effects of PUFAs on cardiolipin remodeling and content, since PUFAs have been shown to be important in maintaining cardiolipin function in oxidative metabolism [148-150], but there is still some debate as to whether n-6 PUFA is more effective than n-3 PUFA in supporting cardiolipin function. Swanson and Kinsella (1986) observed that Sprague-Dawley rats fed diets containing increasing percentages (5%, 10%, and 20%) of menhaden oil over 3 weeks had increasing levels of 22:6n-3 (ranging from ~8% to ~21% of cardiac cardiolipin fatty acids) as well as increasing levels of 20:5n-3 (ranging from an undetectable level to ~3.5%), but contents of 18:2n-6 and 20:4n-6 that decreased by ~one-third to one half [191]. This study observed the largest changes in heart cardiolipin composition with the 10% menhaden oil diet. Unlike changes observed with diets enriched by lard or n-6 PUFA, the changes in molecular cardiolipin species resulting from n-3 diet supplementation can happen quite rapidly and are evident within only a few weeks.

In another report, the work of Pepe *et al*. (1999) reported little effect of a high n-6 PUFA diet relative to a high n-3 PUFA (fish oil supplemented) diet in preventing the age-related decline in cardiac cardiolipin linoleate levels in Wistar rats [192]. In fact, surprisingly, this study reported that increasing the n-6:n-3 PUFA ratio in rat cardiac mitochondria by feeding an n-6 PUFA enriched diet significantly attenuated the calcium-dependent activation of pyruvate dehydrogenase (PDH). This enzyme is important in driving the proton-motive force, which is required in oxidative phosphorylation, suggesting poorer function associated with the supplementation of n-6 PUFA in aging animals. Conversely, this study also reported that the function of pyruvate dehydrogenase was significantly augmented in rats fed an n-3-rich diet, where a higher n-3:n-6 ratio developed in their cardiac mitochondria membranes. In the same study, older rats (age 24 months) fed either standard, low fat reference, or n-6-rich diets, had significantly increased mitochondrial concentrations of calcium, and were more likely to reach calcium overload after ischemia/reperfusion as compared to rats fed n-3-rich diets [192]. The higher n-3:n-6 ratio in rats fed an n-3-rich diet was hypothesized to confer a cardioprotective effect compared to rats with higher n-6:n-3 ratios [192]. This study therefore suggests that n-3 PUFA in heart cardiolipin may play a beneficial role.

However, this role clearly merits significant further investigation, since not all studies identify benefit associated with the diet-mediated enrichment of cardiac cardiolipin with n-3 PUFA. In seven week-old Sprague-Dawley rats supplemented for 4 weeks, an approximate 2-fold

higher linoleate content occurs in cardiac cardiolipin with 20% corn oil feeding relative to 20% sardine oil feeding [193]. Conversely, rats fed sardine oil had ~11-fold higher total n-3 PUFA in cardiolipin relative to those fed corn oil [193]. In this study, it was also shown that mitochondrial respiratory function was significantly impaired in rats fed sardine oil versus corn-oil. The researchers postulated that since cardiolipin is required for cytochrome c oxidase activity, the diet-mediated change in cardiolipin composition in sardine-oil fed rats had decreased cardiolipin content and, thus, mitochondrial respiratory function [193]. A summary of dietary intervention studies that examined alterations in CL content and fatty acyl profile is shown in Table 3.

Table 3: Influence of dietary intervention on cardiolipin content and fatty acyl profile.

	Animal Model	Tissue	Diet length	Diet (%w/w, except where noted)	Diet Type	Effect on CL	Ref.
SFA vs. PUFA							
	Wistar Rats	Liver Liver Liver Liver	12 weeks 12 weeks 12 weeks 12 weeks	5% Lard 5% Fish Oil 30% Lard 30% Fish Oil	SFA n-3 PUFA SFA n-3 PUFA	High-fat (30%) diets increased 18:2n-6 cardiolipin by ~20% DHA in cardiolipin unchanged at low-fat (5%) levels, but decreased at high-fat (30%) levels except in the 30% fish oil rats. AA in cardiolipin decreased by 1% respectively between basal, lard, and fish diets at 5%. No change in cardiolipin AA at 30% diet between basal and lard diets, but decreased in the fish oil diet. No changes in SFA between diets.	[189]
	SHHR Rats	Heart Heart	Until death (~20 wks) Until death (~20 wks)	20% Lard (%kcal) 20% HL-Safflower Oil (%kcal)	SFA n-6 PUFA	Heart 18:2n-6 cardiolipin unchanged by lard diet HL-Safflower diet increased heart 18:2n-6 cardiolipin by 40%	[138]
MUFA vs. PUFA							
	Sprague-Dawley Rats	Heart Heart Heart	16 weeks 16 weeks 16 weeks	20% Corn Oil 20% Olive Oil 20% Soybean Oil	n-6 PUFA MUFA n-6 PUFA	Heart 18:2n-6 cardiolipin increased in rats on corn and soybean oil diets relative to those on the olive oil diet	[137]
n-3 vs. n-6 PUFA							
	Sprague-Dawley Rats	Heart Heart	4 weeks 4 weeks	20% Corn Oil 20% Sardine Oil	n-6 PUFA n-3 PUFA	Corn oil-fed rats had 2-fold higher 18:2n-6 in heart cardiolipin vs. sardine oil-fed rats Sardine oil-fed rats had 11-fold higher n-3 PUFA in heart cardiolipin vs. corn oil-fed rats	[193]
	Sprague-Dawley Rats	Heart Heart Heart	3 weeks 3 weeks 3 weeks	5% Menhaden Oil 10% Menhaden Oil 20% Menhaden Oil	n-3 PUFA n-3 PUFA n-3 PUFA	(5%) Increased menhaden oil increased 22:6n-3 in heart cardiolipin vs. controls (10%) Increased menhaden oil increased 20:5n-3 in heart cardiolipin vs. controls (20%) Increased menhaden oil decreased 18:2n-6 and 20:4n-6 vs. controls	[191]
	Wistar Rats	Heart Heart	6 weeks 6 weeks	15% Animal Fat 15% Fish Oil	n-6 PUFA n-3 PUFA	Increased n-6:n-3 diet had no effect in changing 18:2n-6 levels in heart cardiolipin	[192]

*DHA = docosahexanoic acid, AA = arachidonic acid, HL = high linoleate

Diseases associated with aberrant cardiolipin remodeling

Changes in cardiolipin content or molecular species can have functional and pathological consequences.

Barth Syndrome (BTHS)

BTHS is a rare, X-linked autosomal recessive disorder first described by Peter Barth in 1983 [176]. Because BTHS is an X-linked disorder, it is much more prevalent in males [180], although cases in females have been reported as well [194]. BTHS typically manifests as skeletal- and cardiomyopathies [151, 176, 180], first presenting as heart failure [176, 195-197], and it is also characterized by cyclic neutropenia [176, 195], 3-methylglutaconic aciduria [198], stunted growth [199], and cognitive impairment [179, 196]. The estimated incidence in 2013 was 1.5 cases/1 million births [200], however, there is speculation that the disease as a whole may be under-diagnosed [178]. Recently, a clinical test for cardiolipin species [201] has been developed, which may facilitate detection of this disease.

Traditional testing for Barth Syndrome involves measurement of the MLCL:tetralinoleoyl cardiolipin ratio, as well as sequencing of the *TAZ* gene [202], since BTHS results from loss-of-function mutations in humans of *TAZ* (also known as G4.5) on chromosomal region Xq28 [203], which translates to a loss of function of the phosphatidylcholine transacylase Tafazzin [175]. Mutations in Tafazzin do not decrease the rate of *de novo* cardiolipin synthesis, but rather cause a failure to remodel cardiolipin with linoleoyl residues [152, 174]. Despite this, both a decline in total cardiolipin levels and reduced remodeling of cardiolipin is evident in cells with *TAZ* mutations, because cardiolipin is inadequately reformed from MLCL. Effects of this mutation are most evident in cardiolipin of the heart and skeletal muscle [152]. The loss of tetra-linoleoyl cardiolipin formation through mutations in Tafazzin causes an increase in trilinoleoyl MLCL, an intermediate species found in cardiolipin remodeling [174, 204, 205]. This ultimately changes the mitochondrial ultrastructure in BTHS patients [206]. Thus far, there have been more than 70 disease-causing mutations reported in the *TAZ* gene [205]. These span all exons, although mutations in exon 5 do not seem to be as critical in Tafazzin function as mutations in other exons [207].

Neurodegeneration

Defective cardiolipin remodeling has also been linked to the pathogenesis of neurodegenerative diseases. Alzheimer's and Parkinson's Diseases are associated with increased oxidative stress and lipid peroxidation [144, 145, 208-215]. As a highly unsaturated lipid, cardiolipin is a target for ROS-mediated damage [216, 217]. During aging, the brain content of cardiolipin decreases [148, 158], and this has been attributed to peroxidative destruction of cardiolipin by ROS [218]. Indeed, post-mortem analysis of cardiolipin in brains from Alzheimer's Disease patients demonstrates a reduction in DHA and total PUFA species [115] that is thought to contribute to the early synaptic mitochondrial dysfunction seen in this disease [154].

Cardiolipin is a potent regulator of both mitophagy and mitochondrial-mediated apoptosis, since it functions to anchor cytochrome c to the inner mitochondrial membrane. However, when cardiolipin becomes peroxidized, it translocates to the outer mitochondrial membrane in a process that is mediated by the mitochondrial proteins NPDK-D [124], and PLS3 [125]. Upon externalization to the outer mitochondrial membrane, cardiolipin releases cytochrome c into the cytoplasmic space, which acts as a signal to initiate mitophagy, resulting in the elimination of damaged mitochondria [128]. Failure to achieve the correct acyl species of cardiolipin is detrimental to mitochondrial function [155-157], and may cause the mitochondrial membrane to undergo depolarization, which can induce mitophagy [127, 128]. Decreased mitochondrial content is often seen in neurodegenerative diseases, as well as aging [158, 219]. In Parkinson's Disease, α-synuclein binds to phospholipids associated with mitochondrial membranes [220-223], forming a complex with cardiolipin and cytochrome C that promotes cardiolipin oxidation. This may be a factor in the etiology of the disease [224].

Cardiomyopathies

Abnormalities in cardiolipin, the third most abundant phospholipid in heart [105], are associated with the progression of cardiomyopathies. During the development of heart failure in both rats and humans there is a decrease in total cardiolipin, as well as a loss of the hallmark molecular species tetralinoleoyl-cardiolipin [225]. These losses are also observed in pediatric idiopathic dilated cardiomyopathy, although unique cardiolipin fatty acyl profiles are seen in children with heart failure as compared to adults [226]. Many of these changes are explained by differences in expression of enzymes involved in cardiolipin biosynthesis and remodeling during

heart failure [157]. MLCL-AT1 has 3-5-fold higher expression during heart failure, whereas Tafazzin expression significantly decreases, and ALCAT1 expression does not change [157].

Although the functional significance of changes in cardiolipin fatty acyl profile are currently uncharacterized in most tissues, it is clear that tetra-linoleoyl-cardiolipin is critical for normal heart function [174, 225], and that alterations in the fatty acyl profile of heart cardiolipin can have significant consequences for the development of disease. ALCAT1-mediated remodeling increases the peroxidation index of cardiac cardiolipin, rendering mitochondria more susceptible to oxidative damaged-induced dysfunction [143], which is highlighted by studies in mice. Cardiac overexpression of ALCAT1 in mice causes increases in both oxidative stress and depletion of mitochondrial DNA [227, 228], while genetic ablation of ALCAT1 prevents the onset of cardiomyopathy, and cardiac dysfunction [227]. Future work will be required to determine whether changes in unsaturation of mitochondrial cardiolipin are linked to function of this organelle and susceptibility to damage in tissues other than heart.

Tumor progression

There is increasing evidence that aberrant cardiolipin remodeling, as well as decreased cardiolipin content, are factors in the development of tumors. Studies report significantly increased contents of 'immature' or unremodeled cardiolipin in tumor cells and experimental tumors from murine models [229-233]. Atypical molecular species of cardiolipin are thought to contribute to tumor progression by dysregulating mitochondrial function, since cardiolipin remodelling is required for normal oxidative phosphorylation activity [155-157]. Mature cardiolipin is involved in the formation and stabilization of electron transport chain complexes [123, 234, 235] and has a role as a proton trap during oxidative phosphorylation [13]. Promotion of tumor growth through defects in mitochondrial function was first hypothesized by Otto Warburg in 1956 [236], and is known as the Warburg theory of cancer. This theory remains the subject of controversy and ongoing research. In support, however, Kiebish *et al*. (2008), have observed significant decreases in cardiolipin content and ETC activity in mitochondria from mouse brain tumors as compared to mitochondria from control brain tissue [229]. Additionally, a significant reduction in the diversity of molecular species of cardiolipin has also been reported in mouse brains affected by the presence of microgliomas [229, 233, 237, 238]. In prostate tumors, cardiolipin enriched in palmitoleic acid (16:1n-7) has been found to accelerate prostate tumor growth, and to be significantly higher in

malignant tissue as compared to non-malignant tissue [231]. The functional relationship between cardiolipin species with increased 16:1n7 and mitochondrial function remains, however, to be determined.

Immune function

While cardiolipin in eukaryotic cells is almost exclusively localized to the inner mitochondrial membrane, in prokaryotes it is resident in the outer plasma membrane [129]. Thus, prokaryotic cardiolipin on the plasma membrane of bacteria can act as a potent immunogenic target, which would not typically be detected by immunosurveillance of eukaryotic cells due to the deep internalization of this lipid within intracellular organelles. Glyco- and phospholipids act as natural ligands for major histocompatibility complexes (MHC) and MHC-like proteins, which are involved in presenting antigenic structures to T-cells [239-241]. Specifically, CD1d, an MHC-like protein, has been observed to bind and present cardiolipin to γδ T-cells [242], a subset of T cells that exist in the spleen and livers of healthy mice. Thus, cardiolipin may help to regulate host-pathogen interactions. As well, it has recently been demonstrated that both bacterial and mitochondrial cardiolipin externalization on the plasma membrane serves as an 'eat-me' signal, which is mediated by the CD36 receptor [243]. Thus, externalized cardiolipin generated during the cellular processes of mitophagy and mitochondrial-mediated apoptosis [127, 128, 244] may serve as factors regulating host-host interactions in which mitochondria or the entire cell is phagocytosed [243]. X-ray crystallography has characterized the physical interaction between CD1d and cardiolipin [242]. However, it has not yet been determined whether modification of specific cardiolipin molecular species can alter the immune response to presented lipid.

Chapter Three
Methods and Materials

Materials

Lysophospholipids, phospholipid standards, and non-radiolabeled acyl-CoAs were from Avanti Polar Lipids (Alabaster, AL). Reverse Transcriptase was purchased from Thermo Scientific (Waltham, MA). TRIzol®, cell culture medium and additives were from Life Technologies (Carlsbad, CA). Thin layer chromatography (TLC) plates were from Analtech (Newark, DE). Radiochemicals were purchased from Moravek Biochemicals (Brea, CA) and American Radiolabeled Chemicals (St. Louis, MO). The TnT® Coupled Reticulocyte Lysate System was purchased from Promega (Madison, WI).

Animals

All animal procedures were approved by the University of Waterloo Animal Care Committee under AUPP-13-13 and AUPP-17-18. Animals were housed in a temperature and humidity controlled environment, on a 12:12 hour reversed light/dark cycle. Where possible, littermates were used. Male C57BL/6J mice aged 12 weeks were used for studies on endogenous HRASLS1 content and subcellular localization in brain cellular fractions. All animals had *ad libitum* access to food and water.

Cell Culture and Cell Counting

All cells were maintained at 37°C, with the addition of 5% CO_2. Human embryonic kidney (HEK-293) cells were cultured in 10 cm tissue culture-treated dishes in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Epstein-Barr virus-transformed Barth Syndrome (BTHS) B-lymphocytes from a 10-year-old Caucasian male, and male age- and ethnicity-matched control B-lymphocytes were obtained from Coriell Biorepository. The specific mutation of BTHS B-lymphocytes utilized was a hemizygous change of C>T in exon 3 of the TAZ (G4.5) gene [C.280>T), resuting in the substitution of cysteine for arginine at codon 94. B-lymphocytes were cultured in suspension in 25 mL flasks in Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM L-glutamine, 10% FBS, and 1% penicillin-streptomycin. For experiments requiring the addition of NAE treatments, charcoal-stripped FBS (HyClone, ThermoFisher, Waltham MA, USA) was

utilized in replacement of standard FBS (Gibco, ThermoFisher). Cells were routinely sub-cultured upon reaching 90% confluence by trypsinization with 0.25% trypsin-EDTA.

Cell counting was performed using a Z2 Coulter Counter (Beckman-Coulter) as previously described [245], with minor modifications. Briefly, cells were centrifuged at 1,000 x rpm for 5 minutes at room temperature in order to pellet cells. Cells were then re-suspended in 2 mL of appropriate media, following which 100 μL of cells was aliquoted to a counting vial containing 10 mL of sheath fluid. Cells were counted by the Z2 Coulter Counter, and plated at appropriate seeding densities based on the cell type.

RNA Extraction, Reverse Transcription (RT) and Real-Time (q) PCR

Total RNA was isolated from mouse organs or cell homogenates using TRIzol® Reagent (1 mL/≤100 mg tissue) using a Polytron® homogenizer (VWR, Radnor PA) set at the highest speed. Samples were then incubated at room temperature for 5 minutes, followed by the addition of chloroform (20% of TRIzol® volume) with vigorous shaking for 15-20 seconds, incubation at room temperature for 2 minutes, and centrifugation at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase of each sample was removed and mixed with isopropanol (50% of initial volume of TRIzol®), and incubated at room temperature for 10 minutes to precipitate the RNA. Samples were then centrifuged at 12,000 x g for 10 minutes at 4°C, pelleting the RNA. The supernatant was discarded and the pellet was washed in 1 mL of 75% ethanol-25% DEPC-ddH_2O, centrifuged at 7,500 x g for 5 minutes at 4°C, then air-dried for 5-10 minutes prior to suspension in 50 μL DEPC-ddH_2O. The samples were then incubated at 58°C for 10 minutes to facilitate solubilisation, and stored long-term at -80°C. RNA samples were quantified using a Nanodrop 2000 Spectrophotometer and samples were adjusted to 1 μg/μL. cDNA was synthesized from 1 μg of RNA by oligo(dT) priming using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Thermo Scientific). Briefly, 1 μg of RNA was mixed with 10 μL of a reverse-transcription master mix with pre-added RNAse inhibitors (2 μL of 10X RT Buffer, 0.8 μL of dNTP mix (100 mM), 2 μL of RT Random Primers (oliogoDT), 1 μL of Reverse Transcriptase, 4.2 μL of ddH_2O). Samples were then incubated in a thermocycler at 25°C for 10 minutes, followed by 37°C for 120 minutes, then finally 85°C for 5 minutes. cDNA was stored long term at -20°C. To examine semi-quantitative changes in gene expression, cDNA was diluted

1:4 and 1 μL was mixed with a master mix containing 2 μL of 10X PCR Buffer with added $MgCl_2$, 0.4 μL of 25 mM dNTPs, 1 μL each of Forward and Reverse primers (5 μM each), 0.1 μL of Taq Polymerase, and 14.5 μL of ddH_2O. Samples were then incubated in a thermocycler under the following conditions: 95°C for 4 minutes, followed by 30 cycles at 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. Samples underwent a final extension at 72°C for 7 minutes. Amplicons were resolved on a 2% TAE-agarose gel with ethidium bromide for visualization under UV light by a Chemidoc Touch™ Imaging System (BioRad). A list of primer sequences used for semi-quantitative PCR analysis is found in Table 4 below.

For real-time PCR assays (qPCR), 2 μL of cDNA was added to a 96-well plate pre-loaded in each well containing 7.5 μL of SYBR™ Green (BioRad) Master Mix, 6.67 μM of each forward- and reverse-primer (pre-diluted to 100 μM stock, 1 μL of each added), and 4.5 μL of ddH_2O. In the case of *18S*, expression studies were performed using a TaqMan gene expression assay (Hs99999901_s1) and PerfeCTA qPCR FastMix (Quanta Biosciences, Gaithersburg, MD). Thermal cycling conditions were 95°C for 2 minutes, followed by 40 cycles at 95°C for 10 seconds, then 60°C for 20 seconds. At the end of the cycling protocol, a melt curve analysis was run to ensure primer specificity for the intended product of interest. Gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, expressing results normalized to *18S*, and relative to expression in control samples. A list of primer sequences used for qPCR analysis is found in Table 5 below.

Table 4: List of primer sequences used for semi-quantitative gene expression experiments

Gene	Organism	Primer name	Direction	Primer Sequence (5' → 3')	Product size
Hrasls1	Mouse	Hrasls1-1L	Forward	TGC CTG AGG CGT GCT TGG AGG ATT TG	124 bp
			Reverse	CGC CGC GCC ACC TCT GAT CCC TCC GC	
Hrasls1	Mouse	Hrasls1-1SA	Forward	CTG AGC TGT GAG CAG GCG ATT TGT GTG	166 bp
			Reverse	AGC CAT CAC CCA AGT ACA GTG CCC AG	
Hrasls1	Mouse	Hrasls1-1SB	Forward	TAA GAC AAC CCA GCT TGA GCA GGG AG	115 bp
			Reverse	AGC CAT CAC CCA AGT ACA GTG CCC AG	
18s	Human/ Mouse	18s	Forward	GAT CCA TTG GAG GGC AAG TCA	79 bp
			Reverse	AAC TGC AGC AAC TTT AAT ATA CGC TAT T	
nDNA β2-microglobulin	Human	nDNA β2-microglobulin	Forward	TGC TGT CTC CAT GTT TGA TGT ATC T	86 bp
			Reverse	TCT CTG CTC CCC ACC TCT AAG T	
mtDNA tRNA$^{Leu(UUR)}$	Human	mtDNA-tRNA	Forward	CAC CCA AGA ACA GGG TTT GT	107 bp
			Reverse	TGG CCA TGG GTA TGT TGT TA	
MT-ND1	Human	MT-ND1	Forward	TCC TCT TCT TAA CAA CAT ACC	227 bp
			Reverse	AGA GGG TGA TGG TAG ATG T	

Table 5: List of human primer sequences used in qPCR experiments

Gene	Forward Sequence, 5' → 3'	Reverse Sequence, 5' → 3'	Product size
GPAT1	TCG GGC AGG ACT TTT GTC AG	GGT TTG CCC AGT TGT TCA CC	135 bp
GPAT2	CAT CAG AAG CTC CTG GGG GA	GCC CAG AGA AGC CTA CAT CA	79 bp
GPAT3	GCC TCT GAG GGT TAC CTT GG	GAT CCG GCA GCA AGT CAG AT	133 bp
GPAT4	TGC CGG AAA GGA ATG GAG AC	CCA GTG CTA TCC TGA GCG G	193 bp
AGPAT1	AAC GTG GCG CCT TCC A	GAA GTC TTG GTA GGA GGA CAT GAC T	77 bp
AGPAT2	GGT ACT CGC AAC GAC AAT GG	TTG GTG TTG TAG AAG GAG GAG AAG	125 bp
AGPAT3	CTC TGG AGC CAA CTG GTC AT	GAG GAT GAT GAC TGC GTG CT	114 bp
AGPAT4	GCA CGG AAT GCA CCA TCT TC	CTT GGA GCC CCC TAA CAG C	149 bp
AGPAT5	GGA CGC AGG AAC TCC AAT GT	GCA GCA AAT GCC TGA ACT AGC	99 bp
CDS1	GTG TTT GGA TTC AAT GCT GCC T	AGG GCT CAC ATT CTG TCA CG	106 bp
CDS2	CAG TCA GTC ATT GGC TGG AAA A	ATC CTC CAA AGG GGC CAA TG	100 bp
PGS	GTC AAG CTC CAG AGG CTG TT	CAG CCA TGG TGA CCT AAG GA	95 bp
CLS	CTT GGT GGC AGC TTC TTT GG	GGC CCT GTT CCC ATT GAT GT	210 bp
TAZ	TGG ACC AAG TAC ATG AAC CAC C	TTC AGG TTC CAG ATG TGG CG	169 bp
ALCAT1	CAT GGT GTC ATG GAA AGG GA	TCC AAT AAT GCC ACA GGT AGG G	136 bp
αTFP	TCG GCA TCT GGG TTT TAG TC	GGT CAG CAA AGC AGA AGA CC	234 bp
PPARα	GCG AAC GAT TCG ACT CAA GC	AAA ACG AAT CGC GTT GTG TG	139 bp
RXRa	TTT CCT GCC GCT CGA TTT CT	AGC TGA TGA CCG AGA AAG GC	194 bp
TRPV1	TGC GGT CAA GCA GAG TTT CA	CGT GAC GTC CTC ACT TCT CC	198 bp
GPR55	CAG TCC ACC TGG GGT TCT TC	TGC TTG GCT CTG CAC TCT AC	70 bp

Cloning of Full-length HRASLS1 cDNA, HRASLS1-GFP, and generation of HRASLS1 adenovirus

Full length murine *Hrasls1* (NM_013751.5) was amplified by PCR from mouse whole brain cDNA with the addition of NotI and SalI restriction sites to the N-terminal and C-terminal ends, respectively, using the following primers: Forward: 5'- ACA GGA TTC CTC GGC GTG CTA CC -3', Reverse: 5'- GGT TCT CCT CAT CAG GGA CTC TA -3' The resulting amplicon was subcloned into pGEM-T-Easy resulting in production of pGEM-*Hrasls1* that was verified by direct sequencing, and was then cut with NotI/SalI restriction enzymes for directional subcloning into pET 43.1 resulting in production of pET-*Hrasls1*-6His. Adenovirus was generated using the Ad-Easy® Adenoviral Vector System. A full-length HRASLS1 amplicon with a C-terminal 6 x His tag and Not1 and SalI restriction sites at the N-terminal and C-terminal ends was produced by PCR using 50 ng of pGEM-HRASLS1 as template and the following primers: Forward: 5'ATT TCT AGG GCC CTC CAG GAC CA-3', Reverse: 5'-TGG GTC CTA AAA GCG CTA TTC GTC CAA-3'. The resulting amplicon was subcloned in frame into pShuttle-IRES-hrGFP2. pShuttle-IRES-hrGFP2-HRASLS1 was linearized by digested with PmeI, and transformed into chemically competent BJ-5183 cells that are pre-transformed with Ad-1. Recombinant adenovirus was verified by PacI digest and direct sequencing, amplified in *E. coli*, linearized by PacI digest, then transfected into HEK-293 cells for formation and amplification of active adenoviral HRASLS1. Control adenovirus was produced by the same method, but using pShuttle-IRES-hrGFP2 without DNA insertion into the multiple cloning site for the formation of Ad-1 recombinants in BJ-5183 cells. Amplified virus was titred by serial dilution in cultures of HEK-293 cells grown in 12-well plates, and quantified by fluorescence microscopy-based counting of infected cells in multiple frames per well.

Generation of HRASLS1 Protein in HEK-293 Cells

For production of lysates overexpressing HRASLS1 or control virus, HEK-293 cells grown in 150 mm plates were infected at a multiplicity of infection (MOI) of 20 infectious units (IFU) per cell, then harvested 48 hours later, washed with PBS, and lysed in Lysis Buffer 2 (100 mM Tris-HCl, pH 7.0, 10mM NaCl) by sonication on ice (65% output, 3 x 6 s), which efficiently disrupts mitochondria and other organelles. Unbroken cells and organelles, and cellular debris were cleared by centrifugation (10,000 x g for 10 minutes). This process will pellet any remaining unbroken mitochondria, but not sub-mitochondrial particles generated by sonication. Protein

content in supernatant fractions were quantified using Bradford assay solution and kept at -80°C for long-term storage.

Generation of HRASLS1 Recombinant Protein from Reticulocyte Lysates

Recombinant HRASLS1 was generated in reticulocyte lysates by utilizing a transcription-translation TnT® Coupled Reticulocyte Lysate System under a T3 promoter (Promega) as per the manufacturer's directions. Briefly, 1 μg of either pCMV-3Tag-3A (control) or pCMV-HRASLS1-3FLAG DNA was added to a tube pre-loaded with a master mix containing the following components: 25 μL TnT® Reticulocyte Lysate, 2 μL TnT® Reaction Buffer, 1 μL TnT® T3 RNA Polymerase, 0.5 μL Amino Acid Mixture, Minus Leucine (1 mM stock), 0.5 μL Amino Acid Mixture, Minus Methionine (1 mM stock), and 17 μL ddH_2O. Samples were incubated in a heating block at 30°C for 90 minutes, following which they were immediately placed on ice. Relative protein concentrations were determined by loading 1 μL of sample into a SDS-PAGE stain free acrylamide gel, which was then electrophoresed at 200 V for 40 minutes. Following electrophoresis, a stain-free image was taken on a ChemiDoc Touch™ Imaging System (BioRad), and analysed for total protein per lane by ImageLab™ Software (BioRad). Arbitrary units representing total protein per lane of sample were made relative to units representing total protein per lane for 5 μg of BSA loaded as a control, in order to estimate protein concentrations. To minimize differences in loading between samples, sample concentrations were then adjusted relative to one another (See Appendix A for illustration of this method). Samples were separated into 10 μL aliquots and stored long term at -80°C. For use in enzyme assays, samples were thawed a maximum of two times to minimize loss of enzyme activity due to freeze-thawing.

Protein Extraction and Immunoblot Analysis

Protein lysates were extracted from cultured cells or whole murine tissue. For protein used in enzymatic assays, cells were mechanically scraped in 5 mL of PBS and transferred into a 15 mL tube, upon which samples were centrifuged at 3,000 x rpm for 5 minutes to pellet the cells. The supernatant was removed and the cell pellet was reconstituted in 100 μL Buffer A (100 mM Tris-HCl, pH 7.4, 5 mM NaCl, 3 mM $MgCl_2$, with 1% protease inhibitor cocktail) and lysed by sonication in an ice-slurry bath (65% output, 3 x 6 seconds), which efficiently disrupts mitochondria and other organellar membranes. Unbroken cells and organelles were cleared by

centrifugation at 10,000 x g for 10 minutes at 4°C. This process will pellet any remaining mitochondria, but not sub-mitochondrial particles generated by sonication. Protein in supernatants were quantified using Bradford assay [246] solution for use in radiochemical enzymatic activity assays.

Protein lysates prepared only for use in immunoblotting were prepared in Buffer B (50 mM Tris-HCl, pH 6.8, 1 mM EDTA, and 0.5% Triton X-100). Samples were then mixed with 6X Laemmeli Buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.05% bromophenol blue) and denatured by heating to 95°C for 5 minutes. Samples were electrophoresed on 12% SDS-PAGE stain-free gels at 200 V for 40 minutes, and transferred to nitrocellulose membranes at 350 mA for 90 minutes. Membranes were blocked for 1 hour with 5% skim-milk blocker (*w/v*) in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), then probed overnight in TBST with 3% skim-milk blocker (*w/v*) using primary antibodies (1:1000 dilution) directed against HRASLS1, HA, FLAG, CPT1A, SCD1, ACSL4, Histone H3, Cytochrome C (Cell Signaling). Membranes were washed 3x with TBST then probed with HRP-conjugated antibodies in TBST with 3% skim-milk blocker. Blots were washed 3x with TBST and bands were detected by enhanced chemiluminescence on a ChemiDoc Touch™ Imaging System (Bio-Rad).

Subcellular Fractionation

Subcellular fractions were separated as previously described [247], with minor modifications. Briefly, whole mouse brains from C57BL/6J mice were harvested and homogenized at 1,000 x rpm in Buffer C (250 mM Sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) then centrifuged at 800 x g for 15 minutes to isolate the nuclear fraction. All centrifugation steps were performed at 4°C. Post-nuclear supernatants were removed to a fresh tube on ice for further processing. The nuclear pellet was washed twice by re-suspending in 300 μL of Buffer A, with added protease and phosphatase inhibitor cocktails, followed by 15 minutes of centrifugation at 500 x g, then another 15 minutes at 1000 x g. The final pellet was re-suspended in 300 μL of Buffer B (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 M NaCl, 0.2 mM EDTA, 20% (*w/v*) glycerol, and 1% Triton X-100), incubated on ice for 30 minutes, then sonicated into homogenous suspension while held in an ice bath using three pulses at 65% output for 6 seconds with a 30 second cooling period in between pulses. Sonicated nuclei were spun at 9,000 x g for 30 minutes to pellet debris,

and the supernatant containing the nuclear fraction was recovered. Post-nuclear supernatants from the initial lysis and centrifugation of cells were spun at 11,000 x g for 10 minutes to pellet mitochondria. The post-mitochondrial supernatant was removed to a fresh tube and centrifuged for 60 minutes at 100,000 x g to pellet the microsomal fraction, following which the cytosolic supernatant was removed and placed in a fresh tube. The microsomal pellet was re-suspended in 50 µL of Buffer B by brief suspension of tubes in an ice-cold sonicating water bath. The mitochondrial pellet was re-suspended in 100 µL of Buffer B and sonicated with three 6 second bursts at 65% output in an ice water bath slurry, with a 30 second cooling period in between pulses.

