Small Subunits of Serine Palmitoyltransferase (ssSPTs) and their physiological roles

by

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DEDICATION

To my loving parents, and my family. I will always appreciate all the support and encouragement I got from my sister Gouri, brother-in-law, Manas. I would like to thank a very special man Sambuddha who always believed in me.

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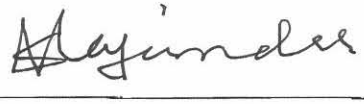
[Signature] 

Saurav Majumder

[Month Day, Year]

ABSTRACT

SMALL SUBUNITS OF SERINE PALMITOYLTRANSFERASE (SSSPTS) AND THEIR PHYSIOLOGICAL ROLES:

Saurav Majumder, PhD, 2014

Thesis directed by: Dr. Teresa M Dunn, Professor, Biochemistry

Sphingolipids are essential components of cells, having diverse functions in cell signaling, survival and other dynamic processes such as trafficking and maintenance of membrane integrity. Serine palmitoyltransferase (SPT) catalyzes the first committed step in sphingolipid synthesis, the condensation of palmitoyl-CoA with serine, to produce 3-ketosphinganine, a precursor of the sphingoid bases that are the backbone of all complex sphingolipids. SPT is a multi-subunit enzyme found in almost all eukaryotes, and even some species of bacteria.

Bacterial SPT is a homodimer of two identical subunits. Yeast SPT is a heterodimer, composed of the Lcb1p and Lcb2p subunits. These SPT subunits are highly conserved throughout evolution. The Lcb1-Lcb2p heterodimers have low basal catalytic activity. In fact, when the LCB1 and LCB2 subunits from higher eukaryotes are expressed in a yeast mutant lacking endogenous SPT, although they are highly expressed and localize to the ER membrane, microsomal SPT activity is much lower than when

measured in the host organism. This observation along with the discovery that yeast SPT has an additional small subunit, Tsc3p, that increases SPT activity more than 50 fold, suggested that the higher eukaryotic enzymes may also require additional subunits for full activity, but blast homology searches of Tsc3p against the human genome showed no candidate homologs. However, recently two functional orthologs of Tsc3p, the small subunits of SPT (ssSPTa and ssSPTb), were identified from humans. These proteins activate the human heterodimer over 100 fold and strongly influence the heterodimer with regard to acyl-CoA substrate preference. Exactly how these small subunits exert their influence on the catalytic activation and substrate selection and whether or not they have other physiological roles is not known. In this study it was hypothesized that such stimulatory subunits of SPT heterodimers are present throughout eukaryotes. Structurally and functionally divergent small stimulatory subunits of SPT from evolutionarily divergent species (budding and fission yeast, mammals, plants flies, fly, and worms*)* may have evolved to provide optimal catalytic activity and allow regulation of SPT as appropriate the host organism. Functional similarities between these highly divergent proteins would indicate that they act by a conserved mechanism.

A phylogenetic approach was used to identify candidate small subunits from evolutionarily divergent species. Here we report that one or more isoforms of the ssSPT subunits exist in each of these different species. All these proteins bind to and significantly increase the enzymatic activity of their cognate heterodimers. In *Arabidopsis thaliana* these proteins are required for pollen development and altering their expression through either silencing or overexpression significantly impacts sphingolipid homeostasis. In contrast to the *S. cerevisiae tsc3Δ*, which is viable, albeit temperature

sensitive, the ssSPT-encoding gene of *S. pombe* has been reported to be essential. A *Drosophila melanogaster* (fly) mutant isolated in a screen for defects in male cytokinesis ('ghiberti mutants') has been reported to be a weak homolog of human ssSPTa. We have shown that this gene does indeed encode a functional small subunit of fly SPT, and that the mutation causes loss of function, consistent with the reported phenotype of reduced sphingolipid levels. Moreover, two additional fly ssSPTs were identified and confirmed to be bona fide ssSPTs. A mutational study of the human ssSPTs revealed that a single amino acid difference between the two isoforms is responsible for dictating the distinct acyl-CoA selectivity of the human heterodimers. Sequence alignment of the Arabidopsis and *S. pombe* small subunits with the human ssSPTs allowed successful prediction of the residue responsible for the acyl-CoA specificity in those proteins, indicating the value of the phylogenetic approach.

It is not yet clear how these small subunits have evolved, because they show a high degree of divergence despite their functional conservation. However our phylogenetic studies indicate that these subunits are the result of coevolution, which coincide with the other highly conserved subunits of SPT. Since they are involved in activation of the heterodimer, dictate substrate specificity, and are important for regulating sphingolipid homeostasis, they might have evolved under different and changing selective pressures.

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