Subfractionation of the MAM was performed according to the protocol that is described in detail by Wieckowski *et al*. [248], with minor modifications. Briefly, whole mouse brains or vHRASLS1-infected HEK-293 cells were harvested and washed 3x in Buffer F (225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4), then homogenized with a Polytron® at 1,500 x rpm in Buffer G (225 mM mannitol, 75 mM sucrose, 0.5% BSA (*w/v*), 0.5% EGTA, 30 mM Tris-HCl, pH 7.4). To isolate crude mitochondria, homogenates were centrifuged at 740 x g for 5 minutes, and the supernatant was removed and placed in a fresh tube, and then centrifuged for 740 x g for an additional 5 minutes, upon which the supernatant from the second spin was removed once more and placed in a fresh tube. That supernatant was then centrifuged at 9,000 x g for 10 minutes to pellet crude mitochondria, and the supernatant containing microsomes, lysosomes, and the cytosol was discarded. The crude mitochondrial pellet was twice re-suspended in 200 µL of Buffer H (225 mM mannitol, 75 mM sucrose, 0.5% BSA, 30 mM Tris-HCl, pH 7.4) and centrifuged for 10 minutes at 10,000 x g. The supernatants were discarded and the final crude mitochondrial pellet was re-suspended in 200 µL of Buffer I (250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EGTA). To isolate MAM from crude mitochondria, 8 mL of Buffer J (225 mM mannitol, 25 mM HEPES, pH 7.4, 1mM EGTA, 30% Percoll (*v/v*)) was pipetted into an ultracentrifugation conical tube. The entire crude mitochondrial homogenate was then carefully layered on top of buffer I and approximately 350 µL of Buffer I was layered on top of the crude mitochondria suspension to fill up the ultracentrifugation tube. Samples were then centrifuged at 95,000 x g for 30 minutes, which separated MAM from purified mitochondria. Both the purified mitochondrial fraction as well as the MAM fraction were collected, and each was diluted 10X with Buffer I. Samples were then centrifuged at 6,300 x g for 10 minutes. To purify the MAM fraction, the supernatant of the MAM samples following the previous centrifugation was removed and

centrifuged at 100,000 x g for 1 h to pellet the MAM, upon which the supernatant was removed, and the pellet was re-suspended in 100 µL of Buffer B. The pellet of the purified mitochondrial fraction was re-suspended in 200 µL of Buffer I and centrifuged at 6,300 x g for 10 minutes. The supernatant was discarded, and the pellet was re-suspended in 100 µL of Buffer B.

Lipid Extraction, Thin Layer Chromatography (TLC), and Gas Chromatography (GC)

Total lipids were extracted from HEK-293 cells, or B-Lymphocytes by the method of Folch [249]. Briefly, brains were homogenized by a Polytron® homogenizer set at the highest speed in 3 mL of 2:1 (*v*/v) chloroform:methanol with added butylated hydroxytoluene as an antioxidant (Sigma-Aldrich, Bellfonte PA). Samples were then vortexed at maximum speed for 15 s, followed by the addition of 500 µL of Na_2PO_4, upon which samples were inverted thrice and centrifuged at 3,000 x rpm for 5 minutes to separate the organic and aqueous layers. The bottom organic layer was removed and placed in a freshly labeled tube. Samples were dried under a gentle stream of N_2, then were reconstituted in 50 µL of chloroform and individual lipid classes resolved by TLC as described below.

Total lipids were extracted from HEK-293 cells following *in vitro* radiochemical enzyme activity assays via Bligh and Dyer extraction [250]. Briefly, quenched reactions were mixed with 0.25 mL of chloroform, vortexed thoroughly, mixed with 0.25 mL of ddH_2O, vortexed again, then centrifuged at 1,000 x g for 5 minutes to separate the aqueous (upper) and organic (lower) phases. The organic phase was recovered and dried under a stream of N_2. Samples were reconstituted in 50 µL of chloroform, applied to a silica gel G plate, and resolved by TLC using a hexane:diethyl ether:glacial acetic acid solvent system (80:20:2, v/v/v) to resolve neutral lipid classes. Bands corresponding to known standards were identified and total phospholipid as well as triacylglycerol bands were scraped for determination of fatty acid composition and content by gas chromatography. To isolate individual phospholipid species, the scraped phospholipid band underwent a double Folch extraction, was dried under N_2 gas, then reconstituted in 50 µL of chloroform. Phospholipids were then resolved by TLC on a silica gel H-plate using a chloroform:methanol:2-propanol:0.25% KCl:trimethylamine solvent front (30:9:25:6:18, v/v/v/v/v). Bands corresponding to individual phospholipid species were identified using known standards. For radiochemical assays, bands were scraped and counted in 10 ml of liquid scintillant (Ecolite, MP Biomedicals, Santa Ana CA, USA). For non-radioactive samples, identified

phospholipids were overlaid with 10 μg of 22:3n-3 ethyl ester internal standard (Nu-Check Prep, Elysian MN), and scraped for determination of fatty acid composition and content by gas chromatography (GC) with flame ionization detection as previously described [251]. Briefly, fatty acid methyl esters were derivatized by transesterification using 14% boron trifluoride in methanol (Thermo Scientific, Bellfonte PA) with hexane on a 95°C heat block for 1 h. Fatty acid methyl esters were analyzed on a Varian 3900 gas chromatograph equipped with a DB-FFAP 15 m x 0.10 mm injected dose x 0.10 μm film thickness, nitroterephthalic acid modified, polyethylene glycol, capillary column (J&W Scientific from Agilent Technologies, Mississauga, Ontario, Canada) with hydrogen as the carrier gas. Samples (2 μL) were introduced by a Varian CP-8400 autosampler into the injector heated to 250°C with a split ratio of 200:1. Initial temperature was 150°C with a 0.25-minute hold followed by a 35°C/minute ramp to 200°C, an 8°C/minute ramp to 225°C with a 3.2-minute hold, and then an 80°C/minute ramp up to 245°C with a 15-minute hold at the end. The flame ionization detector temperature was 300°C with air and nitrogen make-up gas flow rates of 300 and 25 mL/min, respectively, and a sampling frequency of 50 Hz [251].

In-vitro Acyl-CoA:Lysophospholipid Acyltransferase Enzyme Assays

To test direct acyl-CoA-dependent acyltransferase activity by HRASLS1, a substrate mixture containing 80 μM of test lysophospholipid, 200 μM oleoyl-CoA, and 0.025 μCi per reaction [^{14}C]oleoyl-CoA was prepared, dried under a gentle stream of N_2 and reconstituted in 100 μL of acyltransferase reaction buffer (100 mM Tris, pH 7.0, 4 mg/mL BSA) by vortexing at high speed for 30 seconds. To test direct acyl-CoA-independent acyltransferase activity by HRASLS1, a substrate mixture containing 80 μM of monolysocardiolipin, 200 μM of oleate and 0.025 μCi per reaction [^{14}C]oleate was prepared, dried under a gentle stream of N_2, and reconstituted in 100 μL of acyltransferase reaction buffer (100 mM Tris, pH 7.0, 4 mg/mL BSA) by vortexing at high speed for 30 seconds. Unless otherwise indicated, lysophospholipids utilized were all 1-18:1n-9-2-hydroxy-lysophospholipid. Reactions were initiated by addition of 100 μL of pre-warmed substrate mixture to either 100 μg of sonicated, cleared lysates (*i.e.* crude lysates) from HEK-293 cells infected with either adenoviral GFP (control) or adenoviral HRASLS1, prepared as described above, or 10 μg of control or HRASLS1-reticulocyte lysates. Reactions were mixed gently, and incubated in a 37°C water bath for 30 minutes, then quenched by addition of 0.75 mL of methanol:chloroform (2:1, *v/v*). Lipids were extracted by the method of Bligh & Dyer [250], and

resolved by TLC. Individual phospholipid bands were identified using known standards, and corresponding bands were scraped and radioactivity quantified by liquid scintillation counting.

In vitro PC:MLCL Transacylase Activity Assay

To test the transacylase activity of HRASLS1, a protocol similar to that published by Uyama *et al.* was performed, with minor modifications [35]. Briefly, a substrate mixture containing 100 nmol of MLCL, 10 nmol of one of 18:0/18:0 PC, 18:1n-9/18:1n-9 PC, or 16:0/18:2n-6 PC, containing 0.1 μCi (2 nmol) of either [^{3}H-1,2]-18:0/18:0 phosphatidylcholine, [^{3}H-1,2]-18:1n-9/18:1n-9 phosphatidylcholine, or 1-palmitoyl-2-[^{14}C]-linoleoyl phosphatidylcholine, respectively, was mixed and dried under a gentle stream of N_2 and reconstituted in 100 μL of pre-warmed (37°C) Transacylase Reaction Buffer (50 mM Tris-HCl, pH 8, 2 mM DTT, 0.1% Nonidet P-40). Reactions were initiated by addition of 100 μL of pre-warmed substrate mixture to either 100 μg of sonicated, cleared lysates (*i.e.* crude lysates) from HEK-293 cells infected with either adenoviral GFP (control) or adenoviral HRASLS1, prepared as described above, or 10 μg of control or HRASLS1-reticulocyte lysates. Reactions were mixed gently, and incubated in a 37°C water bath for 30 minutes, then quenched by addition of 0.75 mL of methanol:chloroform (2:1, *v/v*). Lipids were extracted by the method of Bligh & Dyer [250], and resolved by TLC. Individual phospholipid bands were identified using known standards, and corresponding bands were scraped and quantified by liquid scintillation counting.

High-Resolution Mitochondrial Respirometry

Mitochondrial fractions were obtained as previously described [252, 253], with minor modifications. Briefly, 90-100% confluent 10 cm culture dishes of HEK-293 cells overexpressing control or HRASLS1 protein were harvested into 2 mL of MiR05 buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM potassium dihydrogenphosphate, 20 mM HEPES, 110 mM sucrose, and 1g/L essential fatty acid free bovine serum albumin). MiR05 buffer is hypertonic to cells, allowing for gentle lysis, and is a mitochondrial respiration buffer developed by Oroboros Instruments (Innsbruck, Austria). For mitochondrial isolation, 10 μL of protease inhibitor cocktail was added per mL of MiR05 buffer. All centrifugation steps were performed at 4°C. Homogenates were centrifuged at 1,400 × g for 7 min, which pellets nuclei and cellular debris, but not mitochondria. The supernatant was removed and spun at 1,400 × g for 3 min to

remove remaining debris. The supernatant was removed to a fresh tube, which was spun at 10,000 × g for 5 minutes to pellet mitochondria. The mitochondrial pellet was washed in 1 mL of MiR05 with protease inhibitor, and spun at 1,400 × g for 3 min to further purify the mitochondrial fraction from any contaminating nuclei or cell debris. The supernatant was then removed into a fresh tube, and spun at 10,000 × g for 5 minutes to pellet the mitochondria again. The supernatant was removed, and the mitochondrial pellet was then re-suspended in 250 µL of MiR05 with protease inhibitor, and 40 µL was immediately injected into a chamber of Oxygraph-2k respirometer. The rest was frozen at -80ºC for subsequent protein and immunoblot analysis.

The rate of mitochondrial oxygen consumption was measured using the Oxygraph-2k respirometer (Oroboros, Austria) equipped with the O2k-Fluorescence LED2-Module for simultaneous measurement of H_2O_2 production using Amplex Red [254]. All measurements were performed at 37ºC. Prior to the addition of mitochondria, 2 mL of MiR05 was equilibrated to the chamber and oxygenated to approximately 300 nmol/mL of O_2. Components of the Amplex Red detection system were added to each chamber (final concentrations; 10 µM Amplex Red, 1 U/mL of horse radish peroxidase, and 5 U/mL of superoxide dismutase), as described previously [254]. After a baseline was recoded, H_2O_2 was injected into each chamber (final concentration of 0.1 µM) to allow for an initial calibration of the fluorescent signal. To examine the components of the electron transport chain in detail, a substrate-uncoupler-inhibitor titration (SUIT) procedure adapted from Afshordel et al. 2015 was performed [252]. Forty µL of the isolated mitochondria solutions were added to each chamber and respiration was allowed to equilibrate (endog). A solution of complex I substrates, including glutamate (5 mM), pyruvate (5 mM) and malate (2 mM), was added to each chamber in the absence of ADP to determine mitochondrial leak (leak (P/G/M). ADP (2 mM) was added to determine the capacity of complex I (CI), followed by the addition of the complex II substrate succinate (10 mM) to determine the capacity of complex I+II (CI + CII). Cytochrome C (10 µM) was subsequently added to test for mitochondrial integrity. The inhibition of ATP synthase with oligomycin (2.5 µM) was then added to determine substrate limited leak state (leak (only)). As a maximal measure of ETS capacity, the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazone CCCP (2.5 µM) was added (ETS). Complex II supported non-coupled respiration (CII) was determined with the addition of rotenone (0.5 µM). The addition of antimycin A (2.5 µM), a complex III inhibitor, allowed for the quantification of residual oxygen consumption (ROX). Finally, tetramethylphenylenediamine (TMPD) (0.5 mM) and ascorbate (2

mM) were added to each chamber to stimulate cytochrome-c oxidase activity (CIV). Autoxidation was determined with the subsequent addition of sodium azide (100 mM). All values were corrected for ROX, and CIV activity was also corrected for autoxidation. All respirometry and Amplex Red fluorescent values were normalized to total protein concentrations, which were determined via Bradford assay.

Metabolic Radiolabelling Experiments in NAE-treated cells

Metabolic labelling experiments were performed similarly as described by Uyama *et al.* (2013) [53], with minor modifications. For radiolabeled metabolic experiments, HEK-293 cells were grown in 10 cm culture dishes in DMEM with the addition of 10% charcoal-stripped FBS, and 1% penicillin. Upon reaching 80% confluence, cells with labelled with the addition of 1 μCi of [^{14}C] palmitate and either 0.1, 1, or 10 μM of PEA, OEA, or SEA. Cells were then incubated for 48 hours, upon which lipids were extracted by the method of Folch [249]. Phospholipids were then resolved on a silica gel H plate in a chloroform:methanol:2-propanol:0.25% KCl:triethylamine (30:9:25:6:18, *v/v/v/v/v*) solvent front. Bands corresponding to cardiolipin as identified by known standards were scraped and counted by scintillation counting.

NAE treatment of HEK-293 cells or B-lymphocytes

For NAE treatment experiments, HEK-293 cells or B-lymphocytes were seeded into 10 cm culture dishes, or 25 mL culture flasks, respectively. Cells were seeded in DMEM (HEK-293) or RPMI1640 (B-lymphocytes) with the addition of 10% charcoal-stripped FBS, 1% pencillin-streptomycin, and NAE added to a final concentration of either 0.1, 1, or 10 μM. Cells were incubated for 48 hours, upon which they were harvested for total lipid and RNA extractions.

N-acylethanolamine and 1-AG and 2-AG sample extractions and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

To quantify changes in NAE species and content, HEK-293 cells were infected with control or HRASLS1 adenovirus for 48 hours, following which cells were collected by trypsinization and pelleted by centrifugation at 1,000 x g for 5 minutes. The supernatant was removed, and the pelleted cells were flash frozen in liquid nitrogen and stored at -80°C for analysis. Quantification of varying NAE species, 1- and 2-arachidonylglycerols (1- and 2-AG), was performed by our

collaborators Lin Lin, MSc and Dr. Richard Bazinet at the University of Toronto, as they have previously described [255, 256].

Statistical Analysis

The results are expressed as means ± S.E.M. Statistically significant differences between two groups were assessed by Student's *t* test. Differences between multiple groups were assessed by one-way analysis of variance with Bonferroni's *post-hoc* test. Significance was accepted at $P < 0.05$.

Chapter Four

Thesis Study I – Cellular and biochemical characterization of HRASLS1

Introduction and Study Rationale

The Harvey-Ras-like tumor suppressor (HRASLS) enzymes are a homologous group of proteins that are a part of a larger papain-like NIpC/P60 superfamily of vertebrate thiol proteases [18, 19]. Growing evidence has indicated a role for HRASLS enzymes in glycerophospholipid metabolism due to their abilities to function as O- and N-transacylases, and as phospholipases A1/2 [35, 45]. While these enzymes have the ability to perform all three enzymatic roles, each isoform has its unique preference of predominant enzymatic activity. HRASLS1 has been demonstrated to have phospholipase A1/2 hydrolytic activity, PC:PE N-transacylase activity, and PC:LPC O-transacylase activities [45]. These *in vitro* observations have been supported as well by metabolic labeling experiments in cells [35, 36, 45, 53]. However, studies have indicated that the O-transacylase activity of HRASLS1 is approximately 8-fold higher than the N-transacylase activity, and approximately 5-fold higher than the PLA1/2 activity, indicating that *in vitro*, HRASLS1 predominately acts as an O-transacylase [45].

While the majority of O-transacylase activity assays of HRASLS enzymes have been performed by utilizing PC or PE as the acyl donor, it is reasonable to speculate that HRASLS1 may be able to hydrolyze and transfer acyl groups from other phospholipids or precursor lipids as well. In fact, investigators have also observed O-transacylase activity for other HRASLS family enzymes when utilizing lysophospholipids as acyl donors [45, 46]. Additionally, the Duncan study (2009) indicated that another HRASLS family member, HRASLS3, shows PLA1 activity with other phospholipids including PS and PI, but not PA, indicating diversity in substrate recognition [48]. Thus, evidence suggests that it would be reasonable to investigate whether HRASLS1 has direct O-transacylase activity in the synthesis of phospholipids other than PC. The O-transacylase activity of HRASLS1 has been previously characterized using PC as an acyl donor, and LPC as an acyl acceptor [45]. This coupled activity suggests that HRASLS1 may function similar to the transacylase Tafazzin, which is a PC:MLCL transacylase that helps to produce tetra-linoleoyl cardiolipin [118]. HRASLS1 is most abundant in tissues that have a relatively high mitochondrial content including heart, brain, and skeletal muscle [33], signifying a potential role for this enzyme in the synthesis and/or remodeling of cardiolipin. **However, no specific study has yet investigated or proposed a role of HRASLS1 in regulating cardiolipin metabolism.** Thus, the

overarching objective of this study was to perform the cellular and biochemical characterization of HRASLS1. Both O-acyltransferase and O-transacylase activities were investigated, as well as the subcellular localization. An overview of study design for this chapter is illustrated in Figure 10.

Objectives and Hypotheses

The objectives and hypotheses for this study were as follows:

1. Objective: To characterize the tissue expression profiles of HRASLS1.

   Hypothesis: HRASLS1 will be ubiquitously expressed, albeit with highest expression in heart and brain tissue.

2. Objective: To characterize the subcellular localization of HRASLS1.

   Hypothesis: Consistent with its putative role in cardiolipin biosynthesis, HRASLS1 will localize to the outer mitochondrial membrane, but not the mitochondria-associated ER membranes (MAM).

3. Objective: To characterize the *in vitro* acyl-CoA-dependent O-acyltransferase and acyl-CoA-independent PC:MLCL O-transacylase activities of HRASLS1 in phospholipid biosynthesis using crude lysates.

   Hypotheses: As compared to controls, crude lysates overexpressing HRASLS1 will:

i. Not have significant *in vitro* oleoyl-CoA-dependent O-acyltransferase activity.

ii. Have significant *in vitro* PC:MLCL transacylase activity using MLCL as an acyl acceptor and [^{3}H-1,2]18:0/18:0-PC, [^{3}H-1,2]18:1n-9/18:1n-9-PC , and 16:0/[^{14}C]18:2n6-PC as acyl donors.

4. Objective: To characterize the direct *in vitro* O-acyltransferase and O-transacylase activities of HRASLS1 in cardiolipin synthesis using reticulocyte lysates.

   Hypotheses: As compared to controls, reticulocyte lysates overexpressing HRASLS1 will:

i. Not have significant *in vitro* oleoyl-CoA-dependent O acyltransferase activity utilizing oleoyl-CoA as an acyl donor and MLCL as an acyl acceptor.

ii. Have significant *in vitro* PC:MLCL transacylase activity using MLCL as an acyl acceptor and [^{3}H-1,2]18:0/18:0-PC, [^{3}H-1,2]18:1n-9/18:1n-9-PC , and 16:0/[^{14}C]18:2n-6-PC as acyl donors

.

Study Design

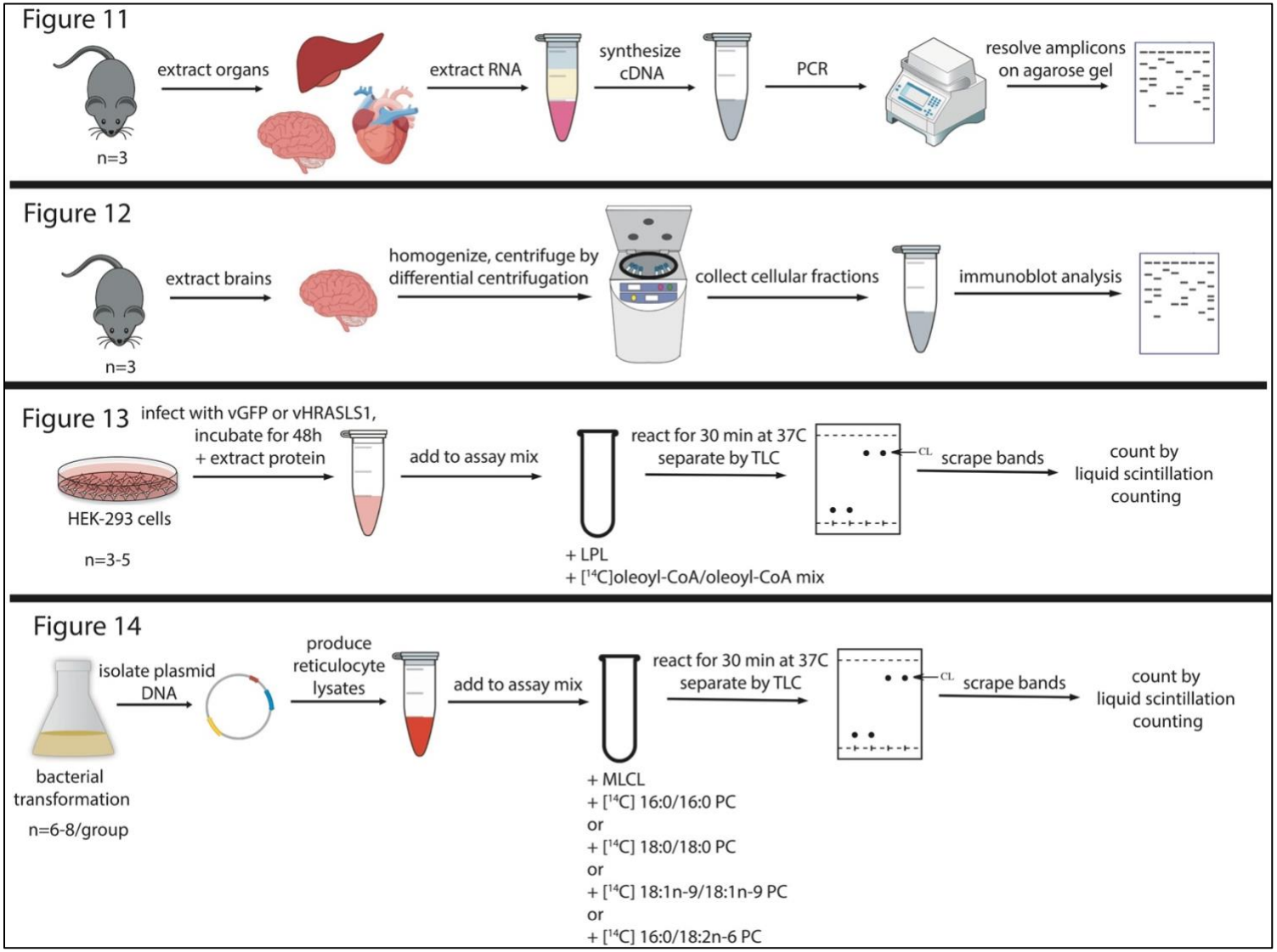

Figure 10: Schematic of the study design of Thesis Study I

Results

HRASLS1 is ubiquitously expressed, but with highest expression in brain and heart

Mouse tissue distribution of *Hrasls1* was analyzed by RT-PCR. Three *Hrasls1* transcript variants of variable lengths were analyzed including a longer isoform (referred to as *Hrasls1-1L*, XM_006522203) that has an additional 110 amino acids at the N-terminus, and two transcripts encoding the shorter forms (referred to as *Hrasls1-1SA* [NM_013751] and *Hrasls1-1SB* [NM_001357092.1], respectively). The tissue distribution of *Hrasls1* mRNA was analyzed and, as expected [33], all three murine transcript variants were found to be highly expressed in mouse brain and heart (Fig. 11). All transcript variants of *Hrasls1* were also present, albeit at variable levels, in the other tissues examined, including liver, lung, kidney, gonadal white adipose tissue (WAT), skeletal muscle, spleen, pancreas, and testes (Fig. 11).

HRASLS1 localizes to the endoplasmic reticulum

Due to the relative abundance of *Hrasls1* in mouse brain, this tissue was used as a source for studies examining subcellular localization of endogenous HRASLS1. Following differential centrifugation and immunoblot analysis, it was evident that endogenous HRASLS1 is predominately found in the microsomal fraction, with a faint band appearing in the mitochondrial fraction, but essentially no expression in the nuclear fraction (Fig. 12A). Fractional purity was assessed by immunoblotting for the mitochondrial protein cytochrome C, the nuclear protein histone H3, and the endoplasmic reticular protein stearoyl-CoA desaturase 1 (SCD1). Although fraction purity was very good, the presence of an HRASLS1 band in the mitochondrial fraction suggested that either slight microsomal contamination had occurred, or that some HRASLS1 was present in a fraction that would potentially co-fractionate with the mitochondria, such as the MAM. As a result, further analysis was performed to test whether HRASLS1 is present in the mitochondria, or in the MAM. First described by Jean Vance in 1990, the MAM is a sub-fraction of the endoplasmic reticulum, which shares contact points with mitochondria, and has been implicated in lipid transfer between the two organelles [17]. Mitochondrial isolates were further processed, in order to separate the purified mitochondrial fraction from a purified microsomal fraction, and a MAM fraction. Immunoblot analysis of isolated fractions indicated that, endogenous HRASLS1 was observed almost exclusively in the microsomal fraction, with a slight band appearing in the MAM fraction, but with no observable detection in the mitochondrial

fraction (Fig. 12B). Fractional purity was assessed by immunoblot analysis for the MAM-specific protein acyl-CoA synthase long chain family member 4 (ACSL4), as well as cytochrome C and SCD1 for the mitochondrial and microsomal fractions, respectively.

Crude lysates prepared from HEK-293 cells overexpressing HRASLS1 have significant acyl-CoA-dependent LPI, MLCL, and DLCL O-acyltransferase activity and di-18:1n-9-PC:MLCL transacylase activity

Prior analysis of HRASLS1 by others indicated that the predominant *in vitro* function of this enzyme is PC:LPC O-transacylase activity (rather than N-transacylase activity, or PLA1/2 activity) [45]. However, the ability of HRASLS1 to catalyze the synthesis of additional classes of glycerophospholipids had not been reported. Additionally, whether HRASLS1 catalyzes acyl-CoA-dependent or acyl-CoA independent reactions had not been directly tested. Therefore, to first test whether HRASLS1 has acyl-CoA-dependent O-acyltransferase activity *in vitro*, crude (post nuclear) cell lysates from HEK-293 cells overexpressing control or HRASLS1 (by adenoviral infection for 48 hours) were produced (Fig. 13A), and acyl-CoA dependent O-acyltransferase activity was assayed. For this work, [^{14}C]oleoyl-CoA was utilized as an acyl donor, and major classes of lysophospholipid acyl acceptors were tested, including lysophosphatidic acid (LPA), LPC, lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), lysophosphatidylglycerol (LPG), lysophosphatidylinositol (LPI), MLCL, and DLCL. Compared to reactions performed with control lysates, reactions with lysates overexpressing HRASLS1 produced significantly more radiolabeled PI from LPI, and CL from both MLCL and DLCL, as quantified by liquid scintillation counting (Fig. 13B). Crude lysates overexpressing HRASLS1 increased esterification of [^{14}C]oleoyl-CoA into LPI by ~2.6-fold compared to control, and increased esterification of [^{14}C]oleoyl-CoA into MLCL and DLCL by ~2.6-fold and ~2.9-fold, respectively. Under these assay conditions, overexpression of HRASLS1 had no significant effect on incorporation of [^{14}C]oleoyl-CoA into LPA, LPC, LPE, LPS, or LPG.

Cellular pH varies significantly across different sub regions of cells, and across organellar membranes. Optimal pH for the oleoyl-CoA-dependent O-acyltransferase activity that was found in crude lysates overexpressing HRASLS1 was assessed by *in vitro* assay of [^{14}C]oleoyl-CoA:MLCL O-acyltransferase activity in reaction buffers at varying pH levels, ranging from pH 6-8. The highest HRASLS1 oleoyl-CoA-dependent O-acyltransferase activity was observed at pH 7 (Fig. 13C). This is in contrast to the finding by Shinohara *et al*. who found that pH 8 is optimal

for the PLA1/2 activity of HRASLS1 [45]. However, as these enzymatic processes differ, the finding of different optimal pH is not entirely surprising.

This finding of significant O-acyltransferase activity with oleoyl-CoA in HEK-293 crude lysates was unexpected, given prior work by others [45, 46], and the homology that HRASLS1 shares with other HRASLS family members that have a well-characterized catalytic mechanism that is CoA-independent [18]. I considered two possible explanations for this phenomenon. The first was that all prior work is incorrect, and HRASLS1 is actually an acyl-CoA-dependent O-acyltransferase, and this was rapidly discarded. The second was that the finding of acyl-CoA-dependent activity is an artefact, related to the use of transduced crude lysates. Crude lysates contain abundant, disrupted organelles, rather than HRASLS1 alone, and therefore also contain abundant phospholipids, as well as other enzymes. I postulated that in this system, other CoA-dependent transacylases naturally present could incorporate oleoyl-CoA into LPC (at the same rate in both *control* and *Hrasls1* infected cells), resulting in the generation of PC substrate that was then used at a higher rate for LPI, MLCL, and DLCL transacylation reactions in lysates overexpressing HRASLS1 versus control. Alternatively, I postulated that during the 48-hour overexpression of HRASLS1, the N-transacylase activity of this enzyme could have generated NAE signaling molecules that may have led to the induction of acyl-CoA-dependent enzymes involved in the synthesis of PI and CL. Additional studies were thus conducted on the CoA-independent O-transacylase activity of crude lysates, and on both the CoA-dependent O-acyltransferase activity, and CoA-independent O-transacylase activity of HRASLS1 using a purer system (*i.e.* reticulocyte lysates).

I therefore next investigated potential acyl-CoA-independent PC:MLCL O-transacylase activity. Based on activity observed in the CoA-dependent reaction, I decided to focus on a potential new role for this enzyme in CL synthesis, specifically. To do this, I assayed these same crude lysates produced from HEK-293 cells that had been infected with adenoviral *Hrasls1*, or control adenovirus, for 48 hours prior to harvest. The PC:MLCL transacylase activity of HRASLS1 was assessed utilizing three different radiolabelled PC acyl donors, and MLCL as an acyl acceptor. As compared to control lysates, lysates produced from HEK-293 cells overexpressing HRASLS1 produced significantly more [^{3}H]-oleate-containing CL when utilizing [^{3}H-1,2]18:1n-9/18:1n-9-PC as an acyl donor (Fig 13E). Specifically, crude lysates overexpressing HRASLS1 increased esterification of [^{3}H] oleate into MLCL by ~1.6-fold compared to control,

P<0.05. In contrast, crude lysates overexpressing HRASLS1 did not produce significantly more radiolabelled cardiolipin than control lysates when utilizing either [^{3}H-1,2]18:0/18:0-PC or 16:0/[^{14}C]18:2n-6-PC as acyl donors (Figs. 13D, and F, respectively).

Preliminary investigation of acyl-CoA-dependent MLCL O-acyltransferase activity and PC:MLCL transacylase activity of HRASLS1 generated using an in vitro reticulocyte lysate transcription/translation system

After observing that crude lysates overexpressing HRASLS1 produced significantly more radiolabeled CL using MLCL as an acyl acceptor, and either oleoyl-CoA or di-oleoyl-PC as acyl donors, we examined if HRASLS1 produced via *in vitro* transcription/translation could also synthesize CL as an acyl-CoA-dependent O-acyltransferase or as a PC:MLCL O-transacylase. To do this, I utilized the TNT® reticulocyte lysate system. There are several benefits to using this system over crude lysates, including that synthesis of HRASLS1 proceeds rapidly (within a few hours) from a plasmid, rather than from viral infection, which can affect background metabolism in cells over the course of days (although this is controlled by use of an adenoviral control), and that NAE-mediated nuclear signaling in the regulation of other enzymes would be abrogated by the use of this anucleate system. Using this coupled transcription/translation system, I first confirmed the production of HRASLS1 protein in reticulocyte lysates by immunoblot analysis of the 3X FLAG tag that is cloned in frame with HRASLS1 (Fig 14A). Next, I performed the same oleoyl-CoA-dependent O-acyltransferase activity assay as I had performed in crude lysates. I utilized [^{14}C]oleoyl-CoA as an acyl donor, and MLCL as an acyl acceptor. However, this time, I did not observe a significantly greater incorporation of [^{14}C]oleoyl-CoA into CL in reticulocyte lysates overexpressing HRASLS1, as compared to control lysates (Fig 14B). Specifically, reticulocyte lysates overexpressing HRASLS1 had 1.08-fold the activity of control reticulocyte lysates (P=0.82, n=3). Examination of the PC:MLCL transacylase activity of reticulocyte lysates overexpressing HRASLS1 was performed next. When [^{3}H-1,2] 18:0/18:0-PC (Fig 14C) was used as an acyl donor, initial investigation demonstrated that the incorporation of [^{3}H]18:0 into CL by reticulocyte lysates overexpressing HRASLS1 was 33% higher than the incorporation by control reticulocyte lysates, and approached significance with n=3 samples (P=0.075), although clearly additional samples should be tested with this PC acyl-donor. Next, [^{3}H-1,2] 18:1n-9/18:1n-9-PC (Fig 14D) was used as an acyl donor. Of note, this lipid was the one species that was incorporated into CL at a significantly greater rate by crude lysates (n=5) overexpressing HRASLS1. The

incorporation of [^{3}H]18:1n-9 into CL by reticulocyte lysates overexpressing HRASLS1 was ~65% higher than the incorporation of [^{3}H]18:1 by control reticulocyte lysates, and also approached significance with n=3 samples (P = 0.18, Fig. 14E), although testing with additional samples is also clearly required. Incorporation of [^{14}C]18:2n-6 from 16:0/[^{14}C]18:2n-6-PC into MLCL to form CL was highly similar (P=0.71) in control and HRASLS1 overexpressing reticulocyte lysates.

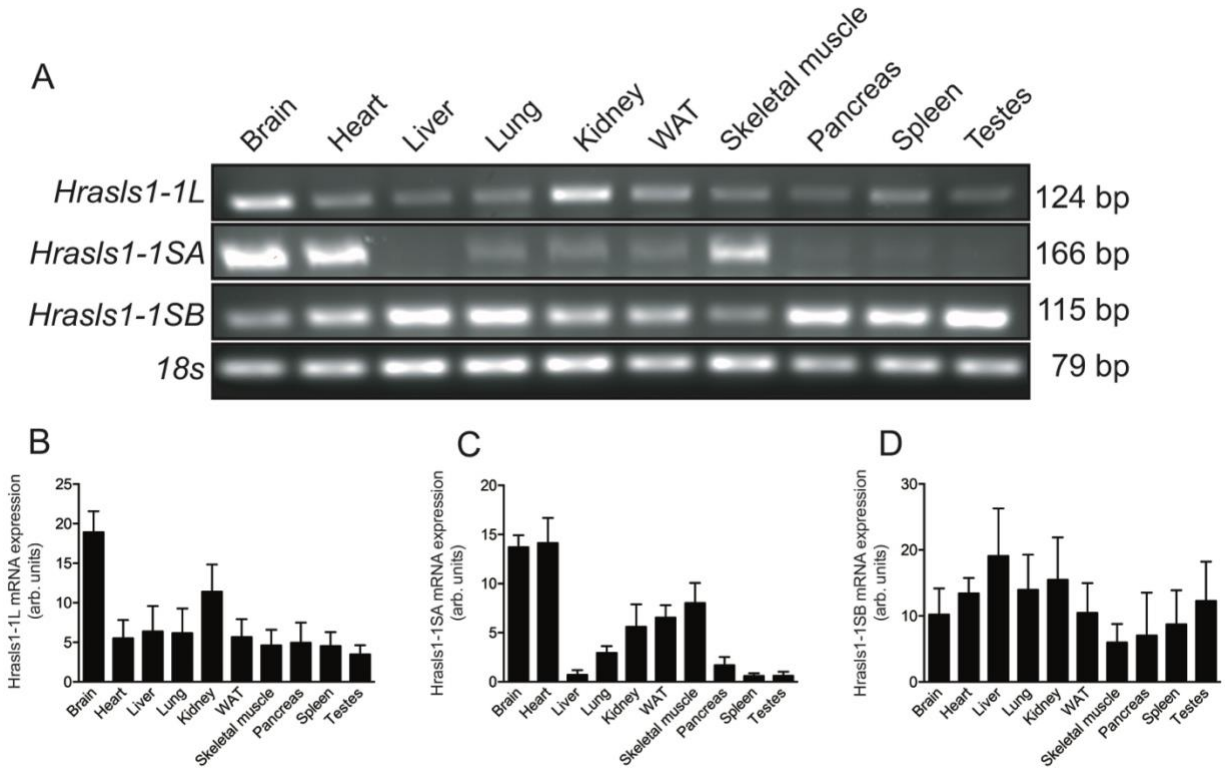

Figure 11: Hrasls1 is highly expressed in the brain and heart.

Representative blot showing mRNA expression of longer (*Hrasls1-1L*) and shorter (*Hrasls1-1SA*, *Hrasls1-1SB*) transcripts of *Hrasls1*, measured by RT-PCR in multiple tissues (A). Quantification of *Hrasls1-1L* mRNA expression (B), Hrasls1-1SA mRNA expression (C), and Hrasls1-1SB expression (D). Data are means ± S.E.M., n = 3.

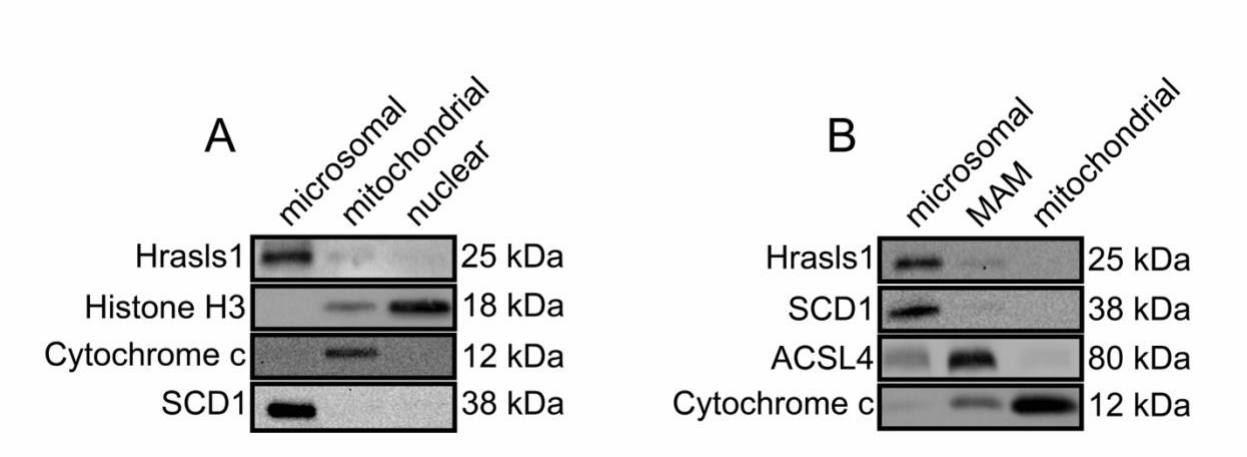

Figure 12: HRASLS1 localizes predominately to the endoplasmic reticulum.

(A) Representative immunoblot of endogenous HRASLS1 in subcellular fractions isolated from mouse brains (n=3). (B) Representative immunoblot of endogenous HRASLS1 in purified brain microsomes, mitochondria, and mitochondria-associated ER membrane (MAM) sub-fractions (n=3).

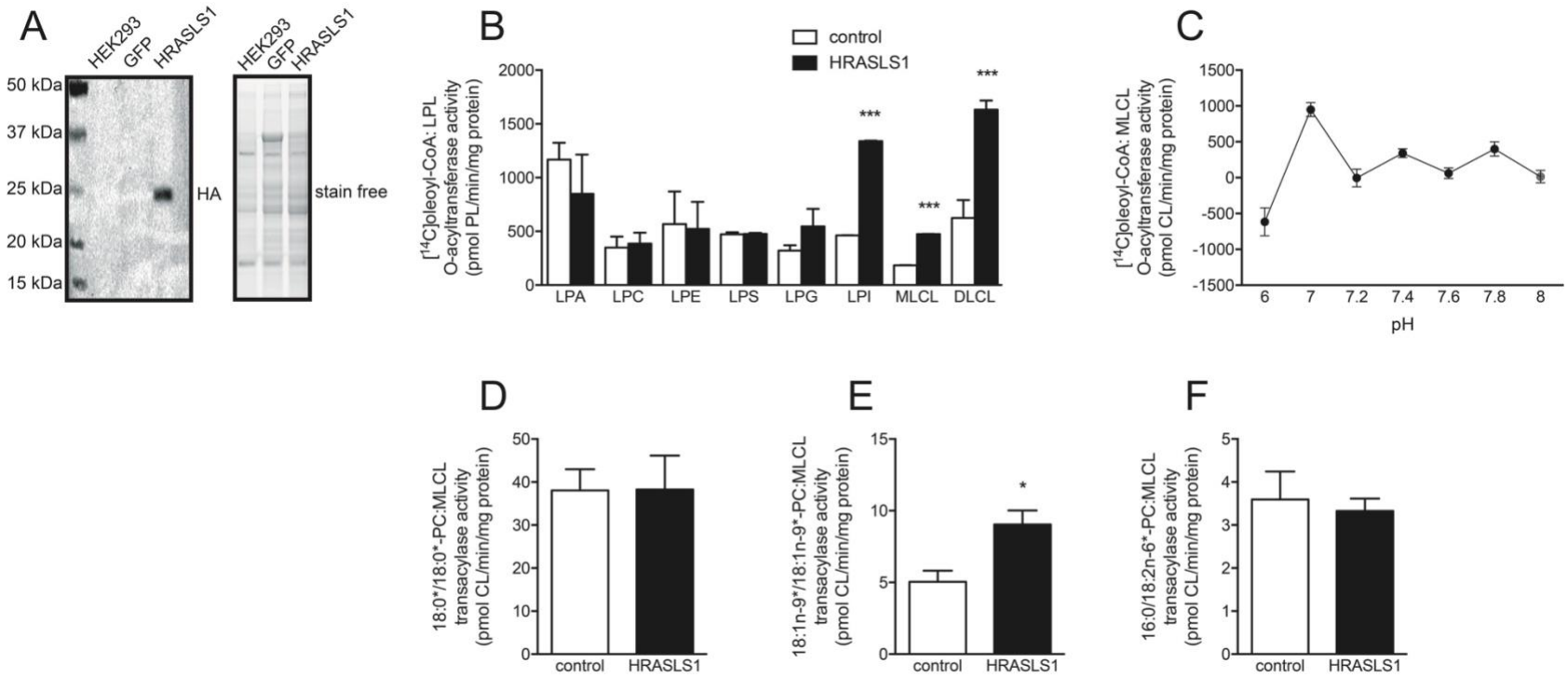

Figure 13: Post-nuclear crude cell lysates derived from HEK293 cells infected with adenoviral *Hrasls1* or *control* adenovirus for 48 hours have both acyl-CoA-dependent LPI, MLCL, and DLCL acyltransferase, and also as a di-oleoyl PC:MLCL transacylase.

(A) Representative immunoblot of HEK-293 cells infected with control adenovirus (expressing GFP alone) or with adenovirus expressing HA-tagged HRASLS1 (as well as a separate GFP reporter protein). (B) Crude post-nuclear lysates from HEK-293 cells infected with either control or HRASLS1 adenoviruses were assayed *in vitro* for acyl-CoA-dependent lysophospholipid O-acyltransferase activity using [^{14}C] oleoyl-CoA and a variety of lysophospholipid acceptors, as indicated. Radiolabeled phospholipids were quantified by liquid scintillation counting (n=3). (C) pH-dependency of HRASLS1 [^{14}C] oleoyl-CoA:MLCL O-acyltransferase activity (n=3). (D) *In vitro* [^{3}H, 1-2]18:0-18:0-PC:MLCL transacylase activity of HEK-293 cell lysates overexpressing control or HRASLS1 protein (n=5). (E) *In vitro* [^{3}H, 1-2]18:1n-9-18:1n-9-PC:MLCL transacylase activity of HEK-293 cell lysates overexpressing control or HRASLS1 protein (n=5). (F) *In vitro* 16:0/[^{14}C]18:2n-6-PC:MLCL transacylase activity of HEK-293 cell lysates overexpressing control or HRASLS1 protein (n=5). Data are means ± S.E.M., *P<0.05, ***P<0.001 *vs.* control.

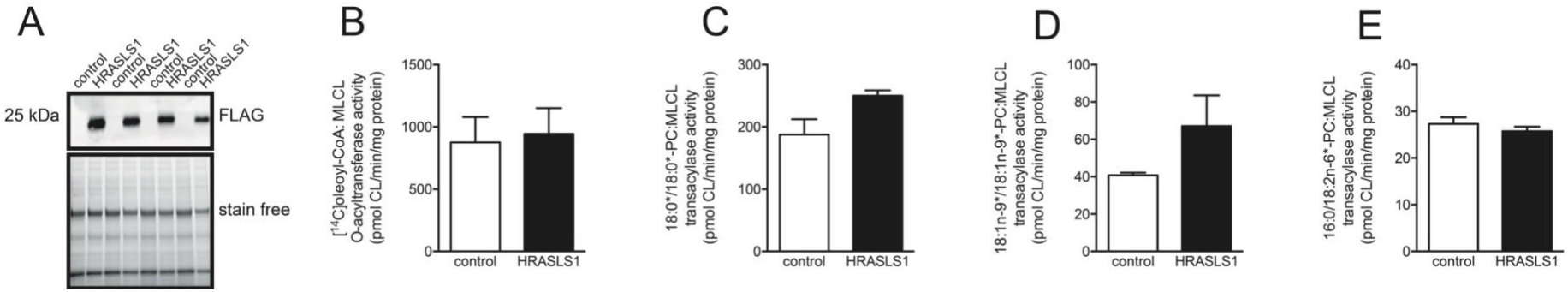

Figure 14: Preliminary testing of acyl-CoA-dependent MLCL O-acyltransferase activity and PC:MLCL O-transacylase activity of reticulocyte lysates overexpressing HRASLS1 or empty vector (control).

(A) Representative immunoblot of reticulocyte lysates overexpressing control (pCMV-3Tag-3A) or HRASLS1 (pCMV-HRASLS1-3Tag-3A) protein. Reticulocyte lysates produced in this immunoblot differ in their protein concentrations. (B) Reticulocyte lysates overexpressing control or HRASLS1 protein were assayed in vitro for acyl-CoA-dependent O-acyltransferase activity using [^{14}C] oleoyl-CoA as an acyl donor and MLCL as an acyl acceptor. (C) Reticulocyte lysates overexpressing control or HRASLS1 protein were assayed *in vitro* for [^{3}H, 1-2]18:0/18:0-PC:MLCL O-transacylase activity. (D) Reticulocyte lysates overexpressing control or HRASLS1 protein were assayed in vitro for [^{3}H, 1-2]18:1n-9/18:1n-9-PC:MLCL O-transacylase activity. (E) Reticulocyte lysates overexpressing control or HRASLS1 protein were assayed in vitro for 16:0/[^{14}C]18:2n-6-PC:MLCL O-transacylase activity. Data are means ± S.E.M., n=3.

Discussion

The major objectives of this study were to characterize the tissue expression profile, subcellular localization, and biochemical activity of HRASLS1. Specifically, I examined the oleoyl-CoA-dependent O-acyltransferase activity of HRASLS1 with a variety of lysophospholipid substrates using crude and reticulocyte lysates, and the PC:MLCL O-transacylase activity of HRASLS1 using reticulocyte lysates. Furthermore, I examined the PC:MLCL transacylase activity of HRASLS1 using both crude lysates and reticulocyte lysates, with di-stearoyl-PC, di-oleoyl-PC, and 16:0/18:2n-6-PC as acyl donors. This investigation has identified a novel function for HRASLS1 in the synthesis of PI and CL. To the best of my knowledge, this is the first time an HRASLS enzyme has been reported to function in CL or PI metabolism.

In agreement with prior observations from the investigation of HRASLS1 tissue distribution [45, 47, 167], this study indicated that longer (*Hrasls-1L*) and shorter (*Hrasls1-1SA, Hrasls1-1SB*) variants of HRASLS1 are ubiquitously expressed, albeit with high expression in brain and heart tissue. The second of the two shorter transcript variants, *Hrasls1-1SB* contains longer 5' and 3' untranslated regions than *Hrasls1-1SA* [47]. A sequence alignment of all three isoforms are shown in Appendix B. The shortest isoform (*i.e. Hrasls1-1SA*) was the isoform studied in subsequent biochemical characterizations in this thesis due to its preferential translation in mice over both *Hrasls1-1L* and *Hrasls1-1SB* [47]. Interestingly, while most studies found significant expression of *Hrasls1* in skeletal muscle as well [47], this study found only modest expression in this tissue. While many factors may contribute to this observed discrepancy, at least two key differences exist between this study and the Hussain study [47], which reports high expression in skeletal muscle. First, the Hussain study utilized 8-week-old mice [47], while this study utilized 12-week-old mice. Gene expression is highly variable throughout aging, and differences in gene transcription with aging are vary in a tissue-to-tissue basis [257]. Therefore, although in both studies the mice utilized have reached adulthood, the discrepancies in expression level may result, at least in part, due to the difference in age between the mice. A second possible explanation may be due to the metabolic state of the animals at the time of sacrifice. Indeed, our prior study has demonstrated that the expression of enzymes involved in phospholipid metabolism is dependent on the metabolic state of the animal [258]. In this study, mice were allowed access to food *ad libitum* prior to sacrifice in the morning. Although the Hussain study does not explicitly state the metabolic conditions of the animals prior to sacrifice [47], it is probable that they also

allowed their mice to have *ad libitum* access to food prior to sacrifice. The time of last feeding, however, may be variable between the two studies, and could vary from mouse-to-mouse. Thus, future studies examining mRNA tissue expression of *Hrasls1* should consider strictly regulating the metabolic state of the animals used prior to sacrifice.

Next, this study investigated the subcellular localization of endogenous HRASLS1. Immunoblot analysis of sub-fractional homogenates from mouse brain indicated that HRASLS1 localizes to the endoplasmic reticulum, and not to the nucleus, mitochondria, or MAM. This finding is in contrast to a previous report by Akiyama *et al*. (1999), which found HRASLS1 present in the cytoplasm, and in the peri-nuclear region of COS-7 cells overexpressing HRASLS1 [33]. The reason for differences in findings between that work and the present study is unknown, but may be a result of the use of overexpressed rather than endogenous protein. Further immunoblot analysis of endogenous HRASLS1 in the cytoplasmic fraction that is obtained after harvesting the microsomal fraction may indicate a minor level of expression, but this would need to be tested directly. This finding identifies HRASLS1 as the second known HRASLS family member to localize to the endoplasmic reticulum. Prior to this work, only HRASLS4 has shown minor localization to the endoplasmic reticulum, as its C-terminal hydrophobic domain is anchored between the ER and the Golgi apparatus [19, 49]. Most other HRASLS family members localize preferentially to the peri-nuclear region, or to the cytoplasm [19].

Based on previous work by others indicating that LRAT and the HRASLS family of enzymes are well-characterized as acyl-CoA-independent O-transacylases [45]. I therefore hypothesized that HRASLS1 would not show acyl-CoA-dependent O-acyltransferase activity. Surprisingly, however, investigation in HRASLS1-overexpressing crude lysates demonstrated significant activity with oleoyl-CoA as an acyl donor, and LPI, MLCL, and DLCL as acyl acceptors. Despite this finding, I hypothesized that the CoA-dependent activity that was evident in crude lysates was likely not a direct function of HRASLS1, but rather may be an artefact related to the use of the specific model, where HRASLS1 is overexpressed for up to 48 hours in HEK-293 cells prior to harvest. Notably, this could lead to additional cellular effects, including changes in other phospholipid metabolizing enzymes, possibly as a result of altered synthesis by HRASLS1 of NAEs, which can modulate nuclear signaling pathways [259, 260]. Thus, I also tested the oleoyl-CoA-dependent MLCL O-acyltransferase activity of HRASLS1 using a reticulocyte lysate *in vitro* transcription/translation system, which would remove confounding caused by any potential

related nuclear activation signaling. As predicted, in that system there was no significant oleoyl-CoA-dependent transacylase activity with MLCL as an acyl donor, suggesting strongly that HRASLS1 does not directly catalyze CL synthesis as an acyl-CoA-dependent O-acyltransferase.

I next tested the acyl-CoA-independent PC O-transacylase activity of HRASLS1 in crude and reticulocyte lysates. Analysis of the PC:MLCL transacylase activity of crude lysates overexpressing HRASLS1 demonstrated greater incorporation of oleate from [^{3}H-1,2] 18:1n-9/18:1n-9-PC into CL using MLCL as an acyl acceptor, but no difference in incorporation of stearate (using [^{3}H-1,2] 18:0/18:0-PC) or linoleate (using 16:0/ [^{14}C]18:2n-6-PC) as compared to crude lysates overexpressing the control adenovirus. In a pilot study using a reticulocyte lysate expression system, there was a preliminary suggestion that PC:MLCL transacylase activity using [^{3}H-1,2] 18:1n-9/18:1n-9-PC and [^{3}H-1,2] 18:0/18:0-PC may be increased by HRASLS1 overexpression, while activity with 1-palmitoyl-2-[^{14}C]-linoleoyl-PC was not affected. Interestingly, this provides some suggestion of a preference for 18-carbon fatty acids found specifically in the *sn-1* position, since there was no evidence that HRASLS1 could utilize 18:2n-6 in the *sn-2* position of 16:0/18:2n-6-PC. To test this, it will be important to investigate activity with other positional isomers of PC, such as [^{14}C]18:2n-6/16:0-PC, as well as other substrates, such as [^{14}C-1,2]18:2n-6/18:2n-6-PC, as acyl donors. There was also some suggestion from the combined data that *sn-1* oleate may be preferred over *sn-1* stearate. It is unclear whether this acyl-preference is due to the specific stereochemical requirement for CL to be enriched in 18-carbon unsaturated fatty acids [261], or if the double bond in oleic acid allows for preferential binding in the catalytic cleft of HRASLS1. Additional investigations are warranted in order to elucidate the exact mechanism, but it is likely a combination of both factors. Overall, however, it is likely that this study was statistically underpowered ($n=3$), and should first be repeated with more samples.

Crude cell lysates overexpressing HRASLS1 synthesized more PI from LPI, and more CL from either MLCL or DLCL, when oleoyl-CoA was used as an acyl donor. Since LPI and DLCL were not tested as substrates in reactions using reticulocyte lysates, it is unknown whether this occurs directly, or whether it is due to induction of other enzymes that have this activity. In particular, ALCAT1 can utilize LPI as an acyl acceptor *in vitro*, in addition to MLCL and DLCL [185]. Interestingly, like HRASLS1, ALCAT1 also predominantly localizes to the endoplasmic reticulum [121], which is an unexpected site of cardiolipin synthesis, a process that is known to occur primarily in the mitochondria [262]. Effects of HRASLS1 overexpression in HEK-293 cells on the

expression of other phospholipid metabolism genes, including *ALCAT1*, is reported in Thesis Study II (Chapter Five).

Conclusion

HRASLS1 is ubiquitously expressed, although it is highly abundant in the brain and the heart, and localizes to the endoplasmic reticulum. Importantly, the current work has demonstrated a new function for HRASLS1 in cardiolipin synthesis. Crude lysates overexpressing HRASLS1 had a greater capacity than control crude lysates to synthesize PI and CL using oleoyl-CoA as an acyl donor. This apparent oleoyl-CoA-dependent MLCL O-acyltransferase activity was not, however, conserved in reticulocyte lysates overexpressing HRASLS1, indicating that HRASLS1 likely does not have direct acyl-CoA-dependent acyltransferase activity. This is in agreement with prior studies on the catalytic mechanism of HRASLS1 [18, 35, 45, 53]. Alternatively, I suggest that HRASLS1 expressed for 48 hours in growing cells may be producing NAPE as a source of NAE, which may in turn signal to increase CL synthesis. Thesis Study II and III will examine the effects of HRASLS1 overexpression on phospholipid levels and phospholipid metabolism *in vivo*, as well as a role for HRASLS1 in NAE production, and a role for NAEs in cellular cardiolipin metabolism.

Chapter Five

Thesis Study II – Investigation of the *in vivo* role of HRASLS1 in cardiolipin metabolism

Introduction and Study Rationale

The HRASLS enzyme family is a group of proteins that share sequence homology with lecithin:retinol acyltransferases (LRATs). Five HRASLS enzymes have been identified in humans, with three enzymes (*Hrasls1,3,5*) conserved in rodents as well. Due to the fact that all members of this enzyme family exhibit varying levels of O-transacylase, N-transacylase, and phospholipase A1/2 activity [26, 45, 46, 53], they are also known as PLA/ATs [19]. A prior study has demonstrated a role for HRASLS1 in the O-transacylation of phospholipids, specifically PC and PE [35]. However, the ability of HRASLS1 to function in synthesizing other phospholipids had not yet been investigated.

Results from this thesis have uncovered a novel role for HRASLS1 in the *in vitro* synthesis of the mitochondrial phospholipid cardiolipin. HRASLS1 may act *in vitro* as a PC:MLCL transacylase, and may prefer PC that has 18-carbon fatty acyl chains in the *sn-1* position. While this novel enzymatic function may have broad implications for understanding cardiolipin metabolism, it is important to determine if these activities are conserved *in vivo*. Crude lysates prepared from HEK293 cells overexpressing HRASLS1 for 48 hours demonstrated an increased capacity to incorporate oleoyl-CoA into MLCL, which was not observed in reticulocyte lysates. This suggests that HRASLS1 may be indirectly involved in CL synthesis through the modulation of cellular CL metabolic pathways as well. The exact mechanism(s) underlying this phenomenon are currently unknown, but the N-acyltransferase activity exhibited by HRASLS1 *in vivo* may play an important role [35]. I speculated that HRASLS1 may be producing NAPE *in vivo*, which can be hydrolyzed into bioactive NAEs that may signal in cellular pathways, including pathways regulating nuclear transcription that is absent in reticulocyte lysates, to increase cellular CL production. In this scenario, identification of the specific NAEs that are produced by HRASLS1 may shed insight into any observed *in vivo* modulation of cellular CL metabolism. Thus, the overarching objective of this chapter was to assess the *in vivo* role of HRASLS1 and NAEs in HEK-293 CL metabolism. An overview of study design for this chapter is illustrated in Figure 15.

.

Objectives and Hypotheses

The objectives and hypotheses for this study were as follows:

1. Objective: To characterize alterations in phospholipid and fatty acyl profiles of HEK-293 cells overexpressing HRASLS1 protein relative to control cells.

   Hypothesis: As compared to controls, HEK-293 cells overexpressing HRASLS1 will have a significantly higher content of PI and CL. Furthermore, fatty acyl analysis of CL will show enrichment with 18-carbon fatty acids (*i.e.* 18:0, 18:1n-9 and 18:2n-6).

2. Objective: To characterize alterations in mitochondrial function and content of mitochondrial DNA in mitochondria isolated from HEK-293 cells overexpressing control or HRASLS1 protein.

   Hypothesis: Consistent with HRASLS1 overexpression resulting in increased CL content, mitochondrial function will be impaired in HEK-293 cells overexpressing HRASLS1. There will be no differences in mitochondrial DNA content between HEK-293 cells overexpressing control or HRASLS1 protein.

3. Objective: To characterize changes in the expression of genes involved in cardiolipin and glycerolipid synthesis following overexpression of HRASLS1 protein in HEK-293 cells.

   Hypothesis: As compared to controls, HEK-293 cells overexpressing HRASLS1 will have a significantly higher expression of genes involved in cardiolipin synthesis. Specifically, *CDS1/2*, *PGS*, *CLS*, and *TAZ* will all be significantly induced as compared to controls.

4. Objective To investigate alterations in the mRNA expression of NAE receptors following HRASLS1 overexpression in HEK-293 cells.

   Hypothesis: As compared to controls, HEK-293 cells overexpressing HRASLS1 will have a significantly higher expression of *PPARα*, *RXRa*, *TRPV1*, and *GPR55*.

5. Objective: To investigate if treatment with OEA, PEA, or SEA can significantly increase endogenous CL content in HEK-293 cells.

   Hypothesis: As compared to controls, HEK-293 cells treated with OEA will have a significantly higher endogenous content of CL.

6. Objective: To characterize any changes in abundant NAEs or 1- or 2-arachidonylglycerol in HEK-293 cells overexpressing control or HRASLS1 protein.
   Hypothesis: As compared to controls, HEK-293 cells overexpressing HRASLS1 will have a significantly higher content of OEA, SEA, and LEA. No differences will be observed in PEA, 1-AG, or 2-AG.

   .

Study Design

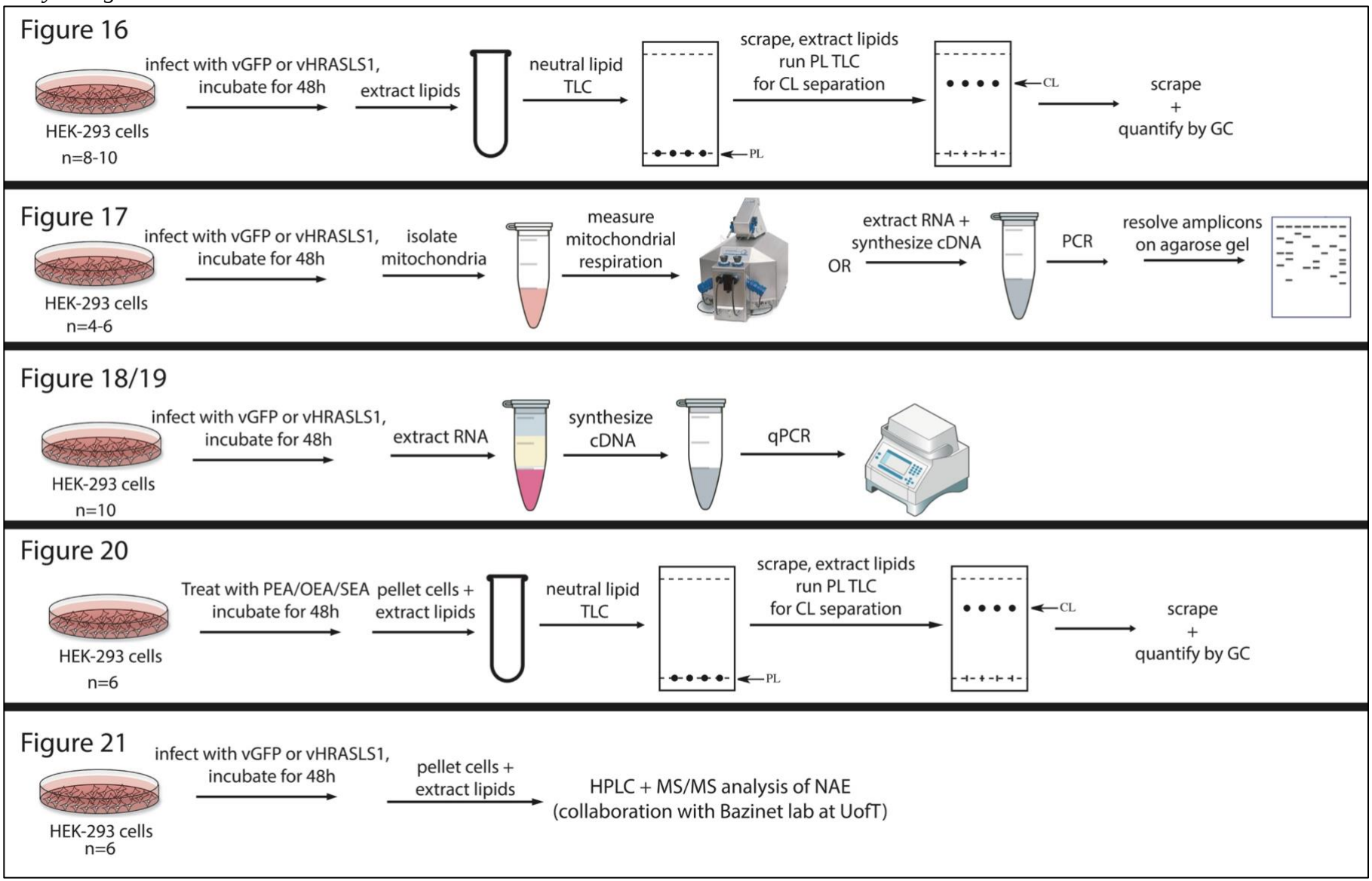

Figure 15: Schematic of the study design of Thesis Study II

Results

HRASLS1 overexpression increases the cellular content of CL in HEK-293 cells

HEK-293 cells were infected with adenoviral HRASLS1 or control virus for 48 hours, then harvested and extracted for total endogenous lipids. Individual phospholipids were resolved by TLC, identified by comparison with known standards, scraped, and analyzed for fatty acyl composition and content, which was also used to calculate the corresponding cellular phospholipid levels. Cells overexpressing HRASLS1 had a 62% greater total cellular CL content compared to control cells (16.43 ± 3.53 nmol CL/10^9 cells versus 26.74 ± 1.87 nmol CL/10^9 cells, respectively, P = 0.0176) (Fig. 16A). There were no other significant differences in total levels of any of the other phospholipids analyzed, including PC, PE, PG, or PI. Data showing cardiolipin content as a percentage of total cellular phospholipid content is shown in Appendix Figure E.

While total cellular CL content is important for mitochondrial metabolism and cellular health, the specific fatty acyl composition of CL is also of critical importance [227, 263]. The fatty acyl composition of CL was determined and quantified from HEK-293 cells overexpressing HRASLS1. There was a significant increase in the total content of saturated fatty acyl species (Fig. 16B). Among individual saturated fatty acyl species, there was a significantly higher content of myristic acid (control, 0.40 ± 0.08 nmol C14:0/mg protein versus HRASLS1, 0.73 ± 0.08 nmol C14:0/mg protein, P = 0.017), palmitic acid (control, 3.99 ± 0.73 nmol C16:0/mg protein versus HRASLS1, 7.54 ± 0.96 nmol C16:0/mg protein, P = 0.011), and stearic acid (control, 6.24 ± 1.07 nmol C18:0/mg protein versus HRASLS1, 11.51 ± 1.57 nmol C18:0/mg protein, P = 0.016) in cells overexpressing HRASLS1 as compared to controls (Fig. 16C). Several other fatty acyl species including lauric acid (C12:0), arachidic acid (C20:0), and tricosylic acid (C23:0) showed an increase that approached significance (P≤0.06). Of the monounsaturated fatty acids analyzed, only erucic acid (C22:1n-9) in CL was higher in HRASLS1 overexpressing cells (Fig. 16D). There were no significant differences in CL content of any of the n-6 PUFA species analyzed, including linoleic acid (C18:2n-6) (Fig. 16E). Although linoleate appeared to be higher (control, 1.21 ± 0.20 nmol C18:2n-6/mg protein versus HRASLS1, 2.65 ± 0.91 nmol C18:2n-6/mg protein, P = 0.1633) in the HRASLS1 overexpressing group, it did not reach significance (P=0.16). However, a few of the n-3 PUFA species were significantly increased in HRASLS1-overexpressing cells compared to controls, including α-linolenic acid (C18:3n-3), eicosatrienoic acid (C20:3n-3), and eicosapentanoic acid (C20:5n-3) (Fig. 16F). Overall, the increased CL in HRASLS1 overexpressing HEK-293 cells was associated primarily with a greater content of saturated, and,

to some extent, also n-3 polyunsaturated fatty acyl species. It is important to note that this data is reflective of the fatty acid concentrations in cardiolipin, and not necessarily the relative abundance of these fatty acids in cardiolipin. No statistically significant differences were observed in the relative content of fatty acids in cardiolipin in HEK-293 cells overexpressing HRASLS1 as compared to controls (data shown in Appendix F).

Adenoviral overexpression of HRASLS1 alters mitochondrial function, but not mitochondrial DNA content

Cardiolipin is found predominantly in the inner mitochondrial membrane, and in most tissues, requires enrichment with C18:2n-6 for optimal function [262]. Since HRASLS1 overexpression in HEK-293 cells increased the cellular content of CL enriched with saturated fatty acids, I investigated whether the function of isolated mitochondria from HEK-293 cells infected with adenoviral control or HRASLS1 had impaired function. As compared to control mitochondria, mitochondria isolated from HRASLS1-overexpressing HEK-293 cells showed a significantly lower endogenous respiration rate (control, 0.067 ± 0.0019 pmol/s/total protein versus HRASLS1, 0.056 ± 0.0020 pmol/s/total protein, P = 0.0040) (Fig. 17A). After the addition of pyruvate, glutamate, and malate (P/G/M), there was no longer a statistically significant difference in the respiratory capacity between the groups. However, assessment of the individual electron transport chain complexes showed distinct differences between mitochondria isolated from control versus HRASLS1-overexpressing cells. Although mitochondria isolated from HRASLS1-overexpressing HEK-293 cells initially showed a significantly decreased level of endogenous respiration, Complex I-supported respiration (assessed by the addition of P/G/M and ADP) was significantly higher in mitochondria isolated from HRASLS1-overexpressing HEK-293 cells versus controls (0.13 ± 0.0046 pmol/s/total protein versus 0.11 ± 0.0003 pmol/s/total protein, respectively, P = 0.0064). However, this phenomenon was reversed upon the addition of succinate, which allows for the assessment of Complex I+II-supported respiration, and which decreased to less than half the control level (0.12 ± 0.004 pmol/s/total protein (HRASLS1-overexpressing) versus 0.26 ± 0.007 pmol/s/total protein (control), respectively, P < 0.001). Upon the final addition of TMPD, which allows for Complex I+II+IV-supported respiration, mitochondria isolated from HEK-293 cells overexpressing HRASLS1 were observed once again to respire at a significantly lower level than controls (0.59 ± 0.017 pmol/s/total protein versus 0.50 ± 0.017 pmol/s/total

protein, respectively, P = 0.0034) (Fig 17A). H_2O_2 production by isolated mitochondria was not significantly different between groups during endogenous respiration, nor during the activation of electron transport chain complexes (Fig 17B).

To estimate if the observed differences in mitochondrial respiration could be attributed to changes in mitochondrial DNA content, and by extension, mitochondrial number, semi-quantitative RT-PCR analysis of two markers of mitochondrial DNA and one marker of nuclear DNA was performed (Fig. 17C), and quantified (Figs. 17D, E, F). No statistically significant differences were observed in the content of either the MT-TL1 gene, which encodes for mitochondrial tRNA, nor the mitochondrial MT-ND1 gene, which encodes for a protein that is a subunit of NADH dehydrogenase (ubiquinone), also known as Complex I. As well, no statistically significant difference was observed in the content of nuclear β2 microglobulin that was analyzed for comparison.

HRASLS1 overexpression significantly increases the expression of genes involved in phospholipid, and specifically, cardiolipin biosynthesis

Real-time PCR was performed to investigate if the observed increased cellular cardiolipin content was possibly related to an induction of phospholipid biosynthetic genes. HEK-293 cells were infected with either control- or HRASLS1-expressing adenovirus for 48h, upon which total RNA was harvested and analyzed by RT-qPCR for relative expression of genes involved in phospholipid synthesis. Gene expression was normalized to *18S*. A general induction of glycerophospholipid biosynthesis genes was observed. The expression of a microsomal GPAT (*GPAT1*, Fig. 18A) and mitochondrial GPAT (*GPAT3*, Fig. 18C) were significantly induced following HRASLS1 overexpression, as were *AGPAT2* (Fig. 18F), *AGPAT4* (Fig. 18H), and *AGPAT5* (Fig. 18I). The product of AGPAT-mediated catalysis, PA, represents a unique branching point in the Kennedy Pathway, where it can either be shuttled into serving as a precursor for DAG and TAG synthesis (along with the synthesis of PC, PE, and PS), or instead be phosphorylated into CDP-DAG, which feeds into CL synthesis. Since HRASLS1 increased the cellular content of CL, alterations in the latter branch were investigated. A highly significant induction was observed in the expression of both *CDS1* (Fig. 18J), and *CDS2* (Fig. 18K), which convert PA into CDP-DAG. Conversion of CDP-DAG into PG is catalyzed by *PGS*, which was also observed to be significantly up-regulated in HRASLS1-overexpressing HEK-293 cells (Fig. 18L). The largest

induction was observed in *CLS*, which was significantly up-regulated approximately ~5.5-fold as compared to controls (Fig. 18M). Following *de novo* synthesis, CL is subject to acyl chain remodeling by one of four known enzymes. Analysis of the expression of these enzymes indicated a significant upregulation in *TAZ* (Fig. 18N), which remodels CL with linoleic acid residues. The expression of the other tested CL remodeling enzymes, ALCAT1 (Fig. 18O) and αTFP (Fig. 18P) were not significantly different from controls.

Adenoviral overexpression of HRASLS1 increases the mRNA expression of PPARα and TRPV1

Changes in gene expression that are evident in cells overexpressing HRASLS1 for 48 hours suggests the activation of a cell signaling pathway or pathways that can affect nuclear transcription. I hypothesized that HRASLS1 may be indirectly involved in CL synthesis through the production of NAPE that are cleaved into NAE, which are bioactive in multiple pathways including transcription regulation. To assess possible alterations in the downstream target receptors of NAEs, real-time PCR was performed. cDNA prepared from HEK-293 cells infected with either control or HRASLS1 adenovirus was analyzed by RT-qPCR for relative expression of *PPARα*, *RXRa*, *TRPV1*, and *GPR55*. As compared to controls, a highly significant upregulation was observed in the expression of *PPARα* ($P<0.05$) (Fig. 19A) and *TRPV1* ($P<0.001$) (Fig. 19C) in HRASLS1-overexpressing HEK-293 cells. The expression of *RXRa*, a known interactor of *PPARα*, verged on statistical significance ($P=0.0589$) (Fig. 19B), whereas the expression of *GPR55* was not significantly different from controls (Fig. 19D).

Content of endogenous CL is not increased following 48 hour OEA, PEA, or SEA treatment of HEK-293 cells

To assess whether candidate NAEs have an effect on cellular CL content, HEK-293 cells were treated with 0.1, 1, or 10 μM PEA, OEA, or SEA (Fig. 20). Cells were incubated for 48 hours, and total lipids were then extracted and resolved by TLC. Phospholipid bands corresponding to known standards were identified, scraped, and fatty acids were quantified by GC-FID. As compared to control cells, endogenous CL content was not significantly higher following treatment with PEA, OEA, or SEA, at any of the concentrations tested. In addition, metabolic labeling experiments utilizing [^{14}C] palmitate along NAE treatment demonstrated a similar effect, as CL

content was not significantly higher following treatment with PEA, OEA, or SEA (data shown in Appendix C).

HRASLS1 overexpression alters the NAE profile of HEK-293 cells

To assess if overexpression of HRASLS1 alters the content of various NAE species, HEK-293 cells were infected with control or HRASLS1 adenovirus for 48 hours, following which they were harvested by trypsinization. Samples were stored at -80ºC until they could be analyzed by our collaborators at the University of Toronto. Surprisingly, as compared to controls, HEK-293 cells overexpressing HRASLS1 had a significantly lower content of PEA (control, 21.58 ± 2.15 pmol PEA/g versus HRASLS1 14.83 ± 1.40 pmol PEA/g, P = 0.0250) (Fig. 21A) and OEA (control, 28.57 ± 4.29 pmol OEA/g versus HRASLS1 16.15 ± 1.02 pmol OEA/g, P = 0.0182) (Fig. 21B). Although linoleoylethanolamide (LEA) appeared to be somewhat (~48%) elevated in HRASLS1-overexpressing HEK-293 cells (6.44 ± 1.99 pmol/g versus control, 4.34 ± 0.68 pmol/g), this difference was also not statistically significant (P = 0.38) in the current samples (Fig 21C). The content of 1-arachidonylglycerol (1-AG) appeared to be lower ~31% lower in cells infected with HRASLS1, but this did not reach significance (P = 0.0956) (Fig. 21D). Likewise, the content of 2-arachidonoylglycerol (2-AG) was not significantly different between control- and HRASLS1-infected cells in the sample tested (n=6) (Fig. 21E). Other NAEs analyzed included SEA and docosahexaenoylethanolamide (DHEA), but these were undetected in most samples. Testing of additional samples is planned to increase statistical power for detection of significant differences.

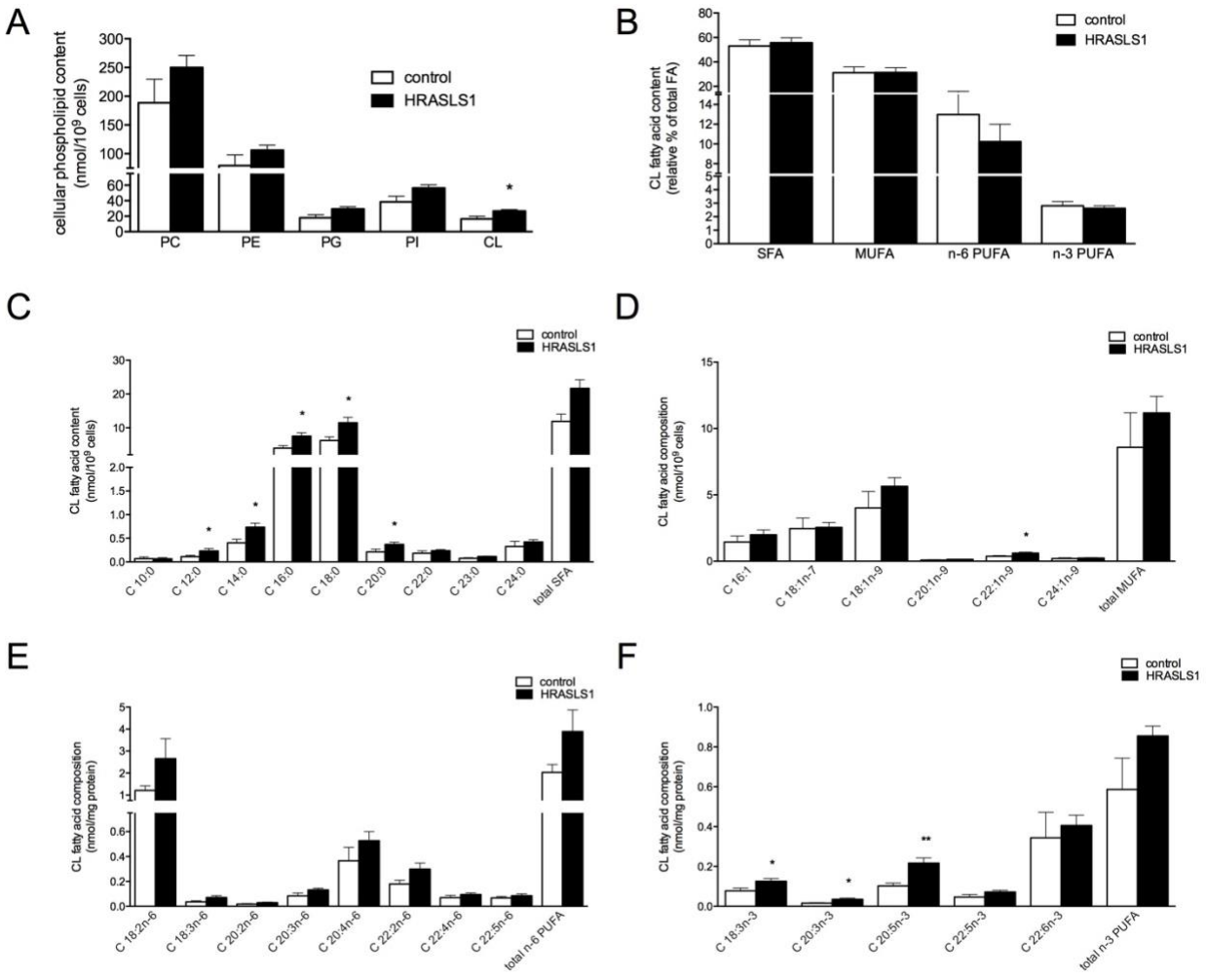

Figure 16: Adenoviral overexpression of HRASLS1 in HEK-293 cells increases cellular content of cardiolipin.

(A) Endogenous phospholipid content of HEK-293 cells overexpressing control or HRASLS1. (B) Relative proportion of major fatty acid classes (*i.e.* saturates (SFA), monounsaturates (MUFA), and n-3 and n-6 polyunsaturated fatty acids (PUFA)) within CL isolated from HEK-293 cells infected with adenoviral HRASLS1 or control virus. Concentrations of individual fatty acids within CL from control or HRASLS1-overexpressing HEK-293 cells including SFA (C), MUFA (D), n-6 PUFA (E), and n-3 PUFA (F). Data are means ± S.E.M. *P<0.05, **P<0.01, vs. controls, n=8=10.

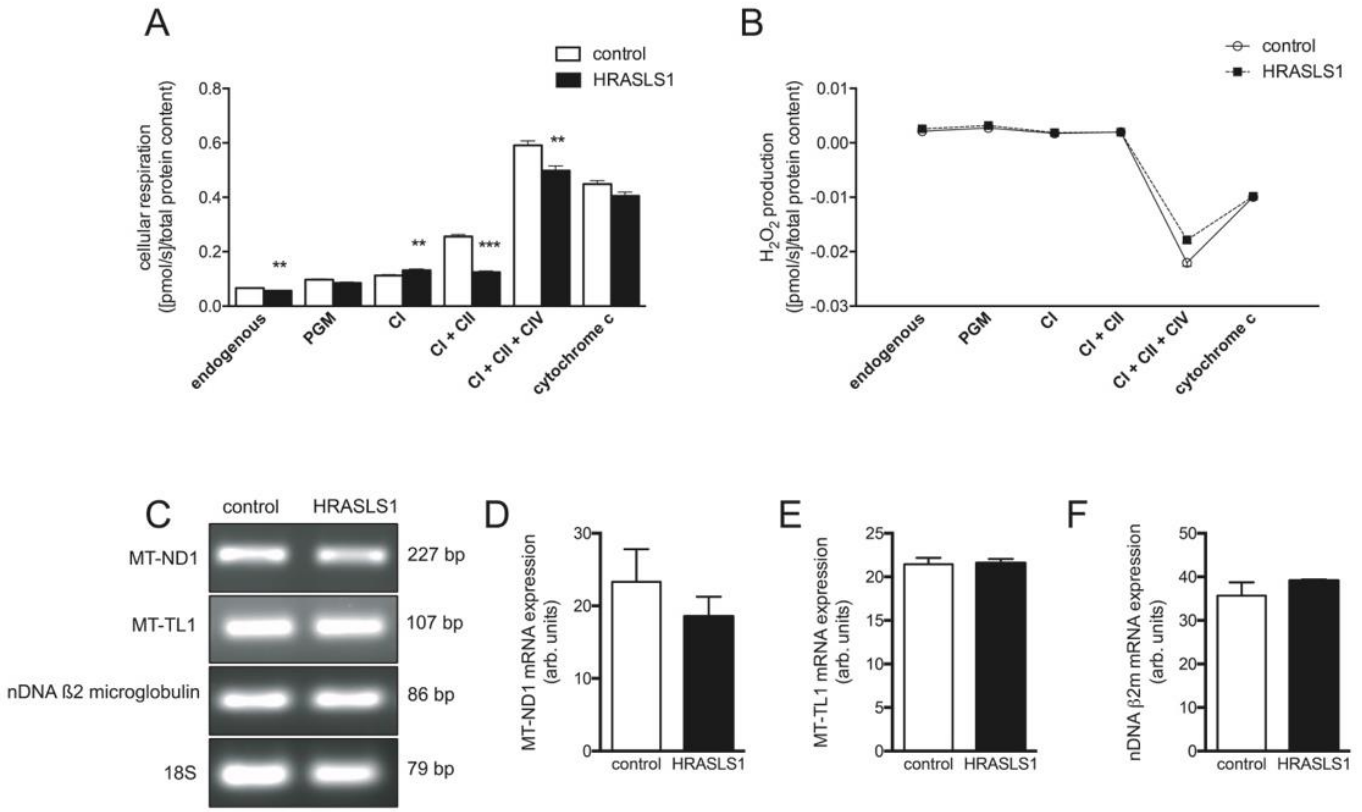

Figure 17: Adenoviral overexpression of HRASLS1 in HEK-293 cells significantly reduces mitochondrial respiration, but does not alter H_2O_2 production or mtDNA content.

(A) Respiratory activities of individual mitochondria electron transport chain complexes (CI, CI plus CII, CI plus CII and CIV) were determined in isolated mitochondria from HEK-293 cells overexpressing control or HRASLS1 protein, along with baseline activity (endogenous), proton leak (PGM), and following the addition of cytochrome c. (B) Hydrogen peroxide production by mitochondria isolated from HEK-293 cells overexpressing control or HRASLS1 protein. (C) Representative RT-PCR of mRNA expression of markers of mitochondrial DNA (MT-ND1, MT-TL1, quantified in (D), and (E), respectively) and nuclear DNA (nDNA β2 microglobulin, quantified in (F)) taken from HEK-293 cells overexpressing control or HRASLS1 protein. Data are means ± S.E.M. **P<0.01, ***P<0.001 vs. controls. (A, B, n=6), (C-F, n=4).

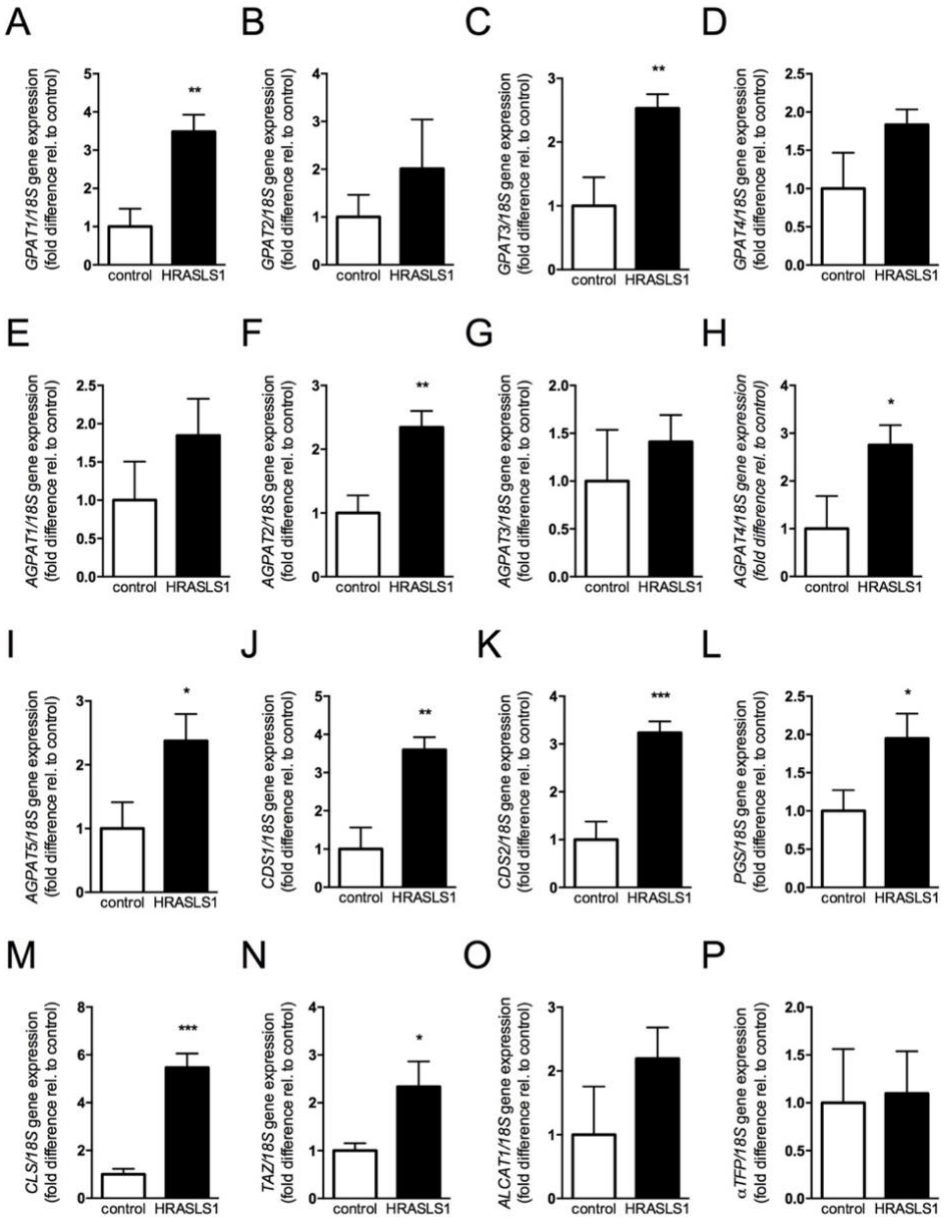

Figure 18: HRASLS1 overexpression in HEK-293 cells significantly increases the expression of genes involved in phospholipid synthesis and remodeling.

Analysis of gene expression from HEK-293 cells overexpressing control or HRASLS1 protein for genes involved in phospholipid, and specifically, cardiolipin synthesis and acyl-chain remodeling. (A-D), Glycerol-3-phosphate acyltransferases 1-4 (*GPAT1-4*). (E-I) Acylglycerophosphate acyltransferases 1-5 (*AGPAT1-5*). (J) Phosphatidate cytidylyltransferase 1 (*CDS1*). (K) Phosphatidate cytidylyltransferase 2 (*CDS2*). (L) Phosphatidylglycerol synthase (*PGS*). (M) Cardiolipin synthase (*CLS*). (N) Tafazzin (*TAZ*). (O) Acyl-CoA:lysocardiolipin acyltransferase 1 (*ALCAT1*). (P) α-trifunctional protein (*αTFP*). Data are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 vs. controls. n=10, performed in triplicate.

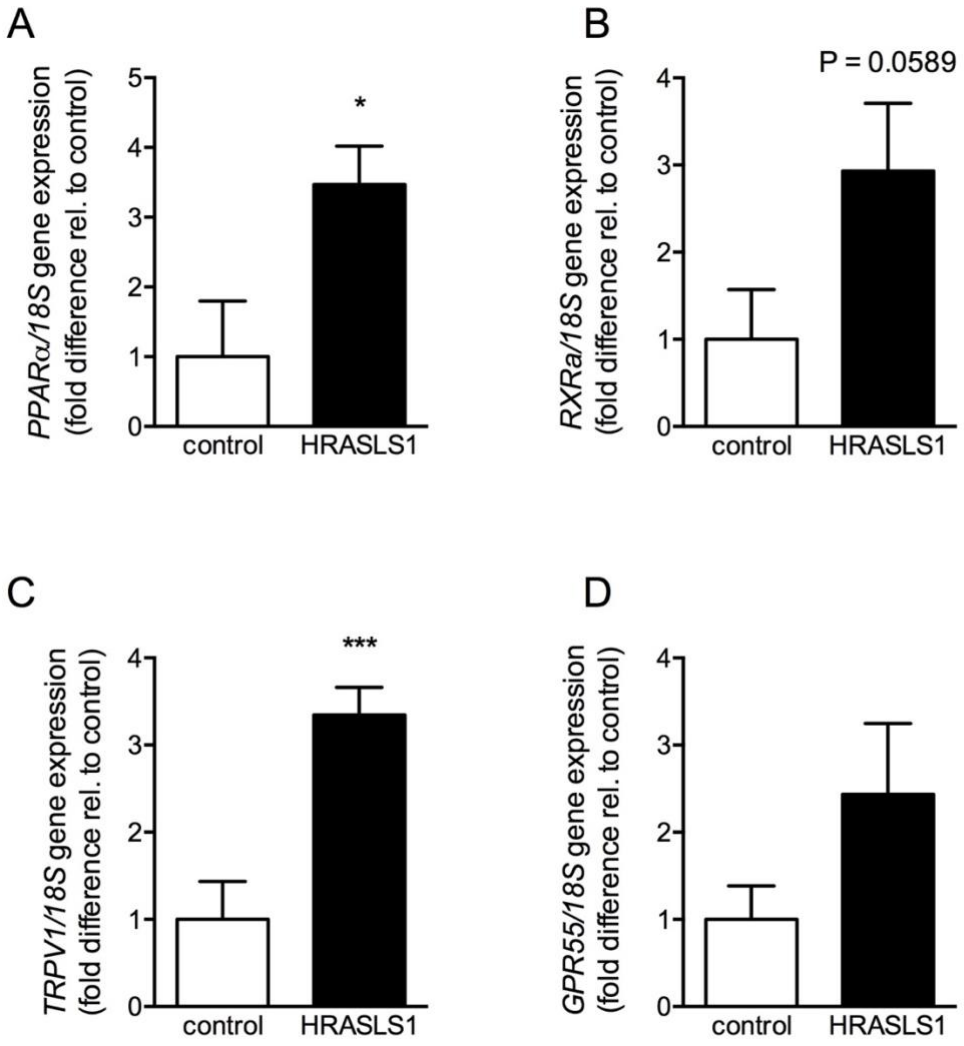

Figure 19: HRASLS1 overexpression in HEK-293 cells significantly increases the mRNA expression of PPARα and TRPV1.

Analysis of gene expression in HEK-293 cells overexpressing control or HRASLS1 protein for known receptors of NAEs including (A) PPARα, (B) RXRa, (C) TRPV1, and (D) GPR55. Data are ± S.E.M. *P<0.05, ***P<0.001 vs. controls. n=10, performed in triplicate.

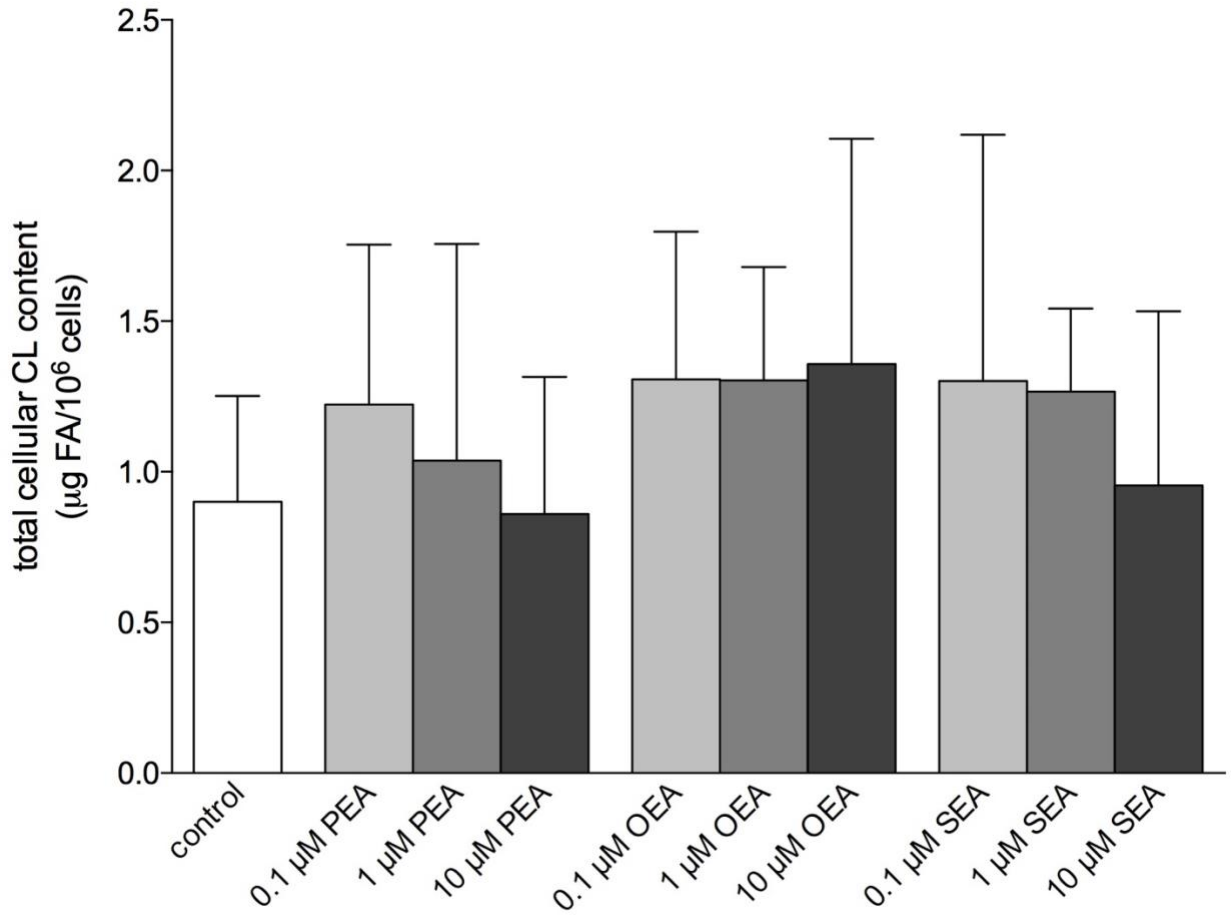

Figure 20: NAE treatment does not increase endogenous CL content in HEK-293 cells.

HEK-293 cells were treated for 48 hours with 0.1, 1, or 10 μM of either PEA, OEA, and SEA. Total lipids were harvested and separated by TLC, and total endogenous CL content was analyzed by GC-FID. No significant differences in CL content were observed in any treatment condition as compared to controls. Data are means ± S.E.M., n=6.

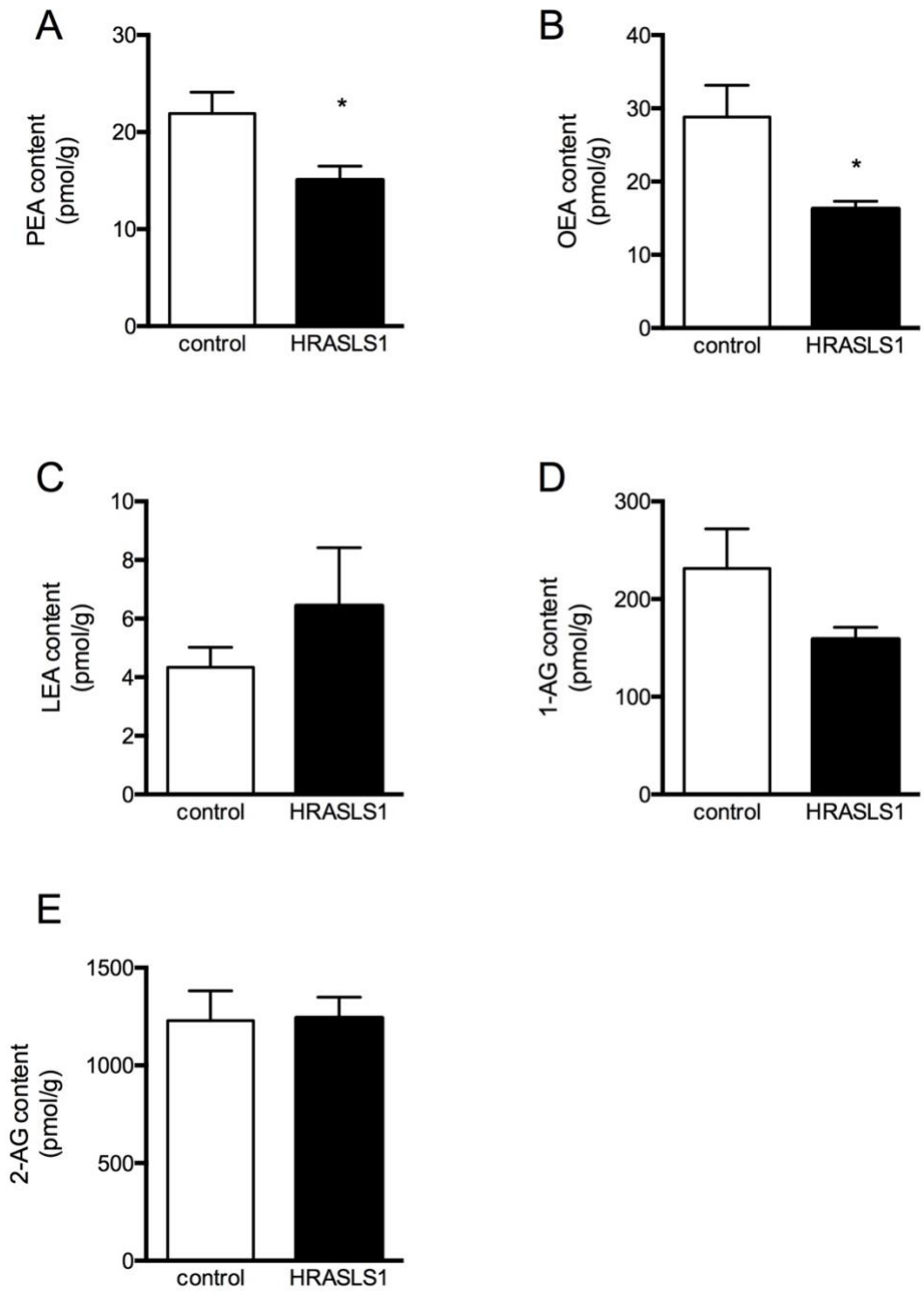

Figure 21: Adenoviral overexpression of HRASLS1 in HEK-293 cells significantly reduces PEA and OEA content.

LC-MS/MS analysis of alterations in the content of major NAE species including (A) PEA, (B) OEA, and (C) LEA, as well as (D) 1-AG and (E) 2-AG in HEK-293 cells overexpressing control or HRASLS1 protein. Data are ± S.E.M. *P<0.05, vs. controls. n=6.

Discussion

In Thesis Study 1, I found that HRASLS1 can play novel roles in cardiolipin synthesis, based on assays conducted *in vitro*. HRASLS1 overexpression in HEK-293 cells indirectly induces oleoyl-CoA-dependent MLCL O-acyltransferase activity, although it does not directly catalyze reactions with these substrates. HRASLS1 overexpression may also indirectly induce PC:MLCL O-transacylase activity in these cells, although evidence from reticulocyte lysates suggests that HRASLS1 may additionally directly catalyze specific PC:MLCL O-transacylase reactions. In the current study, I investigated the effects of 48-hour HRASLS1 overexpression on phospholipid metabolism in HEK-293 cells, including measurement of phospholipid content and composition, and expression of phospholipid synthesis genes. I also analyzed effects of HRASLS1 overexpression on mitochondrial content and function. Finally, I tested potential roles for common NAEs in CL metabolism, and determined effects of HRASLS1 overexpression on the abundance of common NAEs in HEK-293 cells.

In Thesis Study II, I demonstrate for the first time that HRASLS1 overexpression significantly, and specifically, increases cellular cardiolipin content. To the best of my knowledge, this is the first study to-date to provide evidence for an *in vivo* role of HRASLS1 in cardiolipin metabolism. Phospholipid content and acyl composition are highly regulated within the cell [5], and must be maintained in correct relative proportion to one another to preserve homeostasis. Although cardiolipin is a relatively minor constituent of cellular membranes and only accounts for ~2-5% of total phospholipids [5], its content and acyl composition are subject to a high degree of regulation. Cardiolipin is the only known dimeric phospholipid, containing four fatty acyl side chains instead of two. Importantly, the specific composition of cardiolipin is essential for its proper functioning in mitochondria [118, 151, 175, 177], and since this specificity is not achieved initially during *de novo* synthesis, it must be acquired through enzymatic remodeling.

I now show, for the first time, that adenoviral overexpression of HRASLS1 in HEK-293 cells primarily results in a significant increase of endogenous CL that is enriched with saturated and n-3 polyunsaturated fatty acids. One of the largest effects observed was an increase in the 18-carbon saturated fatty acid, stearate. This is different from CL in many tissues including heart [114, 116, 117], liver [142, 159], kidney [142], skeletal muscle [142], and spleen [142], which is enriched in linoleic acid. However, it is notable that HEK-293 cells have a relatively low content of 18:2n-6 in cellular CL, and instead are primarily enriched in CL containing 18:0, 18:1n-9, and

16:0. Interestingly, the linoleate content of endogenous CL in HEK-293 cells nearly doubled following HRASLS1 overexpression compared to control virus overexpression, although this did not reach significance with the current sample size. Increasing the sample size in this experiment would reduce variation and may lead to a statistically significant finding with regards to linoleate content. Overexpression of HRASLS1 did not significantly alter the total MUFA content of CL, nor did it alter the content of most individual MUFA species including oleic acid, which is the second most abundant fatty acyl species found in CL in most tissues [262], and also in CL from control HEK-293 cells.

Previous studies have demonstrated that alterations in CL metabolism can significantly contribute to cellular dysregulation. Decreases in CL content are associated with pathological consequences, the best-characterized of which is the development of Barth Syndrome (BTHS) [176], but CL deficiency is also evident in ischemia-reperfusion injury [149], hypothyroidism [264, 265], and heart failure [266, 267]. On the other hand, increased CL has also been associated with cellular dysfunction, since increases in CL content are evident in studies on hyperthyroidism in both mouse skeletal muscle and rat hearts [268-271]. These studies demonstrate the absolute requirement for the cell to maintain CL, along with other phospholipids, in correct relative abundance and proportion to one another, for the maintenance of health.

Perhaps equally as important as the total cellular content of CL is its acyl chain composition, which is directly regulated by at least four major known enzymes, and can vary by tissue, and diet [262]. Improper acyl-chain remodeling of CL leads to a disease state even if total levels of CL are not reduced, as is evidenced by studies on ALCAT1 [121, 143, 228]. CL that is remodeled by ALCAT1 is significantly enriched in highly unsaturated fatty acids (HUFA: ≥20 carbons, ≥3 double bonds) that are more susceptible to peroxidative damage than fatty acids with a lesser degree of unsaturation [121, 143, 227]. Cardiac overexpression of ALCAT1 in mice causes increases in both oxidative stress and depletion of mitochondrial DNA, while ALCAT1 knockout prevents the onset of cardiomyopathy and cardiac dysfunction [227]. Therefore, I hypothesized that the significant increase in CL content following adenoviral overexpression of HRASLS1 would contribute to mitochondrial dysfunction. Moreover, with the observation that HRASLS1 enriches CL with SFA, I hypothesized that the lack of proper acyl chain remodeling (i.e. to 18:2n-6 or 18:1n-9-containing CL) would also result in significant detrimental consequences for mitochondrial function.

Analysis of results from high-resolution mitochondrial respirometry indicated that mitochondria isolated from HEK-293 cells overexpressing HRASLS1 had significantly lower maximal respiration than controls. Evidence from this work suggests that CL enriched with SFA may not allow for proper functioning of ETC complexes. CL is critical for mitochondrial energy metabolism, since it is required for the proper membrane orientation, assembly, and activity of mitochondrial enzymes including cytochrome c oxidase [272-274], glycerol-3-phosphate dehydrogenase [275], and ATP synthase [234, 276], and for the stabilization of large mitochondrial protein complexes [123, 277]. Moreover, although CL acyl-chain specificity can vary from tissue-to-tissue, a recurrent theme observed in the acyl chain remodeling patterns of this phospholipid is its preference for 18-carbon unsaturated fatty acids (typically either linoleic or oleic acid) [278]. While the precise function of these specific fatty acids in mitochondrial function is not yet fully understood, it has been suggested that repeating chains of oleate or linoleate may create structural uniformity, molecular symmetry, and a relatively high degree of fluidity in the membrane that provides a conducive environment for optimal functioning of ETC complexes [261]. Since overexpression of HRASLS1 both increases CL content, and causes an enrichment of CL with saturated fatty acids, it is not surprising that we observed a significant decrease in mitochondrial respiration.

It is of note that although changes in CL composition were associated with impaired mitochondrial function in this study, the production of SFA-enriched CL may also confer a protective benefit against lipid peroxidation and mitochondrial DNA damage, and therefore be beneficial in some instances. Lipid peroxidation typically affects unsaturated fatty acids as compared to saturates [279], and can further induce damage to mitochondrial DNA [280, 281]. Consistent with this evidence, we observed no difference in H_2O_2 production, nor alterations in markers of mitochondrial DNA content in HRASLS1-overexpressing HEK-293 cells, where CL is enriched in SFA. By comparison, the production of CL by ALCAT1, which is remodeled with HUFA, is targeted by reactive oxygen species that leads to mitochondrial DNA dysfunction [143, 227]. Clearly, a cost-benefit analysis is needed to examine the trade-off between mitochondrial function and maintenance of membrane and mitochondrial DNA integrity in cells. The generation of HRASLS1-overexpressing mice and/or HRASLS1 knockout mice would serve as valuable models to study the downstream effects of altered CL acyl chain composition and mitochondrial function on a cellular, organellar, and organismal basis.

In Thesis Study I, I observed an indirect induction of oleoyl-CoA-dependent MLCL O-acyltransferase activity in lysates from cells that overexpressed HRASLS1 for 48 hours. To explain this, I hypothesized that HRASLS1 may induce the synthesis of NAPE that acts as substrate to increase the synthesis of NAE, which could activate expression of genes involved in phospholipid biosynthesis. To determine if there is any evidence to support this notion, I investigated the mRNA expression of genes involved in phospholipid, and specifically, cardiolipin biosynthesis in HEK-293 cells overexpressing control or HRASLS1 protein. Following overexpression of HRASLS1, I observed a significant increase in the expression of the majority of the Kennedy Pathway genes analyzed. Interestingly, five acyl-CoA-dependent O-acyltransferases – GPAT1, GPAT3, AGPAT2, AGPAT4, and AGPAT5 - were significantly increased. This could lead to increased incorporation of [^{14}C]oleoyl-CoA into PC, which could then act as a substrate for PC:MLCL O-transacylase activity by HRASLS1. Notably, the largest difference in gene expression observed was an ~5.5-fold higher expression of CLS in HRASLS1-overexpressing HEK-293 cells, which could also increase CL content from precursor substrates generated by GPAT and AGPAT enzymes. The magnitude and scope of increases observed in the mRNA expression of nearly every Kennedy Pathway gene supports the idea that HRASLS1 can indirectly regulate cardiolipin biosynthesis through effects on gene expression. The data therefore suggests that *in vivo* N-acyltransferase activity of HRASLS1 may be at least partially responsible for mediating these effects by increasing the synthesis of NAPEs/NAEs. Future studies with NAE receptor antagonists will be important for establishing causality.

NAEs are well-known endogenous activators of transcriptional regulators including TRPV1 [64], GPR55 [65], and GPR119 [65, 66], although perhaps they have been best characterized through their agonism of PPARα [67, 68]. Studies have demonstrated that PPARα can be activated by several highly abundant NAE species including PEA [67], OEA [282], and LEA [260]. I hypothesized that overexpression of HRASLS1 in HEK-293 cells would result in a significant induction in the expression of the transcriptional regulators PPARα, and TRPV1, and consistent with my hypotheses, I observed a significant up-regulation of the mRNA expression of these genes. While this finding does not necessarily confirm a greater content of these receptors as the experiment only measures relative changes in mRNA expression, it does suggest that there may be increased flux through this pathway. NAEs are well-known to play key roles in several physiological processes including nocioception [283], inflammation [284], and food intake [260].

However, their abilities to modulate cardiolipin metabolism have not yet been reported. Although specific research in this area is lacking, it is likely that NAEs signal through the same receptor proteins (*i.e.* PPARα and TRPV1) to mediate phospholipid metabolism. PPARα is a well-known regulator of lipid metabolism and plays a significant role in regulating the phospholipid and TAG content in several murine tissues including the liver [285]. In a study performed by Lee *et al.* (2004) [285], *PPARα*$^{-/-}$ mice exhibited a 45% decrease in the hepatic content of total phospholipids. In addition, PPARα is known to regulate the expression of several Kennedy Pathway genes including the LPAATs/AGPATs [286] and, importantly, PPARα is also able to regulate *de novo* CL biosynthesis via the specific activation of PGPS, CDS1, and CDS2 [287]. Highly abundant NAEs such as PEA and OEA are known to signal through TRPV1 [288], and although TRPV1 does not yet have a defined role in phospholipid biosynthesis, its activation has been shown to stimulate the synthesis of phosphatidylinositol phosphates (PIPs), which are downstream effector lipids that originate from PI [289]. Interestingly, while many different species of NAE are endogenous agonists of TRPV1, evidence has suggested that NAEs comprised of 18-carbon, unsaturated fatty acids including oleic acid and linoleic acid can activate TRPV1 more efficiently than NAEs comprised of 18-carbon saturated fatty acids [290].

Thus, a rationale has been formed for a potential role for NAEs in CL biosynthesis. In particular, PEA, OEA, and SEA are abundant species of NAE in most tissues [291]. Therefore, I examined the ability of these NAEs to modulate CL content in HEK-293 cells, and expected to see a significant increase. However, a 48-hour treatment with any tested NAE species did not result in an increase in endogenous CL content. Results from this experiment suggest that these three species of NAE do not modulate CL in HEK-293 cells. Determination of the effect of HRASLS1 overexpression on NAE levels in HEK-293 cells therefore constitutes a critical next step.

Consequently, I initiated a collaboration to identify alterations in the content of NAEs in HEK-293 cells overexpressing HRASLS1. Surprisingly, a significant decrease in the content of both PEA and of OEA was detected. These data suggest that in HEK-293 cells, PEA and OEA do not play a major role in mediating effects of HRASLS1 on CL synthesis, which helps to explain my finding that treatment of HEK-293 cells with PEA and OEA does not increase endogenous-CL content in HEK-293 cells. SEA was not present at a detectable concentration in HRASLS1-overexpressing HEK-293 cells, suggesting a negligible role in regulation of this cell line. Interestingly, a moderate, although not statistically significant, increase was observed in the

content of LEA. While there exists a deficit in the literature in regards to the role of LEA in CL metabolism, LEA is known to exhibit anti-inflammatory effects [292], in addition to its role in mediating satiety [260]. Like other NAEs, LEA can exert its effects through activation of both PPARα and TRPV1 [260]. While a larger sample size will likely reduce the variation present in the measurement of this NAE species, future studies should aim to investigate the ability of LEA to modulate CL synthesis.

Conclusion

In conclusion, the current study has demonstrated the existence of a role for HRASLS1 in cardiolipin metabolism *in vivo*, extending observations in Thesis Study I on the function of this enzyme in CL metabolism *in vitro*. Overexpression of HRASLS1 in HEK-293 cells significantly increased cellular CL content, and specifically enriched CL with SFA. While the full physiological significance of SFA-enriched CL is unknown, it is likely detrimental to cellular health. Mitochondrial respiration in HEK-293 cells overexpressing HRASLS1 was significantly lower than in controls, while mitochondrial DNA content was not different, suggesting less functional, but not fewer mitochondria. The global increase in the mRNA content of multiple genes involved in phospholipid metabolism, along with PPARα and TRPV1, suggested that HRASLS1 may exert effects on cellular CL levels indirectly, potentially through the signaling of NAEs synthesized downstream of NAPE. While treatment of HEK-293 cells with OEA, PEA, or SEA did not increase CL content, it is likely that other NAE species, such as LEA, may mediate the downstream signaling effects of HRALSL1 on CL in HEK-293 cells.

Future investigations should be performed to test a role for LEA in the synthesis of CL in HEK-293 cells. Data from those studies would provide valuable insight into the potential mechanism(s) of LEA action, and may shed further light into the importance of 18-carbon, unsaturated NAE species in mediating phospholipid metabolism. In addition, the capacity for NAEs to increase CL content in cellular models that exhibit CL deficits should be examined. While decreases in CL are associated with several disease states, individuals suffering from Barth Syndrome can provide a direct model of CL deficiency to study NAE function. Hence, the next chapter of this thesis examines the therapeutic efficacy of NAE treatment in Barth Syndrome.

Chapter Six

Thesis Study III – Effects of OEA on CL metabolism and proliferation of cultured B-Lymphocytes from an Individual with Barth Syndrome

Introduction and Study Rationale

Thesis Study I and Thesis Study II demonstrated new roles for HRASLS1 in the *in vitro* and *in vivo* synthesis and acyl chain remodeling of the mitochondrial phospholipid CL. In that work, I observed widespread increases in the mRNA expression of nearly all genes involved in phospholipid metabolism, and thus hypothesized that HRASLS1 may in fact exert at least some of its CL metabolizing effects through signaling mediated downstream of its N-transacylase activity, and resultant production of NAPEs/NAEs. Direct treatment experiments suggested that OEA, PEA, or SEA are likely not significant regulators of the increase in CL that was evident in HEK-293 cells infected with HRASLS1. Data on changes in major detectable NAE species following HRASLS1 overexpression in HEK-293 cells further supported this notion. Interestingly, these data also suggested a potential role for LEA in HEK-293 cells that suggests it would be a first choice as a therapeutic target in treating CL deficiency in Barth Syndrome, and certainly also merits further investigation in HEK-293 cells. Unfortunately, Thesis Study III was almost completed by the time these data were available. Thus, OEA was selected as a test agent for preliminary therapeutic investigation in BTHS, based on the erroneous prediction that it would turn out to be a major NAE species produced downstream of HRASLS1, which had shown some preference for the use of 18-carbon unsaturated fatty acyls at the *sn-1* position. Regardless, OEA testing in BTHS lymphocytes yielded interesting results.

Thus, at the same time as the NAE experiments in HEK-293 cells were being performed, a parallel set of experiments utilizing cultured B-lymphocytes derived from healthy controls and BTHS patients were carried out simultaneously. B-lymphocytes derived from BTHS patients have been shown to have abnormal CL metabolism, CL deficiency, and impaired cellular function [293], and thus represent a useful experimental model to assess the capacity of NAE to modulate CL metabolism in a disease state. The overarching objective of this chapter was to assess the capacity of OEA to act as a potential therapeutic mediator of CL metabolism and cellular function in BTHS. An overview of study design for this chapter is illustrated in Figure 22.

Study Design

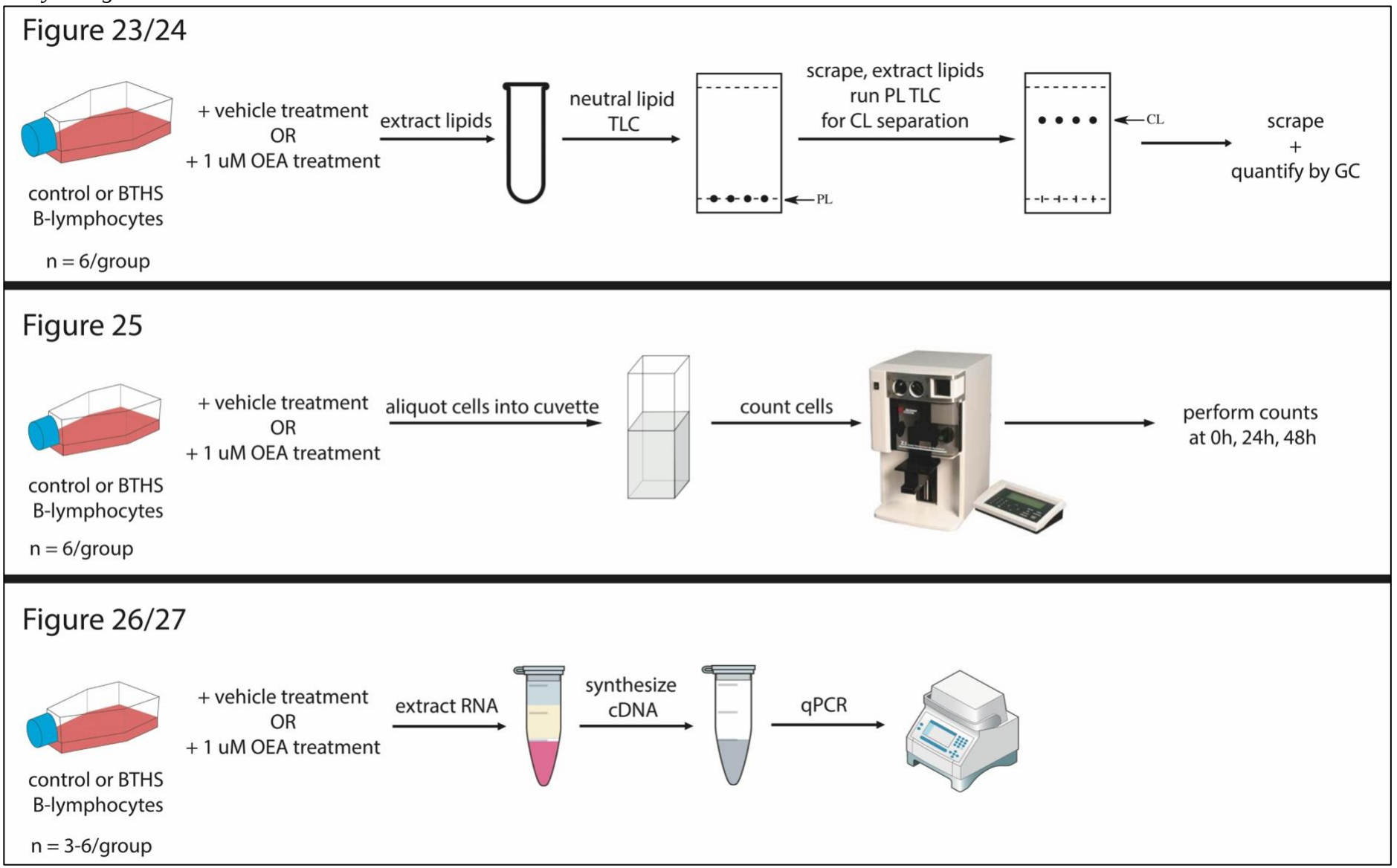

Figure 22: Schematic of the study design of Thesis Study III

Objectives and Hypotheses

The objectives and hypotheses for this study were as follows:

1. Objective: To investigate if treatment for 48 hours with 1 μM OEA can increase the total CL content in cultured B-lymphocytes derived from a 10-year-old male with BTHS relative to the same cells treated with vehicle alone (control), and to a level comparable to that of an age-, sex, and ethnicity-matched control patient.

   Hypothesis: A 48-hour treatment with 1 μM OEA will significantly increase cardiolipin content in BTHS-derived B-lymphocytes as compared to vehicle treated BTHS cells, and will partially restore CL levels to those observed in control patient lymphocytes. Treatment of control B-lymphocytes with OEA will not alter total CL levels.

2. Objective: To characterize the fatty acyl composition of CL in cultured B-lymphocytes derived from age-, sex-, and ethnicity-matched control and Barth Syndrome individuals that have been treated for 48-hour with vehicle or with 1 μM OEA.

   Hypothesis: A 48-hour treatment with 1 μM OEA will significantly enrich cardiolipin with saturated fatty acids and n-3 polyunsaturated fatty acids in BTHS B-lymphocytes as compared to mock treated and treated control B-lymphocytes.

3. Objective: To investigate if treatment for 48 hours with 1 μM OEA can increase the proliferation of cultured B-lymphocytes derived from a 10-year-old male with BTHS relative to the same cells treated with vehicle alone (control), and can rescue cellular proliferation to a level comparable to that of an age-, sex, and ethnicity-matched control patient.

   Hypothesis: A 48-hour treatment with 1 μM OEA will significantly increase the proliferative rate of BTHS-derived B-lymphocytes as compared to vehicle treated BTHS cells, and will partially restore proliferation to levels observed in control patient lymphocytes.

4. Objective: To assess changes in mRNA expression in cultured B-lymphocytes derived from an age-, sex-, and ethnicity-matched control and Barth Syndrome patient of genes involved in phospholipid biosynthesis following a 48-hour treatment with 1 μM OEA.

Hypothesis: A 48-hour treatment with 1 μM OEA will significantly increase the expression of CDS1/2, PGS, and CLS in BTHS-derived B-lymphocytes as compared to vehicle-treated BTHS-derived B-lymphocytes. This treatment will have no effect significant effect on the expression of these genes in control lymphocytes.

Results

OEA treatment of BTHS B-lymphocytes increases total cellular CL content

B-lymphocytes derived from a healthy control or BTHS patient were treated either with vehicle control, or 1 μM OEA for 48 hours, then harvested and extracted for total endogenous lipids. Individual phospholipids were resolved by TLC, and endogenous CL was identified by comparison with known standards, scraped, and analyzed for fatty acyl composition and content. As expected, vehicle-treated BTHS cells displayed an ~40% lower content of CL than control vehicle-treated cells (control vehicle, 2.07 ± 0.16 μg FA of CL/10^6 cells versus BTHS vehicle, 1.25 ± 0.13 μg FA of CL/10^6cells, $P<0.05$) (Fig. 23A). However, this difference was no longer significant following treatment of cells with 1 μM OEA (control OEA, 1.85 ± 0.27 μg FA of CL/10^6 cells versus BTHS OEA, 1.63 ± 0.19 μg FA of CL/10^6 cells, $P = 0.528$). It is important to note that although treatment with 1 μM OEA partially rescued CL content in BTHS B-lymphocytes, it did not do so at a statistically significant level (BTHS vehicle, 1.25 ± 0.13 μg FA of CL/10^6 cells versus BTHS OEA, 1.63 ± 0.19 μg FA of CL/10^6cells, $P=0.13$).

OEA treatment of BTHS B-lymphocytes significantly alters the relative content of individual fatty acids in CL

The fatty acid composition of CL in B-lymphocytes derived from a healthy control subject or a BTHS patient treated with either vehicle or 1 μM OEA was determined and quantified by GC-FID. Analysis of the relative changes in the major fatty acyl classes within CL displayed an overall higher relative content of SFA, and a lower relative content of MUFA, in both vehicle- and OEA-treated BTHS cells as compared to both vehicle- and OEA-treated control cells (Fig. 23B).

Several differences were observed between the groups upon examination of individual fatty acyl species within CL. In particular, there was a significantly higher relative content of both palmitic acid (C 16:0) and stearic acid (C 18:0) in both vehicle- and OEA-treated BTHS cells as compared to both vehicle- and OEA-treated controls (Fig. 24A). Of the monounsaturated fatty acyl species analyzed, a significantly lower relative content of palmitoleic acid (C 16:1) was observed in CL from the vehicle-treated BTHS cells as compared to vehicle-treated controls. However, this difference was not conserved in the OEA-treated BTHS cells as compared to the OEA-treated control cells. A significantly lower relative content of oleic acid (C18:1n-9) was observed in both BTHS treatment conditions as compared to both control treatment conditions, although it is notable

that treatment with OEA helped to restore C18:1n-9 in CL from the BTHS cells to levels seen in vehicle-treated control cells (Fig. 24B).

The relative percent of linoleic acid (C18:2n-6) was highly significantly lower in content in both BTHS vehicle and OEA-treated cells as compared to both control vehicle- and OEA-treated cells (Fig. 24C). Notably, treatment of BTHS lymphocytes with OEA did not reduce the relative abundance of linoleic acid in CL relative to control lymphocytes, and may even have slightly, but non-significantly, enriched this acyl species. Of the n-3 PUFA species, only a difference in α-linolenic acid, (ALA, C 18:3n-3) was observed, where vehicle- and OEA-treated BTHS cells had a significantly higher relative content of ALA as compared to both vehicle-treated control cells (Fig. 24D). Interestingly, the ALA content of control lymphocytes did not differ significantly from BTHS lymphocytes after treatment with OEA due to a slight increase in the content of this fatty acyl in control cell CL, and a slight decrease in the content in BTHS cell CL.

OEA treatment of BTHS B-lymphocytes reverses growth impairments

Control or BTHS-derived B-lymphocytes were seeded in 15 mL culture flasks at a density of approximately ~1 million cells/flask. Growth media was RPMI-1640 with 10% charcoal-stripped FBS, 1% penicillin-streptomycin, and cells were treated with either OEA at a final concentration of 1 μM (treated), or with only the vehicle control (ethanol). Baseline measurements of cell numbers were taken at Day 0 to ensure accurate seeding densities, and counts were performed subsequently at 24 hour and 48 hours. On Day 0, all groups of control and BTHS B-lymphocytes were seeded at approximately 1 million cells/flask. After 24 hours (Day 1), cell counts in the control (vehicle) groups began to demonstrate the expected growth impairments of BTHS B-lymphocytes as compared to control B-lymphocytes. At the 48-hour time-point (i.e. Day 2), differences in cell growth were statistically significant. As compared to the vehicle-treated control B-lymphocytes, there were significantly fewer vehicle-treated BTHS-derived B-lymphocytes (i.e. control vehicle-treated, $5.04 \times 10^6 \pm 5.30 \times 10^5$ total cells versus BTHS vehicle-treated $2.94 \times 10^6 \pm 3.86 \times 10^5$ total cells, $P = 0.0094$) (Fig. 25). Treatment of BTHS-derived B-lymphocytes with 1 μM OEA reduced this difference, so that after two days, the number of BTHS lymphocytes treated with OEA ($4.23 \times 10^6 \pm 5.45 \times 10^5$ total cells) was not significantly different from the number of control lymphocytes either treated ($4.64 \times 10^6 \pm 3.77 \times 10^5$ total cells), or untreated ($5.04 \times 10^6 \pm 5.30 \times 10^5$ total cells) with OEA. However, the rescue of cell proliferation

was only partial, since at 48 hours, the number of BTHS lymphocytes treated with OEA was not statistically different from the number of vehicle-treated BTHS lymphocytes (1 μM OEA BTHS $4.23 \times 10^6 \pm 5.45 \times 10^5$ total cells total cells, versus BTHS vehicle-treated $2.94 \times 10^6 \pm 3.86 \times 10^5$ total cells, P=0.0832).

BTHS B-lymphocytes have significantly higher mRNA expression of GPAT3 and CDS1 as compared to control B-lymphocytes

Real-time PCR was performed to investigate any underlying differences in the mRNA expression of genes involved in phospholipid biosynthesis between control and BTHS B-lymphocytes. Cells were treated with vehicle or OEA, grown for 48 hours, following which total RNA was harvested and analyzed by RT-qPCR for relative mRNA expression of genes involved in phospholipid biosynthesis. All data was normalized to the mRNA expression of *18S*. Vehicle-treated lymphocytes from control subjects were compared with vehicle-treated lymphocytes from BTHS subjects (Fig. 26), and it was determined that the majority of phospholipid and CL synthesis genes were not differently expressed. Only two significant differences were evident. *GPAT3* mRNA was ~7-fold more abundant in BTHS lymphocytes versus control subject lymphocytes (control, 1.00 ± 0.672 versus BTHS, 7.46 ± 0.836, P = 0.0038) (Fig. 26C). And, similarly, *CDS1*, was 18-fold more abundant in BTHS cells as compared to controls (control, 1.00 ± 1.048 versus BTHS, 18.25 ± 1.141, P = 0.0004) (Fig. 26J).

OEA treatment of BTHS B-lymphocytes increases the expression of Agpat4 as compared to OEA-treated controls

OEA-treated lymphocytes from control subjects were compared with OEA-treated lymphocytes from BTHS subjects (Fig. 27), and it was determined that, similar to effects observed in vehicle-treated cells, the majority of phospholipid and CL synthesis genes were not differently expressed. The only significant difference observed was an ~2.5-fold increase in the mRNA expression of *AGPAT4* in OEA-treated BTHS cells compared to OEA-treated controls (control, 1.00 ± 1.048 versus BTHS, 2.68 ± 0.597, P = 0.0490) (Fig. 27H).

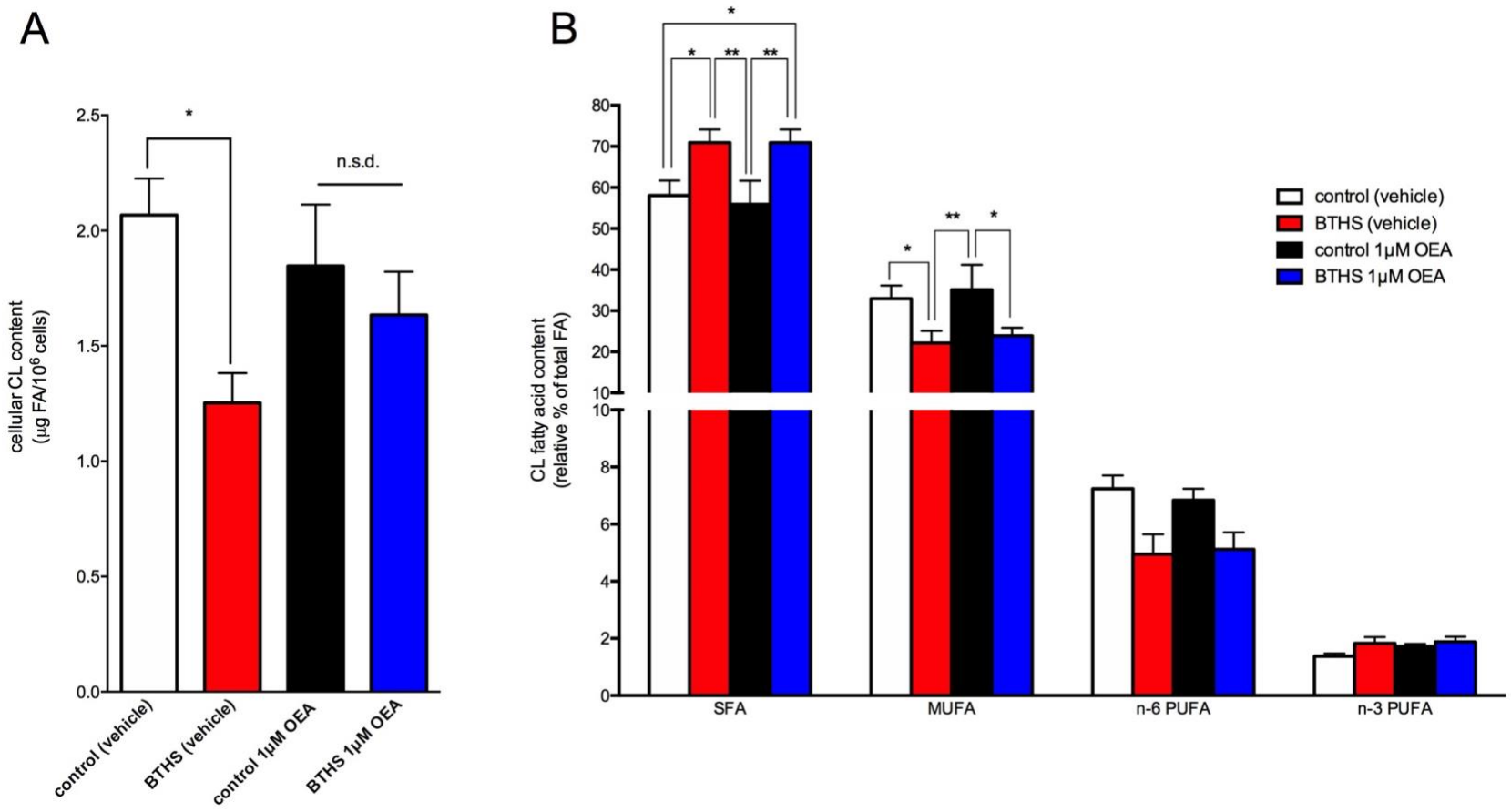

Figure 23: OEA treatment partially rescues CL content in BTHS-derived B-lymphocytes

(A) Endogenous CL content of B-lymphocytes derived from healthy controls or BTHS patients treated with vehicle (control) or 1 µM OEA for 48 hours. (B) Relative proportion of major fatty acid classes (*i.e.*, SFA, MUFA, n-6 PUFA, n-3 PUFA) within CL isolated from healthy controls and BTHS patients treated either with vehicle alone (control) or 1 µM OEA. Data are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, n=6.

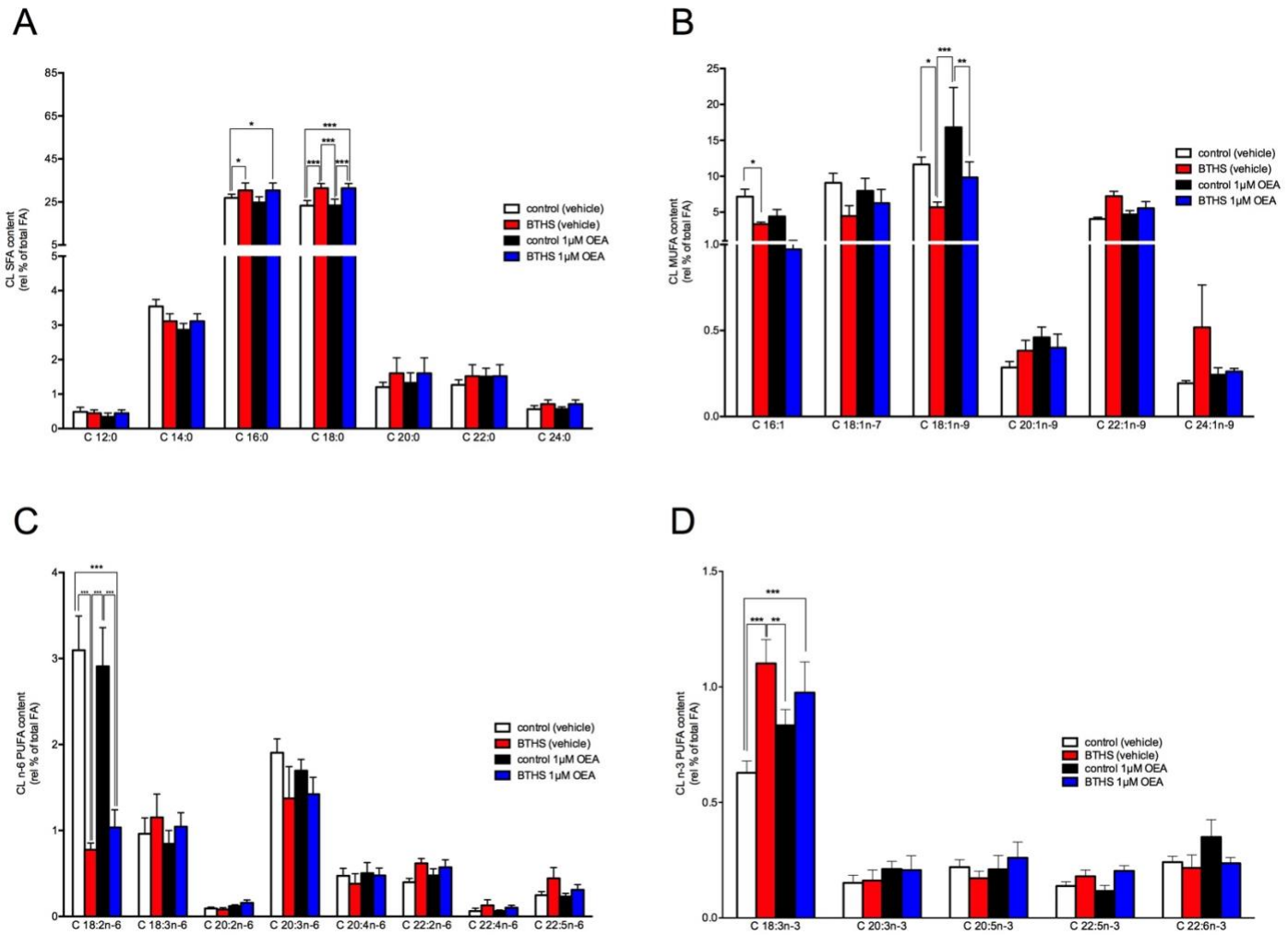

Figure 24: Fatty acyl profiles of CL isolated from healthy control B-lymphocytes and BTHS B-lymphocytes with or without OEA treatment.

Relative proportion of fatty acyls within CL from cultured B-lymphocytes of healthy controls or BTHS patients treated with vehicle alone, or 1 μM OEA for 48 hours, including SFA (A), MUFA (B), n-6 PUFA (C), and n-3 PUFA (D). Data are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, n=6.

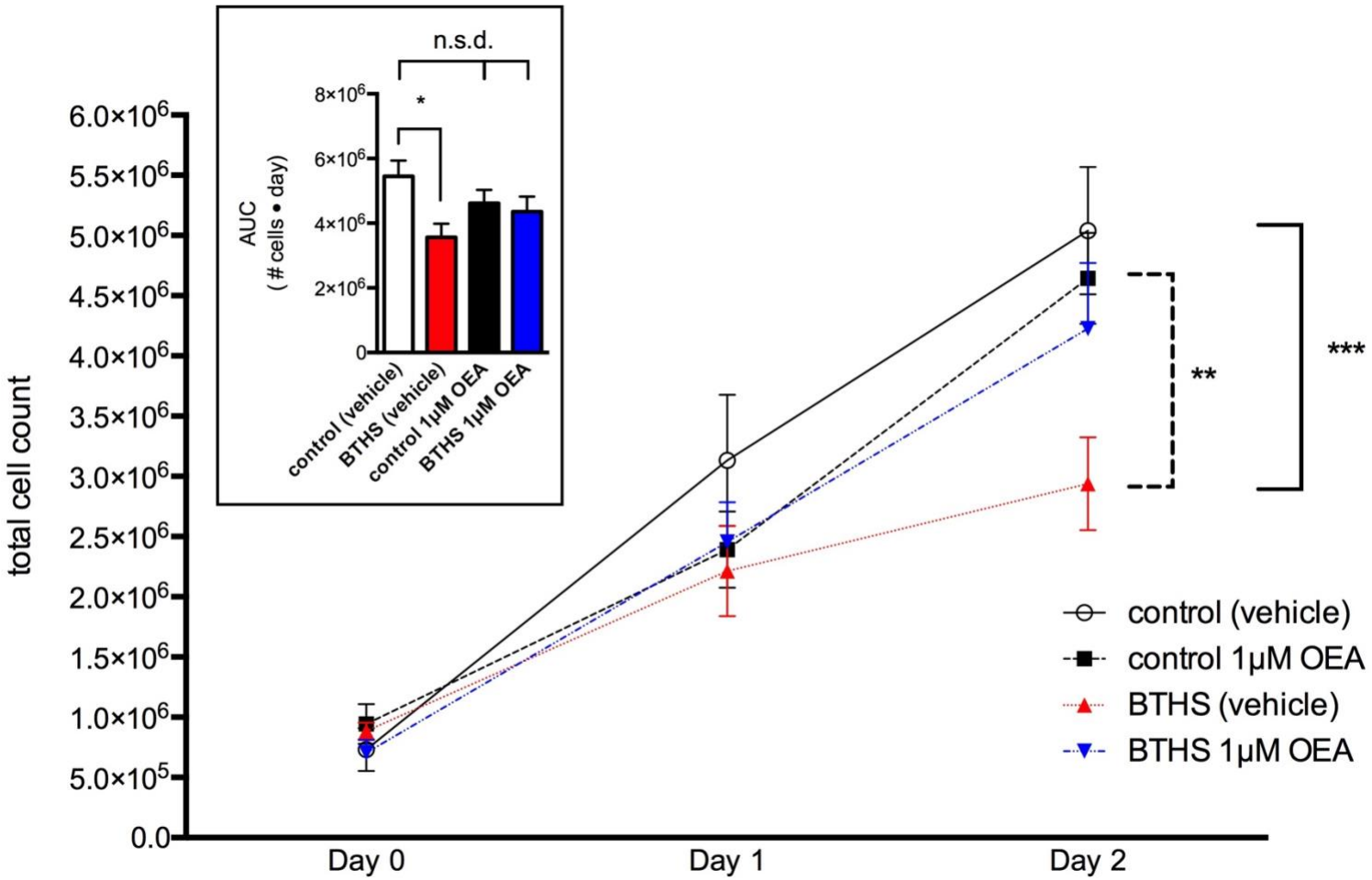

Figure 25: OEA treatment partially rescues the growth deficit observed in BTHS B-lymphocytes

Growth curves of B-lymphocytes derived from a healthy control or BTHS patient, treated either with vehicle alone, or 1 µM OEA for 48 hours. Cellular proliferation rate, as calculated by area under the curve (AUC) is shown in the inset figure. Data are means ± S.E.M. *P<0.05, **P<0.01, ***P<0.001, n=6.

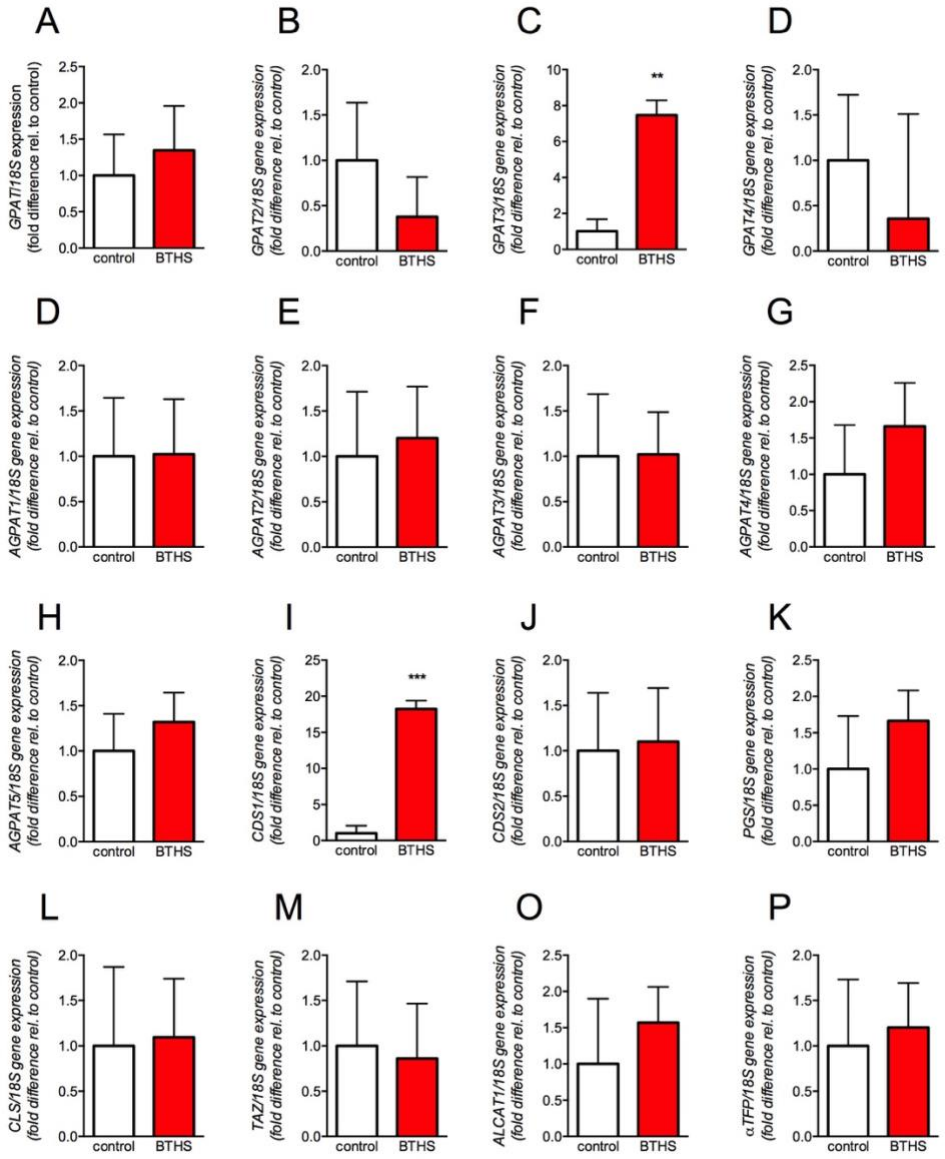

Figure 26: Expression of phospholipid biosynthetic genes in vehicle-treated healthy control and BTHS B-lymphocytes.

Analysis of B-lymphocytes derived from vehicle-treated healthy controls or BTHS patients for expression of genes involved in phospholipid, and specifically, CL synthesis and acyl-chain remodeling. (A-D), Glycerol-3-phosphate acyltransferases 1-4 (*GPAT1-4*). (E-I) Acylglycerophosphate acyltransferases 1-5 (*AGPAT1-5*). (J) Phosphatidate cytidylyltransferase 1 (*CDS1*). (K) Phosphatidate cytidylyltransferase 2 (*CDS2*). (L) Phosphatidylglycerol synthase (*PGS*). (M) Cardiolipin synthase (*CLS*). (N) Tafazzin (*TAZ*). (O) Acyl-CoA:lysocardiolipin acyltransferase 1 (*ALCAT1*). (P) α-trifunctional protein (*αTFP*). Data are means ± S.E.M. **P<0.01, ***P<0.001, n=3, performed in triplicate.

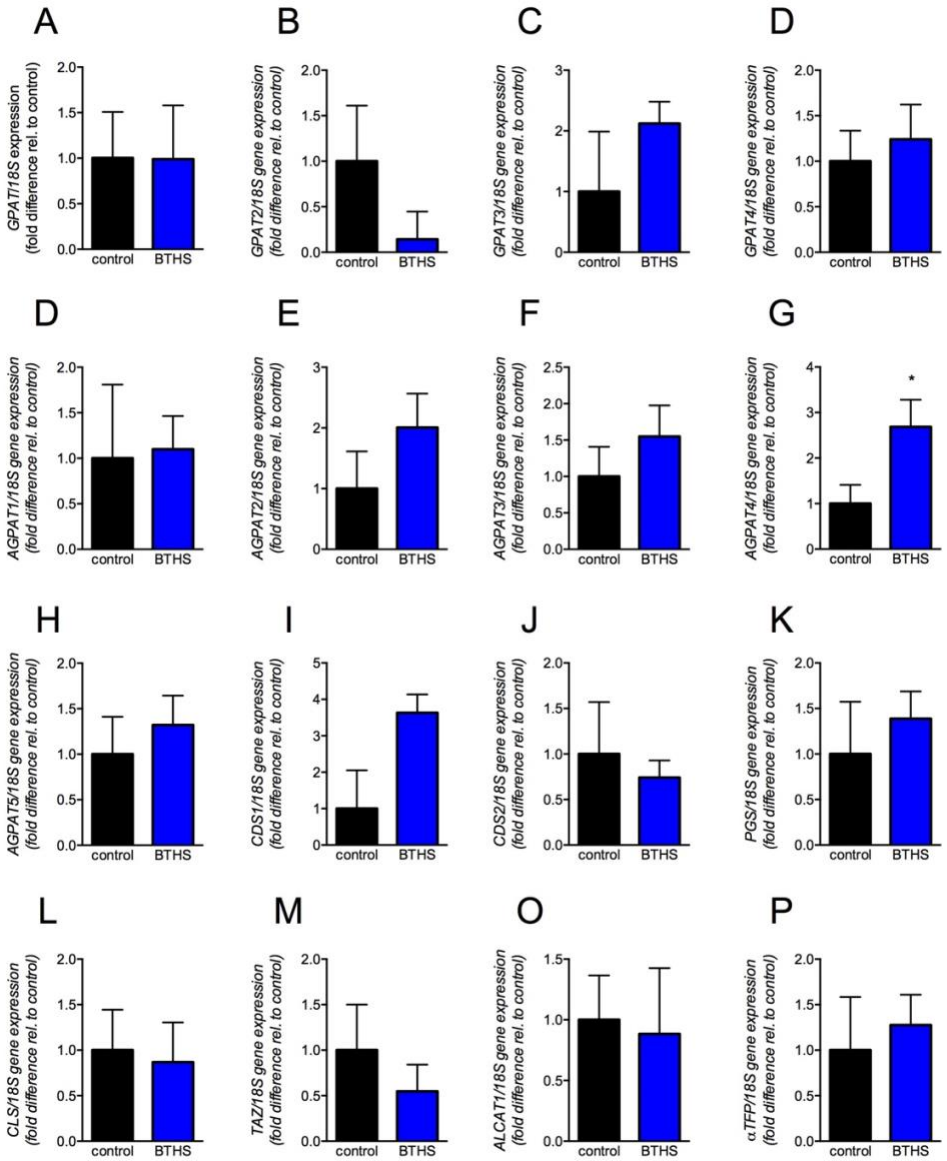

Figure 27: Expression of phospholipid biosynthetic genes in healthy control and BTHS B-lymphocytes treated with 1 μM OEA.

Analysis of B-lymphocytes derived from a healthy control subject or BTHS patient and treated with 1 μM OEA, for genes involved in phospholipid, and specifically, CL synthesis and acyl-chain remodeling. (A-D), Glycerol-3-phosphate acyltransferases 1-4 (*GPAT1-4*). (E-I) Acylglycerophosphate acyltransferases 1-5 (*AGPAT1-5*). (J) Phosphatidate cytidylyltransferase 1 (*CDS1*). (K) Phosphatidate cytidylyltransferase 2 (*CDS2*). (L) Phosphatidylglycerol synthase (*PGS*). (M) Cardiolipin synthase (*CLS*). (N) Tafazzin (*TAZ*). (O) Acyl-CoA:lysocardiolipin acyltransferase 1 (*ALCAT1*). (P) α-trifunctional protein (*αTFP*). Data are means ± S.E.M. *P<0.05 vs. controls. n=5, performed in triplicate.

Discussion

The current study examined the ability of OEA to modulate CL metabolism in B-lymphocytes taken from a healthy control subject and an individual with BTHS. This work demonstrates, for the first time, that treatment with an NAE (specifically OEA) can partially rescue CL content and cellular growth of BTHS B-lymphocytes relative to controls.

Since this study was performed in parallel with the previous study (Thesis Study II) examining an *in vivo* role for HRASLS1 in CL metabolism, the protocol was not informed by results from our collaborator on relative changes in species of NAEs occurring as a result of HRASLS1 infection. Quantifiably, OEA is amongst the most abundant NAE species produced in many cell types [294], and is produced in greater amounts than the well-studied anandamide [295]. In addition, NAE receptors such as PPARα and TRPV1, which may regulate phospholipid metabolism, have shown preference for activation by species which contain an 18-carbon, unsaturated fatty acid, such as oleic acid [290]. Thus, OEA was selected for use as a potential candidate modulator of CL in both HEK-293 cells, and B-lymphocytes.

Although HRASLS1 increased CL in HEK-293 cells, it decreased OEA levels significantly, strongly suggesting that this NAE is not involved in the modulation of CL by HRASLS1, and that other NAEs should be investigated. This was substantiated by the finding that treatment of HEK-293 cells with OEA did not alter the endogenous cardiolipin content. Regardless, since this was not known when studies with BTHS lymphocytes were initiated, OEA was tested as a potential therapeutic. Although results from this work do not assist in explaining the effects of HRASLS1 on CL metabolism, as was originally intended, they are highly novel, and of strongly related interest to this thesis in the context of exploring the broader regulation of CL synthesis.

The study was performed with the overarching hypothesis that candidate NAEs, such as OEA, could be useful as potential therapeutics in disorders of impaired CL metabolism. This is not the first time that an NAE species has been tested for a therapeutic purpose in the treatment of a disease. Indeed, several studies have examined the use of OEA and PEA in modulating disease conditions including intestinal hyperpermeability and inflammation [296], obesity [297, 298], inflammatory bowel disease [299], and neurodegeneration [300]. In fact, the focus of a growing number of pharmacological studies is the development of inhibitors against N-acylethanolamine acid amidase (NAAA), an enzyme which hydrolyzes, and therefore degrades, endogenous NAEs

[301]. This is, however, the first time that an NAE has been tested for the purpose of modulating CL synthesis to ameliorate a disease associated with deficiency of this mitochondrial lipid.

Analysis of the CL content of vehicle-treated control and BTHS lymphocytes demonstrated an expected deficit in CL content in BTHS cells that has already been well characterized [174, 278, 293]. Following a 48-hour treatment with 1 μM OEA, however, an ~30% increase in endogenous CL was observed in the OEA-treated BTHS cells as compared to vehicle-treated BTHS cells. While the increased level in OEA-treated BTHS cells did not fully restore CL to levels found in either treated or untreated control cells, the significant difference between the groups was lost, due to the increase in CL in the OEA-treated BTHS cells. These data therefore indicate that treatment with OEA can, at least in part, increase the cellular content of CL in cells that exhibit CL deficiencies. This outcome is in contrast to the findings that were observed in OEA-treated HEK-293 cells, which did not show an increase in CL content. It is not clear whether the efficacy of OEA in BTHS B-Lymphocytes is due to the difference in cell type (lymphocytes versus embryonic kidney cells), or due to the CL-deficient state of these cells, a combination, or some other factor.

Increasing CL content in BTHS patients is a primary objective in the treatment of this disorder. At the present time, several potential therapies are under investigation including TAZ-enzyme replacement therapy (ERT), and gene therapy via the use of recombinant adeno-associated viruses (rAAV). ERT has already shown effectiveness in treating other metabolic disorders including Fabry disease [302], and Pompe disease [303]. In addition, rAAV gene therapy is a viable therapeutic option for mouse models of both Fabry disease [304-306] and Pompe disease [307, 308]. Unfortunately, however, while they are the focus of current investigation by several laboratories, no viable ERT or rAAV therapies have been developed that are yet effective or used in BTHS. Another potential therapy to restore CL in BTHS is lipid replacement therapy (LRT), which was recently investigated by Ikon *et al*. (2017) in BTHS [309]. In that study, the use of exogenous CL nanodisk (CL-ND) delivery particles was examined for its ability to increase CL content in TAZ-KD mice [309]. While CL-ND treatment had been previously found to be effective in increasing CL content in cultured cells [310], it failed to alter the CL profile of TAZ-knockdown mice, indicating that CL-ND therapy is not suitable for *in vivo* treatment of BTHS [309]. Thus, the discovery that exogenous OEA treatment can increase CL content in cultured BTHS B-lymphocytes represents an exciting opportunity. Further studies should aim to investigate whether

this effect is conserved *in vivo*, beginning with *Taz*-knockout mice. In addition, future studies should investigate the use of a wider range of NAE species, to attempt to achieve a more complete rescue of the CL content in BTHS B-lymphocytes. One such NAE species that may be of particular interest is LEA, due to the prior observation that LEA content is modestly elevated in HEK-293 cells overexpressing HRASLS1. NAEs are produced at varying levels in a cell-specific manner [311, 312]. In this regard, the characterization of major NAE species that are present in BTHS B-lymphocytes would likely provide valuable insight for future therapeutic applications within these cells.

Perhaps equally as exciting as the finding that exogenous OEA treatment can increase CL content in BTHS B-lymphocytes is the observation that it can also partially rescue the growth deficiency that is observed in these cells. While BTHS is highly variable in both its clinical presentation and progression [195], a common feature of this disease is growth delay [199], especially during early childhood [202, 313]. This deficit was easily observed after 48 hours of growth by comparing the number of vehicle-treated or OEA-treated control cells to the number of vehicle-treated BTHS B-lymphocytes, which was almost half, despite equivalent initial (day 0) seeding densities. Treatment of BTHS B-lymphocytes with OEA caused a striking improvement in cell proliferation by ~44%, such that the number of treated BTHS cells was not significantly different from the number of treated or untreated controls.

Thus, OEA represents an exciting therapeutic option for the treatment of BTHS that may help to ameliorate the CL deficits and growth deficiency that is present in these individuals, and that can contribute to serious adverse health effects including neutropenia. Interestingly, OEA treatment only restored both CL content and cellular growth to approximately 80-85% of the levels that are seen in healthy untreated controls, respectively. While these data are exciting, it is unclear why OEA treatment does not result in a full rescue of CL content or growth. While I had previously observed a significant upregulation of the mRNA content of genes involved in phospholipid biosynthesis in HEK-293 cells overexpressing HRASLS1, the same effects were not observed in B-lymphocytes treated with OEA (again supporting the idea that increased OEA does not regulate effects on CL observed in HRASLS1-overexpressing cells). Indeed, there were no significant differences between OEA-treated control and BTHS B-lymphocytes in the mRNA expression of almost all Kennedy Pathway or CL remodeling genes, with the exception of *AGPAT4*. This may suggest that OEA does not induce a significant increase in the *de novo* biosynthesis of CL.

However, it is interesting that only a single *AGPAT* is upregulated following OEA treatment, since previous work from our laboratory has suggested that *AGPAT* enzymes may produce individual pools of PA that are shunted into the synthesis of specific downstream phospholipids [3, 4, 314, 315]. Thus, the upregulation of *AGPAT4* in BTHS B-lymphocytes following OEA treatment may be the factor that leads to increased CL content in these cells. Further work, however, is clearly needed to properly examine what role, if any, *AGPAT4* plays in BTHS B-lymphocytes.

CL isolated from BTHS B-lymphocytes has a substantial deficiency in 18:2n-6. Treatment with OEA resulted in a slight, but not statistically significant increase in the relative content of linoleate in BTHS cells, as compared to BTHS cells treated with vehicle alone. However, linoleate in BTHS CL was only observed at a third of the level that is normally found in healthy control cells. This suggests that the incomplete rescue in cellular growth may be due to an increased total CL content that could not fully compensate functionally, since it was not appropriately enriched in sufficient linoleate. This contention is supported by a prior case study performed by Bowron *et al*. (2015), which identified seven BTHS patients from three unrelated families, whose linoleate-enriched CL was present within the control range [316]. These individuals present with a mild form of BTHS. Most have excellent exercise tolerance that is atypical in BTHS [151, 317], and two individuals are even asymptomatic [316]. Thus, CL acyl chain remodeling with linoleate may not be necessarily required to improve growth in BTHS B-lymphocytes, but may be required for fully functional compensation. Further work is clearly needed to substantiate this notion.

Conclusion

In conclusion, the current study has demonstrated that treatment with OEA may improve major biochemical and functional defects stemming from Taz-deficiency in BTHS B-lymphocytes. Specifically, OEA treatment was found to partially rescue CL content and cellular proliferation in BTHS B-lymphocytes relative to B-lymphocytes from a healthy matched donor. While OEA did not cause any major differences in the mRNA expression of genes specifically involved in CL biosynthesis, CL content was higher in these cells. The content of linoleate in CL was not significantly increased by OEA treatment of BTHS cells, suggesting that at least in B-lymphocytes, linoleate-enriched CL may not be required for cells to derive a functional benefit, such as enhanced growth capacity. Clearly, future studies are warranted to elucidate the exact mechanisms by which OEA treatment can increase CL content and cellular growth in these cells. In addition, the analysis of major NAE species present in BTHS B-lymphocytes may lead to the identification of other NAE species, that may result in a more complete restoration of CL content and cellular growth. Nonetheless, these findings provide exciting initial data supporting investigation of the use of NAEs in the treatment of BTHS and other metabolic disorders that feature dysregulated CL metabolism.

Chapter Seven
Thesis Summary, Integrated Discussion, and Future Perspectives

Thesis Summary

The major objective of this thesis was to conduct an initial characterization of the role of HRASLS1 in cellular physiology and metabolism, and to extend understanding of the role of HRASLS1 in phospholipid metabolism. While HRASLS1 has previously been shown to participate directly in the synthesis of PC and PE [35], no study had investigated its ability to synthesize other phospholipids, including CL. I hypothesized that since HRASLS1 can act as a PC O-transacylase by transferring a fatty acyl from the *sn-1* position (and, to a lesser extent, the *sn-2* position) from either PC or PE to LPC, it may also be able to function as a PC:PL O-transacylase in the synthesis of other phospholipids that had not yet been tested.

I found that crude lysates harvested from HEK-293 cells adenovirally transduced with HRASLS1 for 48 hours developed an increased ability to acylate MLCL, DLCL, and LPI when radiolabeled oleoyl-CoA was added as the acyl-donor source. However, based on extensive prior biochemical and molecular studies [45, 46, 53], including X-ray crystallographic characterization of the catalytic domain of homologous enzymes[18, 51], it seemed highly unlikely that the catalytic action of HRASLS1 would be acyl-CoA-dependent. Rather, HRASLS1 is expected to use a phospholipid such as PC as an acyl donor in transacylase reactions. A study with reticulocyte lysates confirmed that acyl-CoA-dependent activity of HRASLS1 is absent when the enzyme is produced through *in vitro* transcription/translation in a system that lacks a nucleus. Importantly, the discrepancy between activity in crude lysates and reticulocyte lysates overexpressing HRASLS1 strongly suggested that this enzyme may function to increase CL synthesis in cells (i.e. *in vivo*) through the activation of alternate cellular pathways, including transcription.

Investigation of a direct PC:MLCL O-transacylase activity for HRASLS1 in a pilot study using the reticulocyte lysate expression system yielded promising results with two PC acyl donors species, that will need to be confirmed with additional samples. This finding suggests that HRASLS1 may function similar to the CL remodeling enzyme Tafazzin [118]. However, while my results tentatively suggest that HRASLS1 may have some specificity for 18-carbon fatty acyl chains at the *sn-1* position of PC, it is not yet known if this enzyme has a preference for linoleate. This should also be tested directly. It is of interest that, similar to Tafazzin, HRASLS1 also shows high levels of expression in the heart and brain. Interestingly, however, although HRASLS1 has a

putative role in the synthesis of CL, a mitochondrial phospholipid, it localizes endogenously to the endoplasmic reticulum. This is not the first enzyme involved in cardiolipin metabolism to localize to the ER, as the CL remodeling enzyme ALCAT1 was shown to localize there by Cao *et al.* in 2004 [121].

The next major objective of this thesis was to investigate the role of HRASLS1 in cells. Following overexpression of HRASLS1 in HEK-293 cells, I observed an ~50% increase in endogenous CL content. The CL produced from HRASLS1 overexpression was enriched in SFA, which I hypothesized may have a detrimental effect on mitochondrial function. This hypothesis was substantiated. Mitochondria isolated from HEK-293 cells respired significantly less than control mitochondria, although the content of mitochondrial DNA was found to be the same, strongly suggesting that mitochondria containing SFA-enriched CL are present in similar numbers, but are less functional.

Although only the increase in CL reached significance, all groups of phospholipids appeared to increase somewhat in cells overexpressing HRASLS1 for 48 hours. This suggested that HRASLS1 may act to promote CL synthesis both directly as a PC:MLCL (or PC:DLCL) O-transacylase, and indirectly through the regulation of cell signaling affecting phospholipid pathways associated with CL production. Previous work performed by Uyama *et al*. (2012) demonstrated that HRASLS1 has significant PC:PE N-transacylase activity *in vivo*. I therefore hypothesized that HRASLS1 may exert indirect effects on CL metabolism through the increased production of NAPE and, consequently, NAE, which would signal for the cell to increase CL synthesis. I expected that HRASLS1 overexpression would cause an increase in the mRNA expression of enzymes involved in CL synthesis and remodeling, namely *CLS*, *TAZ*, *ALCAT1*, and *αTFP*. While this was observed, I also found that overexpression of HRASLS1 in HEK-293 cells caused a significant increase in the mRNA expression of the majority of Kennedy Pathway genes. These data supported the notion that HRASLS1 was likely exerting at least some of its effects on CL via an indirect signaling mechanism.

Based on the known N-transacylase activity of HRASLS1, it was hypothesized that changes in the cellular concentrations of specific NAEs were most likely responsible for this signaling. NAEs are present at exceedingly low levels, and unfortunately, we do not currently have the capacity to identify them at the University of Waterloo. A collaborative investigation with

Dr. Richard Bazinet's lab at the University of Toronto was initiated to identify these changes. However, these data were only completed a few weeks ago. Thus, studies on the effects of NAEs were conducted with candidate species that were predicted (but not known) to increase with HRASLS1 activity. Three of the most abundant NAE species were selected for testing. HRASLS1 is reported to show some preference for activity at the *sn-1* position of PC, that is typically enriched in SFA and MUFA. I therefore tested the effect of PEA, OEA, and SEA treatment of HEK-293 cells on CL levels. Unfortunately, later data from the Bazinet Lab indicated that PEA and OEA were not increased, and instead *decreased* significantly in HRASLS1-overexpressing HEK-293 cells, and SEA was undetected in most samples. It was therefore not surprising that these NAE species had no significant effect on CL synthesis in HEK-293 cells. Of interest, one species of NAE, LEA was slightly increased, although the assayed concentration was variable, and levels were not significantly different from control. It is therefore suggested that the LEA content of HRASLS1-overexpressing cells be analyzed in a larger sample size to decrease variability in means, and also that LEA is tested for effects on CL production in HEK-293 cells.

At the same time as I was investigating the ability of NAE treatment to increase CL in HEK-293 cells, I performed a parallel set of experiments with OEA treatment only, using B-lymphocytes taken from a healthy control, and an individual suffering from BTHS. These studies formed the third major study in this thesis. Although OEA treatment did not increase CL synthesis in HEK-293 cells, it increased the CL content in BTHS B-lymphocytes, partially rescuing the CL deficit that is present in cells of individuals afflicted with BTHS [174]. This finding is in contrast to our previous finding that NAE treatment does not increase CL in HEK-293 cells, suggesting either a different function for OEA in different cell types, or that OEA effects on CL synthesis may be more effective when a CL deficiency is present. Regardless, the functional consequences of increasing CL synthesis were significant. Perhaps the most exciting finding from this study is that treatment with OEA improved cellular growth of BTHS B-lymphocytes, matching nearly 85% of the growth observed in healthy controls. These data therefore show that NAE treatment is effective in partially restoring both CL content and cellular growth, to a similar extent, in B-lymphocytes from an individual with BTHS. A summary of the key findings and novel contributions of each thesis study is shown in Table 6.

Table 6: Summary of key findings and novel contributions

Thesis Study I: Cellular and biochemical characterization of HRASLS1	
Key Findings	• HRASLS1 is ubiquitously expressed, but has high expression in the heart and brain • HRASLS1 localizes to the ER, and not the mitochondria, nor the MAM • HRASLS1 overexpression in cells for 48 hours results in increased oleoyl-CoA-dependent O-acyltransferase activity with MLCL, DLCL, and LPI as acyl acceptors, and also increased PC:MLCL O-transacylase activity in lysates • HRASLS1 likely has di-oleoyl PC:MLCL transacylase activity *in vitro*
Conclusions and Novel Contributions	• A novel role for HRASLS1 in cardiolipin metabolism has been discovered • Since significant oleoyl-CoA-dependent O-acyltransferase activity was only observed in crude lysates and not reticulocyte lysates, data suggests that the N-acyltransferase activity of HRASLS1 may play an important role in CL synthesis.
Thesis Study II: Investigation of the *in vivo* role of HRASLS1 in cardiolipin metabolism	
Key Findings	• HRASLS1 overexpression in HEK-293 cells increases total CL content • CL produced by HRASLS1 overexpression is enriched in SFA • Mitochondrial respiration is impaired in HEK-293 cells overexpressing HRASLS1, but content of mtDNA is not different • Overexpression of HRASLS1 in HEK-293 cells causes a significant upregulation in the expression of Kennedy Pathway genes. These data suggest that HRASLS1 may mediate CL metabolism through the generation of NAPE, and therefore downstream NAE. • The mRNA content of PPARα and TRPV1, targets of NAE signaling, are increased following HRASLS1 overexpression in HEK-293 cells. • 48-hour treatment of HEK-293 cells with 0.1, 1, or 10 μM of PEA, OEA, or SEA does not increase endogenous CL content • Overexpression of HRASLS1 in HEK-293 cells causes a significant decrease in the content of OEA and PEA, with a slight, but not statistically significant increase in LEA
Conclusions and Novel Contributions	• Discovery that HRASLS1 overexpression regulates CL metabolism *in vivo* • Overexpression of HRASLS1 significantly upregulates mRNA expression of genes involved in phospholipid biosynthesis and in CL metabolism • Overexpression of HRASLS1 alters the NAE profile of HEK-293 cells
Thesis Study III: Effects of OEA on CL metabolism and growth of Cultured B-Lymphocytes from an Individual with Barth Syndrome	
Key Findings	• OEA treatment partially rescues CL content and cellular proliferation in BTHS B-lymphocytes
Conclusions and Novel Contributions	• NAE treatment may be a therapeutic option to restore CL deficits in CL deficiency diseases

Integrated Discussion and Future Perspectives

The results from this thesis have identified HRASLS1 as potentially the first new CL remodeling enzyme that has been discovered in over a decade [121]. Prior to this finding, only four enzymes were known to participate directly in CL remodeling including *TAZ*, *ALCAT1*, *MLCL-AT1*, and *αTFP* [262]. As such, HRASLS1 will likely be the subject of additional studies. Although this thesis tested the enzymatic activity for HRASLS1 in the synthesis of CL, because of the small sample size in the reticulocyte lysate work, it is still unclear if it has both a direct PC:MLCL O-transacylase, and indirect (via NAE production) role in this process. Experiments from this thesis demonstrated that HRASLS1 overexpression for 48 hours induces oleoyl-CoA-dependent MLCL, DLCL, and LPI O-acyltransferase activity in crude lysates produced from those cells. However, this activity was not detected in reticulocyte lysates, indicating that HRASLS1 is unable to catalyze this reaction directly. Rather, it is most likely that HRASLS1 mediates at least some of its effects on CL metabolism through the generation of NAPE, and NAE. The finding of a general induction of lipid biosynthetic genes in cells overexpressing HRASLS1 supports this. It is important, nonetheless, to either include or exclude a direct role for HRASLS1 in the catalysis of CL, and therefore additional study of HRASLS1 PC:MLCL O-transacylase activity in reticulocyte lysates is critical.

Future studies should seek to further investigate the physiological role of HRASLS1 in the context of CL metabolism. In this sense, the generation of either *Hrasls1*$^{-/+}$ and/or *Hrasls1*$^{-/-}$ mice would be invaluable. While I observed that HRASLS1 overexpression increases CL content in HEK-293 cells *in vivo*, it would be interesting to see if these effects are conserved in an animal model as well. Previous studies, including a relatively recent investigation by Ikon *et al*. (2015), have shown that CL content modulation is possible in cultured cells [310], but may not be conserved in more complex models, such as mice [309]. In this regard, the generation of a liver-specific *Hrasls1* transgenic mouse would likely provide rapid insight into this question, and could be readily achieved through tail-vein injections of purified adenoviral HRASLS1 into C57BL/6J mice.

Interestingly, this thesis demonstrated that HRASLS1 overexpression produces CL that is enriched in SFA. It is not known whether this indicates substrate preference by HRASLS1, either for SFA specifically, or for transacylase activity at the *sn-1* position, which is typically enriched in SFA, or if it merely reflects the increased synthesis of immature (*i.e.* under-remodeled) CL

through the 'indirect' induction of phospholipid and CL synthesis genes, mediated by HRASLS1-NAPE-NAE signaling. Regardless, an important insight has been generated from this finding, wherein it was demonstrated that mitochondrial respiration is impaired in HEK-293 cells that contain SFA-enriched CL. While this finding in an isolated system is of interest, the physiological consequences bear further investigation. I hypothesized that since SFA are generally less susceptible to peroxidation than MUFA or PUFA, the production of SFA-enriched CL by HRASLS1 may, under certain conditions, and in some tissues, be partially protective against ROS attack. Thus, this altered CL profile may confer a benefit in specific instances, although the increased content of SFA in CL likely provides an unfavourable typical environment for ATP production, and cellular function as a whole. Future work is warranted to understand the physiological consequences stemming from production of CL enriched in SFA, and the role of HRASLS1 in this process in specific tissues.

Future work on the role and regulation of HRASLS1 in CL deficiency disorders is also merited. In certain disease states, such as BTHS, the increase of CL content that may be mediated by HRASLS1 overexpression could provide an overall benefit to the cell that outweighs the potential impairments in optimal cellular function due to enrichment with SFA. While the loss of linoleate-containing CL has been shown to be critical in the development of BTHS [174], an overall increase in total CL content may reduce the severity of the disease. In this sense, the generation of *Taz*$^{-/-}$/*Hrasls1-transgenic* mice will likely provide valuable insights.

An important finding from this thesis is that exogenous OEA treatment can partially rescue CL content and cellular proliferation in BTHS B-lymphocytes. To the best of my knowledge, this is the first time NAEs have been demonstrated to play a role in CL metabolism. While future work should address if alternative NAE species such as LEA may cause a greater effect, the notion that exogenous NAEs can be used as therapeutics in BTHS is exciting nonetheless. Currently, there is no known cure for BTHS, and treatment is often restricted to symptom management [318]. While progress is being made in the development of ERT and rAAV-mediated gene therapies for use in BTHS, no commercial therapies are available on the market. In addition, both ERT and rAAV-mediated gene therapy are extremely costly therapeutic options. A report by van Dussen *et al.* estimated that ERT typically averages a cost upwards of ~$250,000 USD per patient, per year for treatment of type 1 Gaucher disease [319], and Rombach *et al.* have estimated that ERT for Fabry disease can cost approximately $115,000 – $200,000 USD per patient, per year [320].

Thus, the realization that exogenous NAE treatment can partially rescue CL content and cellular growth in BTHS is exciting, as NAEs are currently utilized for several pharmacological therapies, and are widely available at a significantly lower cost than ERT. Historically, pharmacological agents that have targeted the endocannaboid system have been formulated to inhibit the CB1 receptor in an attempt to restrict food intake for the treatment of obesity, and obesity-related disorders [298, 321]. A well-known pharmacological anorectic agent that was developed was known as Rimonabant [321], and while this drug showed promising results in reducing obesity and improving cardio-metabolic outcomes in overweight and obese individuals [322], its use has been banned in several countries due to adverse psychiatric side-effects including anxiety and depression [298, 323]. Consequently, research has focused on the use of exogenously-administered NAEs that do not inhibit the CB1 receptor [298]. Perhaps the most well-studied is PEA, which when administered exogenously has demonstrated therapeutic effects in a wide variety of disorders including multiple sclerosis [324], eczema [325], and resolving inflammation and providing chronic pain relief [326-328]. Currently, OEA is under investigation for its anorectic effects as a therapy for obesity [298], although it may mediate beneficial effects on atherosclerosis as well [329]. However, precaution should be exercised before implementing OEA treatment in BTHS, as its anorectic effects may pose a serious risk to BTHS patients, who already have difficulties in eating [318, 330, 331]. OEA may therefore be a beneficial therapy for BTHS if it is used in combination with a pharmacological agent that could mediate its anorectic effects. In order to better understand the mechanism by which OEA exerts its effects in restoring CL content and cell growth in BTHS, future studies should investigate oral supplementation of exogenous NAE in a mouse model of BTHS, namely, TAZ knockdown (KD) or knockout (KO) mice [151, 332]. Both the TAZ-KD and TAZ-KO mice display aberrant CL metabolism, along with cardiac and skeletal muscle defects, recapitulating the biochemical and physiological deficiencies that are observed in human BTHS [151, 332, 333]. Identification of decreased NAE species found in the hearts and skeletal muscle of BTHS patients or TAZ-deficient mice may be helpful in guiding the selection of candidate NAEs with the greatest likelihood of therapeutic benefit.

The use of exogenous NAE may also have therapeutic applicability beyond the treatment of BTHS. Although this thesis only examined the use of NAE in the treatment of B-Lymphocytes from a subject with BTHS, several disorders are associated with impairments in CL metabolism including, but not limited to, diabetes [136, 334], obesity [334], cardiovascular disorders [136,

334], neurodegeneration [154, 212], and cancers [229, 231, 335]. In addition, major alterations are known to occur in CL metabolism, even in the absence of overt disease throughout the normal course of aging [336-338]. Thus, future studies should investigate the utility of exogenous NAE supplementation in treatment of these disorders, and perhaps also as a preventative measure to maintain optimal CL metabolism, prior to the onset of disease.

There are several strengths to the studies that have been performed in this thesis. First, by testing multiple substrates the *in vitro* investigation of HRASLS1 activity identified a novel role for the enzyme in increasing the capacity of cells to catalyze the oleoyl-CoA-dependent acylation of MLCL, DLCL, and LPI, as well as PC:MLCL O-transacylation. Enzyme assays performed in reticulocyte lysates narrowed the MLCL acylation activity of HRASLS1 to, likely PC:MLCL O-transacylase activity, and excluded oleoyl-CoA-dependent acylation as a direct function of this enzyme. Next, transacylase experiments utilized three different species of PC, and demonstrated that HRASLS1 likely prefers the *sn-1* position, and likely shows acyl specificity for 18-carbon fatty acids. Prior to this work, only PC and PE had been investigated as possible acyl acceptors [35]. These data will help inform the design of future studies to investigate the substrate-specificity of this enzyme. In addition, the *in vivo* investigations characterized most genes involved in phospholipid, and cardiolipin biosynthesis. This is significant. The Kennedy Pathway represents a complex series of acylation and de-acylation events catalyzed by at least thirteen distinct enzymes, with an additional four enzymes involved in CL acyl chain remodeling. The extent of characterization of the mRNA expression of HEK-293 cells overexpressing HRASLS1, as well as treated and untreated control and BTHS B-lymphocytes, helps to illustrate cellular phospholipid synthesis in these cells. The identification of the alterations in NAE content following HRASLS1 overexpression also provide important information for future studies. Finally, in any experiment assessing the effects of exogenous NAE treatment, charcoal-stripped FBS was used in order to remove exogenous NAEs in serum, which could confound the results.

However, this thesis is not without its limitations as well. Studies II and III utilized cultured cells as an *in vivo* measurement of CL content and cellular growth. While cultured cells are more widely available and less expensive than animal models, future studies should confirm these findings in mice, and if successful, eventually in humans as well. Generation of a liver-specific transgenic model of HRASLS1 overexpression using available adenovirus would potentially confirm the findings in Study II, while utilization of either TAZ-KD or TAZ-KO mice would

confirm the findings in Study III. Next, while data demonstrating that mitochondrial respiration is impaired in HRASLS1-overexpressing HEK-293 cells provides novel insights, the respiration protocol was not performed with ETC-complex inhibitors. Performing a substrate-uncoupler-inhibitor titration (SUIT) procedure, as previously described [2, 252] would allow for assessment of possible alterations in individual ETC complexes. Finally, NAE experiments in HEK-293 cells were performed by adding exogenous PEA, OEA, and SEA. Unfortunately, these turned out to be a poor choice, since these NAEs were actually decreased (or undetected, in the case of SEA) following HRASLS1-overexpression in these cells. Due to differences in experimental timing with my collaborators, I was unaware of this fact while performing these experiments. Nevertheless, if possible, future experiments should investigate changes in the NAE profile of the cell culture or animal model being utilized, prior to assessing if certain exogenously-added NAE species have any effects.

Thus, this thesis expands on the current literature by demonstrating for the first time that HRASLS1 plays indirect and, likely, direct roles in cellular CL metabolism. Importantly, the concept of multiple synergistic enzymatic activities falling on a single pathway is novel and should be further delineated. Although the exact mechanism by which HRASLS1 exerts its effects are still unknown, it is likely that it produces NAPEs, and by extension NAEs that signal for increases in phospholipid synthesis. Future work with NAE-receptor antagonists will be important to test this directly, and the discovery of this novel function will likely be the focus of several fundamental studies to follow. Furthermore, this thesis shows that treatment with OEA can partially rescue CL content and cellular growth in BTHS B-lymphocytes. Consequently, this work now provides an avenue for future studies to examine the use of exogenous NAEs for the treatment and prevention of disorders associated with CL deficiency.

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Appendices

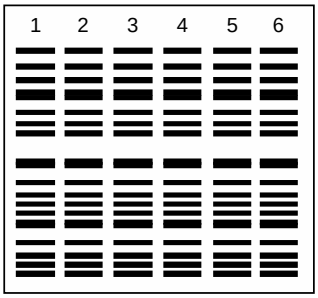
Appendix A: Schematic of determining relative protein concentrations in protein generated from the TnT® Coupled Reticulocyte Lysate System

Method: Determining relative protein concentrations betweeen reticulocyte lysate samples

1

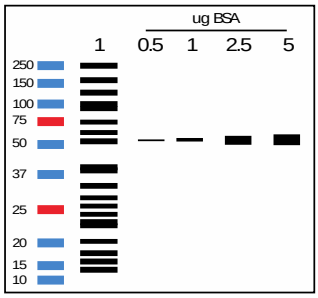
- produce reticulocyte lysates
- load 1ul of each sample into a stain-free acrylamide gel, and electrophorese gel

2



- quantify total protein per lane
- normalize all samples to the sample in lane 1
- this will give **normalization values**

3



- load 1 ul of sample 1 in a stain-free acrylamide gel
- load increasing concentrations of BSA as well
- electrophorese, quantify total protein/lane of sample 1, and quantify total protein content of BSA bands

4 Perform calculations:

First, to get protein concentration of sample 1:

$$\frac{\text{[total protein/lane of sample 1]}}{\text{[total protein of 1ug BSA band]}}$$

this will give the concentration of sample 1 in ug/uL

Next, multiply the concentration of sample 1 (ug/ul) by the normalization factors that have been obtained in step **2**

-- This method will give relative protein concentrations between samples --

Appendix Figure 1: Schematic to determine relative protein concentrations from reticulocyte lysates.

Appendix B: Sequence alignment of Hrasls1 long and short transcript variants

Protein alignment:

```
Hrasls1_1L      MPEACLEDLGGGEGAGLGRAPKTRGRRQGPQDAVWQARARNPRRDQRWRGARTLALTQRR   60
Hrasls1_1SA     ------------------------------------------------------------   0
Hrasls1_1SB     ------------------------------------------------------------   0

Hrasls1_1L      EAVVALAVALASGRCGWSHPGSASGTAASPWRTREARHCLQGTKTTQLEQMAVNDCFSLT   120
Hrasls1_1SA     ----------------------------------------------MAVNDCFSLT   10
Hrasls1_1SB     ----------------------------------------------MAVNDCFSLT   10
                                                               **********

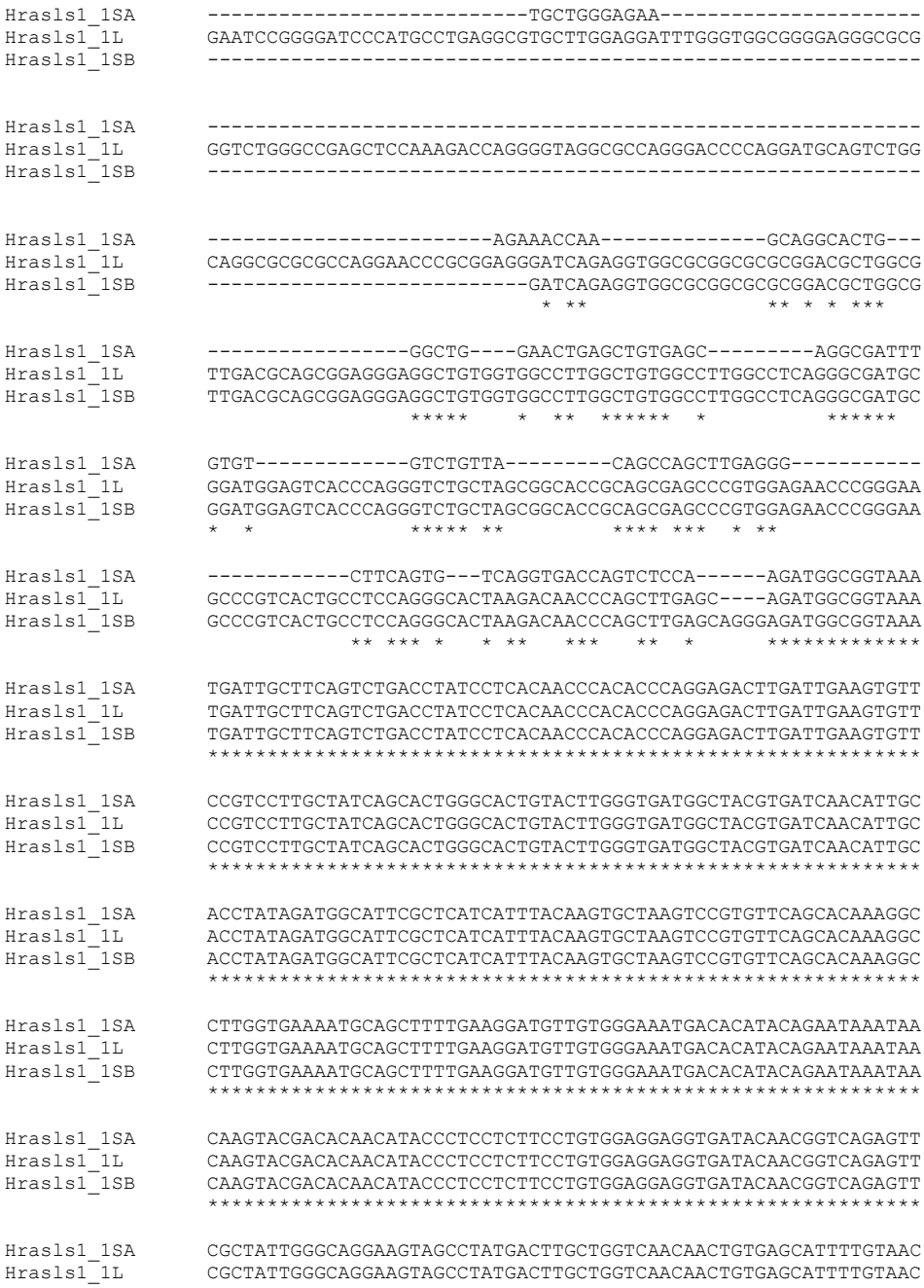
Hrasls1_1L      YPHNPHPGDLIEVFRPCYQHWALYLGDGYVINIAPIDGIRSSFTSAKSVFSTKALVKMQL   180
Hrasls1_1SA     YPHNPHPGDLIEVFRPCYQHWALYLGDGYVINIAPIDGIRSSFTSAKSVFSTKALVKMQL   70
Hrasls1_1SB     YPHNPHPGDLIEVFRPCYQHWALYLGDGYVINIAPIDGIRSSFTSAKSVFSTKALVKMQL   70
                ************************************************************

Hrasls1_1L      LKDVVGNDTYRINNKYDTTYPPLPVEEVIQRSEFAIGQEVAYDLLVNNCEHFVTLLRYGE   240
Hrasls1_1SA     LKDVVGNDTYRINNKYDTTYPPLPVEEVIQRSEFAIGQEVAYDLLVNNCEHFVTLLRYGE   130
Hrasls1_1SB     LKDVVGNDTYRINNKYDTTYPPLPVEEVIQRSEFAIGQEVAYDLLVNNCEHFVTLLRYGE   130
                ************************************************************

Hrasls1_1L      GVSEQANRAIGTIGLVAAGIDIFTFLGLFPKRQRTKY  277
Hrasls1_1SA     GVSEQANRAIGTIGLVAAGIDIFTFLGLFPKRQRTKY  167
Hrasls1_1SB     GVSEQANRAIGTIGLVAAGIDIFTFLGLFPKRQRTKY  167
```

Appendix Figure 2: Protein alignment of long and short Hrasls1 transcript variants

Nucleotide alignment:



Appendix Figure 3: Nucleotide alignment of long and short Hrasls1 transcript variants.

```
Hrasls1_1SB      CGCTATTGGGCAGGAAGTAGCCTATGACTTGCTGGTCAACAACTGTGAGCATTTTGTAAC
                 ************************************************************

Hrasls1_1SA      CTTGCTGCGCTATGGAGAAGGAGTGTCAGAGCAGGCCAACCGAGCAATCGGCACCATCGG
Hrasls1_1L       CTTGCTGCGCTATGGAGAAGGAGTGTCAGAGCAGGCCAACCGAGCAATCGGCACCATCGG
Hrasls1_1SB      CTTGCTGCGCTATGGAGAAGGAGTGTCAGAGCAGGCCAACCGAGCAATCGGCACCATCGG
                 ************************************************************

Hrasls1_1SA      ATTGGTGGCAGCTGGTATTGATATCTTCACATTCCTCGGCTTGTTTCCCAAAAGACAAAG
Hrasls1_1L       ATTGGTGGCAGCTGGTATTGATATCTTCACATTCCTCGGCTTGTTTCCCAAAAGACAAAG
Hrasls1_1SB      ATTGGTGGCAGCTGGTATTGATATCTTCACATTCCTCGGCTTGTTTCCCAAAAGACAAAG
                 ************************************************************

Hrasls1_1SA      AACGAAATATTAGCAGTTTATTGAAGAGATTGGAATGAAGAAATTTGTGAGGAGAAAAAA
Hrasls1_1L       AACGAAATATTAGCAGTTTATTGAAGAGATTGGAATGAAGAAATTTGTGAGGAGAAAAAA
Hrasls1_1SB      AACGAAATATTAGCAGTTTATTGAAGAGATTGGAATGAAGAAATTTGTGAGGAGAAAAAA
                 ************************************************************

Hrasls1_1SA      AAATCCTAGGGTGAATACTTATTTTGAATGCATCATTATTGCTCATGGTCCCCATGATGG
Hrasls1_1L       AAATCCTAGGGTGAATACTTATTTTGAATGCATCATTATTGCTCATGGTCCCCATGATGG
Hrasls1_1SB      AAATCCTAGGGTGAATACTTATTTTGAATGCATCATTATTGCTCATGGTCCCCATGATGG
                 ************************************************************

Hrasls1_1SA      ATGGCAGACTCTGTAATAAATTGCTTGCTGATTTTAATCTTATCATTGAGCCAAGAAAAG
Hrasls1_1L       ATGGCAGACTCTGTAATAAATTGCTTGCTGATTTTAATCTTATCATTGAGCCAAGAAAAG
Hrasls1_1SB      ATGGCAGACTCTGTAATAAATTGCTTGCTGATTTTAATCTTATCATTGAGCCAAGAAAAG
                 ************************************************************

Hrasls1_1SA      TTTTTCCCAACTAGCAGAGATTTGCCGTGGCAGCTTGAACAAAATGCAATTGCCTTTTGA
Hrasls1_1L       TTTTTCCCAACTAGCAGAGATTTGCCGTGGCAGCTTGAACAAAATGCAATTGCCTTTTGA
Hrasls1_1SB      TTTTTCCCAACTAGCAGAGATTTGCCGTGGCAGCTTGAACAAAATGCAATTGCCTTTTGA
                 ************************************************************

Hrasls1_1SA      TCGAGCCAGCTGAGGATCTTAACAGAACCAAGACCACATTTTATCTTCTGCTGTAAATAT
Hrasls1_1L       TCGAGCCAGCTGAGGATCTTAACAGAACCAAGACCACATTTTATCTTCTGCTGTAAATAT
Hrasls1_1SB      TCGAGCCAGCTGAGGATCTTAACAGAACCAAGACCACATTTTATCTTCTGCTGTAAATAT
                 ************************************************************

Hrasls1_1SA      TGTTTTCCTTTTCCCTAAGGACAGCTGTTTTGCCAGAGGTGTGGAGAACCATTTGCATAC
Hrasls1_1L       TGTTTTCCTTTTCCCTAAGGACAGCTGTTTTGCCAGAGGTGTGGAGAACCATTTGCATAC
Hrasls1_1SB      TGTTTTCCTTTTCCCTAAGGACAGCTGTTTTGCCAGAGGTGTGGAGAACCATTTGCATAC
                 ************************************************************

Hrasls1_1SA      ACTGCTGAGAGACAGTTGTTAGGGCCAACATCTAAATTCCTTTTGCTTTCTTTGTCAGAA
Hrasls1_1L       ACTGCTGAGAGACAGTTGTTAGGGCCAACATCTAAATTCCTTTTGCTTTCTTTGTCAGAA
Hrasls1_1SB      ACTGCTGAGAGACAGTTGTTAGGGCCAACATCTAAATTCCTTTTGCTTTCTTTGTCAGAA
                 ************************************************************

Hrasls1_1SA      AAAGGAGCGTGAACATATCCAGTAGTTTGGATACATGGATATATATCCATGATATATCCA
Hrasls1_1L       AAAGGAGCGTGAACATATCCAGTAGTTTGGATACATGGATATATATCCATGATATATCCA
Hrasls1_1SB      AAAGGAGCGTGAACATATCCAGTAGTTTGGATACATGGATATATATCCATGATATATCCA
                 ************************************************************

Hrasls1_1SA      AGTACTTAAGGATCTCTGCATGACAAAGCATTTGAGGTGGTACCCCCATATTGCTGCACC
Hrasls1_1L       AGTACTTAAGGATCTCTGCATGACAAAGCATTTGAGGTGGTACCCCCATATTGCTGCACC
Hrasls1_1SB      AGTACTTAAGGATCTCTGCATGACAAAGCATTTGAGGTGGTACCCCCATATTGCTGCACC
                 ************************************************************
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Hrasls1_1SA      TGTGTGGGTGGAGCATGTAAGAAGGAGTGGGGGACTTTTGTGTGTGCATATGTATGGAAT
Hrasls1_1L       TGTGTGGGTGGAGCATGTAAGAAGGAGTGGGGGACTTTTGTGTGTGCATATGTATGGAAT
Hrasls1_1SB      TGTGTGGGTGGAGCATGTAAGAAGGAGTGGGGGACTTTTGTGTGTGCATATGTATGGAAT
                 ************************************************************

Hrasls1_1SA      TCTTATGAATGGTGTGCATGTATAGGGACGGTCTTGGTCTCACCTTTTTTATTCTTATTT
Hrasls1_1L       TCTTATGAATGGTGTGCATGTATAGGGACGGTCTTGGTCTCACCTTTTTTATTCTTATTT
Hrasls1_1SB      TCTTATGAATGGTGTGCATGTATAGGGACGGTCTTGGTCTCACCTTTTTTATTCTTATTT
                 ************************************************************

Hrasls1_1SA      TATTCTTATTCTTATTTTATTTTATAATTTATAAAAGGAGACAGGGTATGCTGTCCTTTA
Hrasls1_1L       TATTCTTATTCTTATTTTATTTTATAATTTATAAAAGGAGACAGGGTATGCTGTCCTTTA
Hrasls1_1SB      TATTCTTATTCTTATTTTATTTTATAATTTATAAAAGGAGACAGGGTATGCTGTCCTTTA
                 ************************************************************

Hrasls1_1SA      TAGCTTATGGCAAACTAGCTAGCCTGTGGGCATCCAGAGAGTCTCCTGTCTCTGCCTCCA
Hrasls1_1L       TAGCTTATGGCAAACTAGCTAGCCTGTGGGCATCCAGAGAGTCTCCTGTCTCTGCCTCCA
Hrasls1_1SB      TAGCTTATGGCAAACTAGCTAGCCTGTGGGCATCCAGAGAGTCTCCTGTCTCTGCCTCCA
                 ************************************************************

Hrasls1_1SA      TTTCACGGTAGGAGTTCTGGAGTTACCGAAGTGAACTAACAACCCAAGCTTTCTGTGGAC
Hrasls1_1L       TTTCACGGTAGGAGTTCTGGAGTTACCGAAGTGAACTAACAACCCAAGCTTTCTGTGGAC
Hrasls1_1SB      TTTCACGGTAGGAGTTCTGGAGTTACCGAAGTGAACTAACAACCCAAGCTTTCTGTGGAC
                 ************************************************************

Hrasls1_1SA      TCTGGGGATCTGAGCTCAAGACATCATGCTTATGTTTAAGATGCTTTACCCACTGAGCCC
Hrasls1_1L       TCTGGGGATCTGAGCTCAAGACATCATGCTTATGTTTAAGATGCTTTACCCACTGAGCCC
Hrasls1_1SB      TCTGGGGATCTGAGCTCAAGACATCATGCTTATGTTTAAGATGCTTTACCCACTGAGCCC
                 ************************************************************

Hrasls1_1SA      CTCTCCAATCTCTCTCTCTCTCTTTCTTTCTCTCTCTCTCTCCCTTGTACGTTCAGACCC
Hrasls1_1L       CTCTCCAATCTCTCTCTCTCTCTTTCTTTCTCTCTCTCTCTCCCTTGTACGTTCAGACCC
Hrasls1_1SB      CTCTCCAATCTCTCTCTCTCTCTTTCTTTCTCTCTCTCTCTCCCTTGTACGTTCAGACCC
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Hrasls1_1SA      AGTGTTCAAGACTCAGTGATGAACAATGGGCTGTCGTTTTATTGATTTAAATGGATCCCT
Hrasls1_1L       AGTGTTCAAGACTCAGTGATGAACAATGGGCTGTCGTTTTATTGATTTAAATGGATCCCT
Hrasls1_1SB      AGTGTTCAAGACTCAGTGATGAACAATGGGCTGTCGTTTTATTGATTTAAATGGATCCCT
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Hrasls1_1SA      CCACATTGTGTAGTAGCTCAGTGGTTTGACCTTTGGGGCTTGGCAAGCAATCATTAATAT
Hrasls1_1L       CCACATTGTGTAGTAGCTCAGTGGTTTGACCTTTGGGGCTTGGCAAGCAATCATTAATAT
Hrasls1_1SB      CCACATTGTGTAGTAGCTCAGTGGTTTGACCTTTGGGGCTTGGCAAGCAATCATTAATAT
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Hrasls1_1SA      TAATGCAAAGAGCTATATTTAAAACACAAAATTCCTCATTCCTTTAAAGATGTGAACTGT
Hrasls1_1L       TAATGCAAAGAGCTATATTTAAAACACAAAATTCCTCATTCCTTTAAAGATGTGAACTGT
Hrasls1_1SB      TAATGCAAAGAGCTATATTTAAAACACAAAATTCCTCATTCCTTTAAAGATGTGAACTGT
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Hrasls1_1SA      AAATCAAGGAATCCGCTTATTTTATTCTTTTATTTTACTGATTATCCGTTGAATTAAACT
Hrasls1_1L       AAATCAAGGAATCCGCTTATTTTATTCTTTTATTTTACTGATTATCCGTTGAATTAAACT
Hrasls1_1SB      AAATCAAGGAATCCGCTTATTTTATTCTTTTATTTTACTGATTATCCGTTGAATTAAACT
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Hrasls1_1SA      CCTTCTTAATGCCATTTCTGCAGAATTCTTGATAGCAGGTCAGGAGAGGTGGGACGTTAG
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Hrasls1_1L       CCTTCTTAATGCCATTTCTGCAGAATTCTTGATAGCAGGTCAGGAGAGGTGGGACGTTAG
Hrasls1_1SB      CCTTCTTAATGCCATTTCTGCAGAATTCTTGATAGCAGGTCAGGAGAGGTGGGACGTTAG
                 ************************************************************

Hrasls1_1SA      TCTTTCTACATGAAGGTAGTATAAGGGCATCATGACATTACCTCATAAACCACACAGCTA
Hrasls1_1L       TCTTTCTACATGAAGGTAGTATAAGGGCATCATGACATTACCTCATAAACCACACAGCTA
Hrasls1_1SB      TCTTTCTACATGAAGGTAGTATAAGGGCATCATGACATTACCTCATAAACCACACAGCTA
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Hrasls1_1SA      GCCCGGCATCCTTTTGTGCCATTATCAAAAGCATGTCAATCAATAAGGAAAGAGTGCCAG
Hrasls1_1L       GCCCGGCATCCTTTTGTGCCATTATCAAAAGCATGTCAATCAATAAGGAAAGAGTGCCAG
Hrasls1_1SB      GCCCGGCATCCTTTTGTGCCATTATCAAAAGCATGTCAATCAATAAGGAAAGAGTGCCAG
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Hrasls1_1SA      TGACCATGGGGATAGAACGTGATGGAGTATGGTAAAGTCAAGTGCACAAGACTCGGTGGA
Hrasls1_1L       TGACCATGGGGATAGAACGTGATGGAGTATGGTAAAGTCAAGTGCACAAGACTCGGTGGA
Hrasls1_1SB      TGACCATGGGGATAGAACGTGATGGAGTATGGTAAAGTCAAGTGCACAAGACTCGGTGGA
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Hrasls1_1SA      AAGCTCAGACCTACTTAACTGATTTGACCTTGGTGCTGGAAACACTCACACTGATGAGGG
Hrasls1_1L       AAGCTCAGACCTACTTAACTGATTTGACCTTGGTGCTGGAAACACTCACACTGATGAGGG
Hrasls1_1SB      AAGCTCAGACCTACTTAACTGATTTGACCTTGGTGCTGGAAACACTCACACTGATGAGGG
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Hrasls1_1SA      TAACAGTAATGTGAACAGAACTATGTGCTGCCACTCCCATCCAGACAGAGTCCCCTCTGT
Hrasls1_1L       TAACAGTAATGTGAACAGAACTATGTGCTGCCACTCCCATCCAGACAGAGTCCCCTCTGT
Hrasls1_1SB      TAACAGTAATGTGAACAGAACTATGTGCTGCCACTCCCATCCAGACAGAGTCCCCTCTGT
                 ************************************************************

Hrasls1_1SA      CCCTTCTTTACTTTTCTAACAGGCCCTTCTTGTCCGAACCTCCAGACTCATTCTTTAGTT
Hrasls1_1L       CCCTTCTTTACTTTTCTAACAGGCCCTTCTTGTCCGAACCTCCAGACTCATTCTTTAGTT
Hrasls1_1SB      CCCTTCTTTACTTTTCTAACAGGCCCTTCTTGTCCGAACCTCCAGACTCATTCTTTAGTT
                 ************************************************************

Hrasls1_1SA      GTCTCAGAAGGCACGAGGTTACTTTTATCCTGAATGCAGGTCTCTGTTCCCAAATAGTAA
Hrasls1_1L       GTCTCAGAAGGCACGAGGTTACTTTTATCCTGAATGCAGGTCTCTGTTCCCAAATAGTAA
Hrasls1_1SB      GTCTCAGAAGGCACGAGGTTACTTTTATCCTGAATGCAGGTCTCTGTTCCCAAATAGTAA
                 ************************************************************

Hrasls1_1SA      AGAATATTCAGTTTTCTAGAAGTTTCCTATTAAGGTAAGGGCAGCTGCAGCCTCTGGGCT
Hrasls1_1L       AGAATATTCAGTTTTCTAGAAGTTTCCTATTAAGGTAAGGGCAGCTGCAGCCTCTGGGCT
Hrasls1_1SB      AGAATATTCAGTTTTCTAGAAGTTTCCTATTAAGGTAAGGGCAGCTGCAGCCTCTGGGCT
                 ************************************************************

Hrasls1_1SA      ACTTTCCCCTGGTGTGCCACCTTCTCAATAAACTATATTTGCAGGGCATTTGTCTCTGGC
Hrasls1_1L       ACTTTCCCCTGGTGTGCCACCTTCTCAATAAACTATATTTGCAGGGCATTTGTCTCTGGC
Hrasls1_1SB      ACTTTCCCCTGGTGTGCCACCTTCTCAATAAACTATATTTGCAGGGCATTTGTCTCTGGC
                 ************************************************************

Hrasls1_1SA      AGAATTACACCGCCTGTCAGGAAAGGGACTAGGACTGAGAACCAAATGCCTCTTAGGGAT
Hrasls1_1L       AGAATTACACCGCCTGTCAGGAAAGGGACTAGGACTGAGAACCAAATGCCTCTTAGGGAT
Hrasls1_1SB      AGAATTACACCGCCTGTCAGGAAAGGGACTAGGACTGAGAACCAAATGCCTCTTAGGGAT
                 ************************************************************

Hrasls1_1SA      TCAAATGAAACCTTTCTAGAGGACTCTGACATCTCCACTCCAGAAGAAAAGCAAAATGTT
Hrasls1_1L       TCAAATGAAACCTTTCTAGAGGACTCTGACATCTCCACTCCAGAAGAAAAGCAAAATGTT
Hrasls1_1SB      TCAAATGAAACCTTTCTAGAGGACTCTGACATCTCCACTCCAGAAGAAAAGCAAAATGTT
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Hrasls1_1SA     AAAAGGAAAACGTCAGAAATGCACTTTTTAATCCATGGGTTTATTTCCTTATCAGTTATG
Hrasls1_1L      AAAAGGAAAACGTCAGAAATGCACTTTTTAATCCATGGGTTTATTTCCTTATCAGTTATG
Hrasls1_1SB     AAAAGGAAAACGTCAGAAATGCACTTTTTAATCCATGGGTTTATTTCCTTATCAGTTATG
                ************************************************************

Hrasls1_1SA     TGAAGCTTTGGCAGCCTTGGTTTGCTGTAAACATGGCCAACCTTAAAATGGGCAGCCCTA
Hrasls1_1L      TGAAGCTTTGGCAGCCTTGGTTTGCTGTAAACATGGCCAACCTTAAAATGGGCAGCCCTA
Hrasls1_1SB     TGAAGCTTTGGCAGCCTTGGTTTGCTGTAAACATGGCCAACCTTAAAATGGGCAGCCCTA
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Hrasls1_1SA     AATAGCTGTGGTTGTTTTCCTGCAGGTACTCAATACCCACACGTTCAATGCAACTTATAC
Hrasls1_1L      AATAGCTGTGGTTGTTTTCCTGCAGGTACTCAATACCCACACGTTCAATGCAACTTATAC
Hrasls1_1SB     AATAGCTGTGGTTGTTTTCCTGCAGGTACTCAATACCCACACGTTCAATGCAACTTATAC
                ************************************************************

Hrasls1_1SA     TTGGATCAGCTATTTCCCCAACACTATGAAATATTGTCTGGGCATATTTTCAGCAGTCAG
Hrasls1_1L      TTGGATCAGCTATTTCCCCAACACTATGAAATATTGTCTGGGCATATTTTCAGCAGTCAG
Hrasls1_1SB     TTGGATCAGCTATTTCCCCAACACTATGAAATATTGTCTGGGCATATTTTCAGCAGTCAG
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Hrasls1_1SA     GAATGGAGTCCACTTCTATCATGTTTCAAAAAATGGAATGTAAAGATCACCTGCATCAAG
Hrasls1_1L      GAATGGAGTCCACTTCTATCATGTTTCAAAAAATGGAATGTAAAGATCACCTGCATCAAG
Hrasls1_1SB     GAATGGAGTCCACTTCTATCATGTTTCAAAAAATGGAATGTAAAGATCACCTGCATCAAG
                ************************************************************

Hrasls1_1SA     TGATAGGTTTTTGGTAATACAAGGAAAGAAATGTCTGTATTTTTATTGAATATGTACATA
Hrasls1_1L      TGATAGGTTTTTGGTAATACAAGGAAAGAAATGTCTGTATTTTTATTGAATATGTACATA
Hrasls1_1SB     TGATAGGTTTTTGGTAATACAAGGAAAGAAATGTCTGTATTTTTATTGAATATGTACATA
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Hrasls1_1SA     TAACAAATAAAATATGCTATCTAAATGTCA
Hrasls1_1L      TAACAAATAAAATATGCTATCTAAATGTCA
Hrasls1_1SB     TAACAAATAAAATATGCTATCTAAATGTC-
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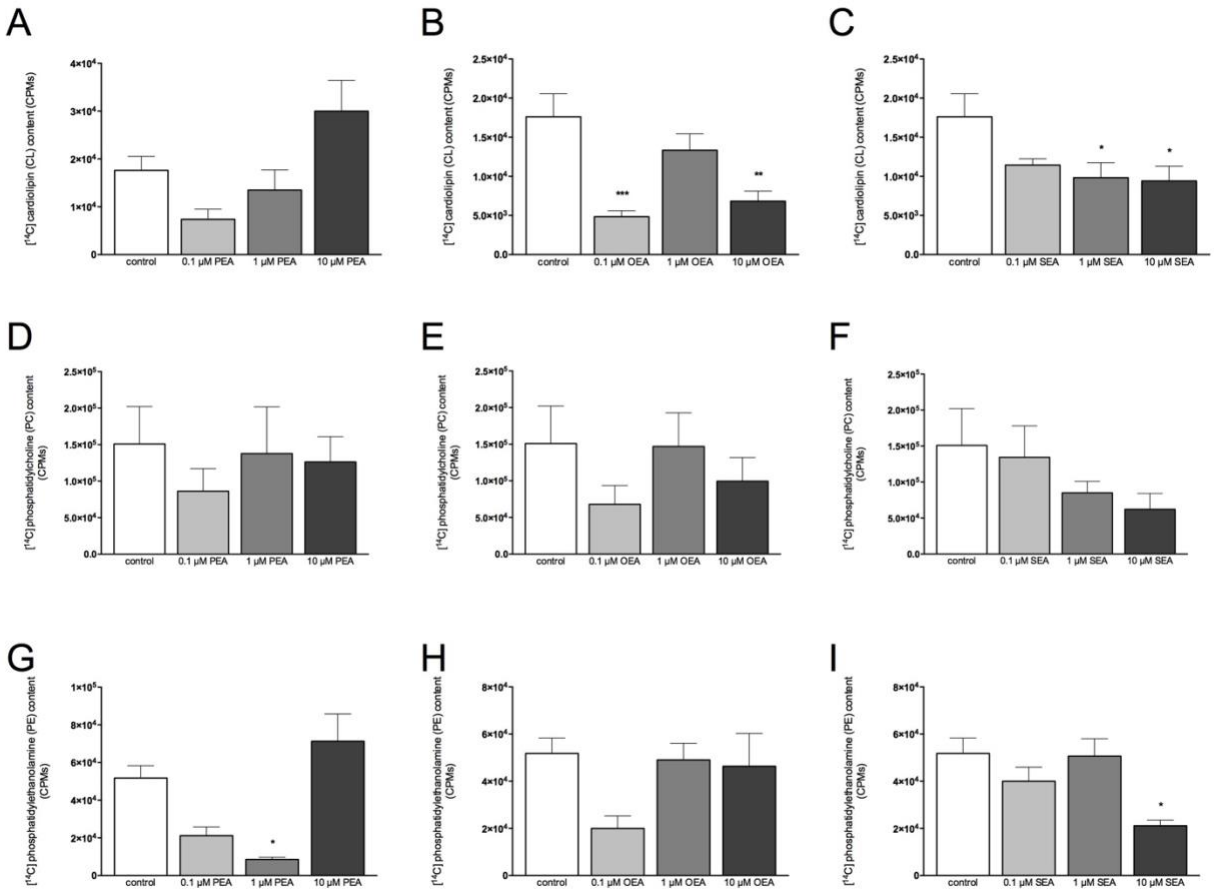
Appendix C: Metabolic label of HEK-293 cells with [^{14}C] palmitate and treatment with NAE



Appendix Figure 4: Metabolic treatment of HEK-293 cells with [^{14}C] palmitate and NAE does not significantly increase [^{14}C]cardiolipin content.

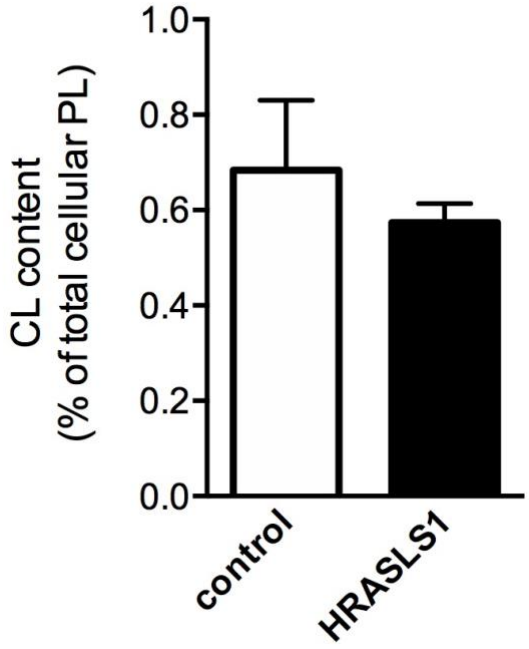
HEK-293 cells were treated for 48 hours with [^{14}C] palmitate and 0.1, 1, or 10 µM of PEA, OEA, or SEA. Total lipids were harvested and phospholipids separated by TLC. Cellular [^{14}C] cardiolipin content in HEK-293 cells were treated with (A) PEA, (B) OEA, or (C) SEA. Cellular [^{14}C] phosphatidylcholine content in HEK-293 cells were treated with (D) PEA, (E) OEA, or (F) SEA. Cellular [^{14}C] phosphatidylethanolamine content in HEK-293 cells were treated with (G) PEA, (H) OEA, or (I) SEA. Data are means ± S.E.M., n=3-6, *P<0.05, **P<0.01, ***P<0.001 vs. controls.

Appendix D: List of buffers and components

- Lysis Buffer 1
    - 50 mM Tris, pH 8.0, 2.4 mM EDTA, 100 mM NaCl, 0.5% *w/v* SDS with proteinase K added at a concentration of 1.0 mg/mL
- Lysis Buffer 2
    - 100 mM Tris-HCl, pH 7.0, 10mM NaCl
- Buffer A
    - 100 mM Tris-HCl, pH 7.4, 5 mM NaCl, 3 mM $MgCl_2$, with 1% protease inhibitor cocktail
- Buffer B
    - 50 mM Tris-HCl, pH 6.8, 1 mM EDTA, and 0.5% Triton X-100
- Laemmeli Buffer
    - 125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% 2-mercaptoethanol, and 0.05% bromophenol blue
- Tris-Buffered Saline with Tween (TBST)
    - 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20
- Buffer C
    - 250 mM Sucrose, 50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$
- Buffer D
    - 20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.5M NaCl, 0.2 mM EDTA, 20% (*v/v*) glycerol, 1% Triton X-100
- Buffer E
    - 225 mM mannitol, 75 mM sucrose, 0.5 mM EGTA, 2 mM MOPS, pH 7.4
- Buffer F
    - 225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl, pH 7.4
- Buffer G
    - 225 mM mannitol, 75 mM sucrose, 0.5% BSA (*w/v*), 0.5% EGTA, 30 mM Tris-HCl, pH 7.4
- Buffer H
    - 225 mM mannitol, 75 mM sucrose, 0.5% BSA (*w/v*), 30 mM Tris-HCl, pH 7.4
- Buffer I
    - 250 mM mannitol, 5 mM HEPES, pH 7.4, 0.5 mM EGTA
- Buffer J
    - 225 mM mannitol, 25 mM HEPES, pH 7.4, 1 mM EGTA, 30% Percoll (*w/v*)
- Acyltransferase Reaction Buffer
    - 100 mM Tris, pH 7.0, 4 mg/mL BSA
- Transacylase Reaction Buffer
    - 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 0.1% Nonidet P-40
- Native Binding Buffer
    - 50 mM NaH_2PO_4, pH 8.0, 0.5 M NaCl, 10 mM imidazole, pH 6.0, final solution adjusted to pH 8.0
- Native Wash Buffer
    - 50 mM NaH_2PO_4, pH 8.0, 0.5 M NaCl, 20 mM imidazole, pH 6.0, final solution adjusted to pH 8.0
- Native Elution Buffer

    - 50 mM NaH_2PO_4, pH 8.0, 0.5 M NaCl, 250 mM imidazole, pH 6.0, final solution adjusted to pH 8.0
- MiR05 buffer
    - 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM lactobionic acid, 20 mM taurine, 10 mM potassium dihydrogenphosphate, 20 mM HEPES, 110 mM sucrose, and 1g/L essential fatty acid free bovine serum albumin
- Hank's Buffered Salt Solution
    - 0.137M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4, 0.1g glucose, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, final solution pH adjusted to 7.1
- Tyrode's solution
    - 95% O_2, 5% CO_2; 121 mM $NaCl_2$, 5 mM KCl, 24 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 5.5 mM glucose, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 7.3

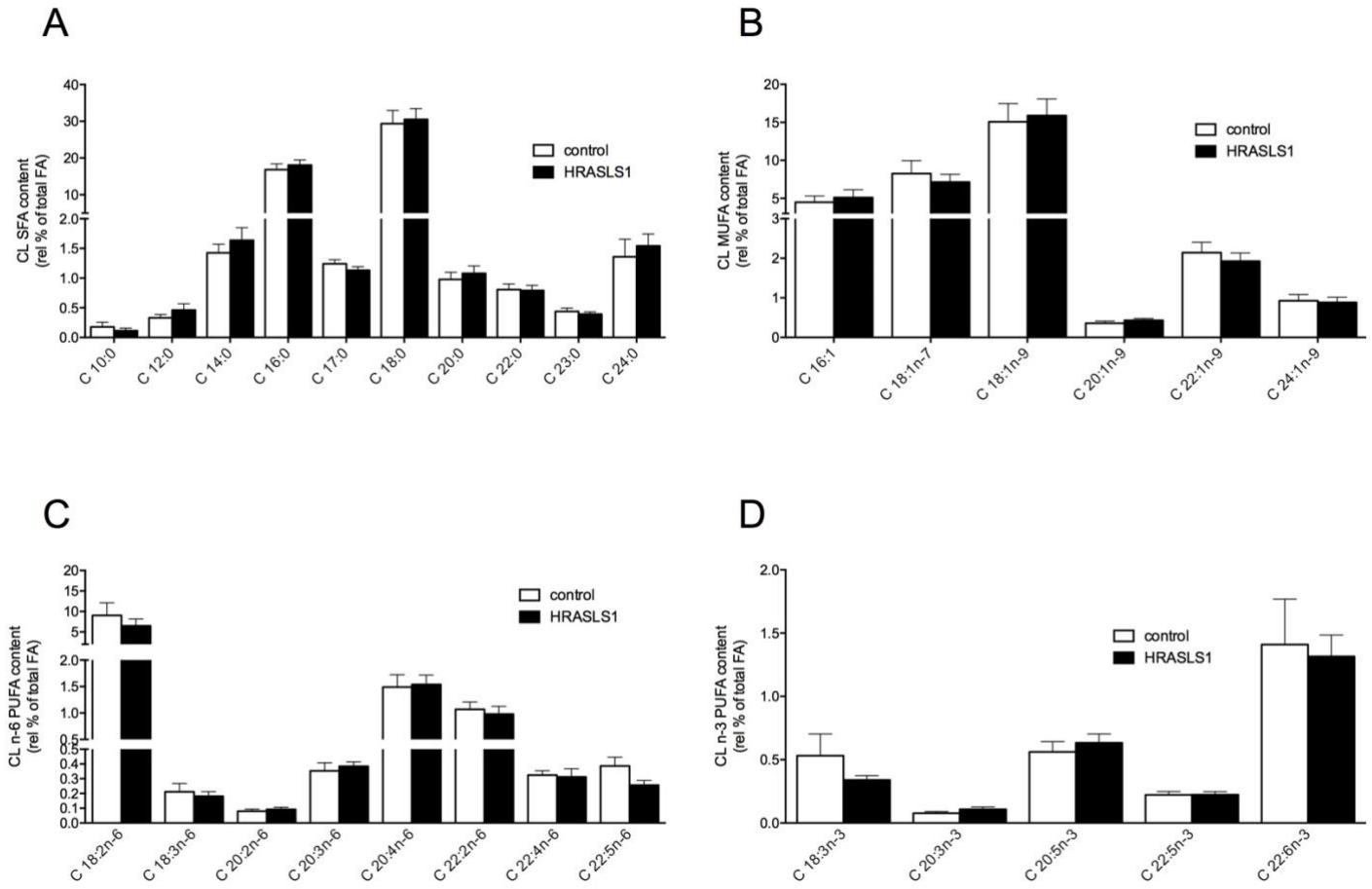
Appendix E: CL in HEK-293 cells as expressed as a percentage of total cellular PL



Appendix Figure 5: CL as a percentage of total cellular PL in HEK-293 cells overexpressing control or HRASLS1

Endogenous cardiolipin content of HEK-293 cells overexpressing control or HRASLS1 as a percentage of total cellular phospholipid. Data are means ± S.E.M. n=8-10.

Appendix E: Relative percent of fatty acid content in HEK-293 cells overexpressing control or HRASLS1



Appendix Figure 6: HRASLS1 overexpression in HEK-293 cells does not significantly alter relative fatty acyl composition of cardiolipin versus controls.

Relative proportion of individual fatty acids within cardiolipin from control or HRASLS1-overexpressing HEK-293 cells including SFA (A), MUFA (B), n-6 PUFA (C), and n-3 PUFA (D). Data are means ± S.E.M. n=8-10.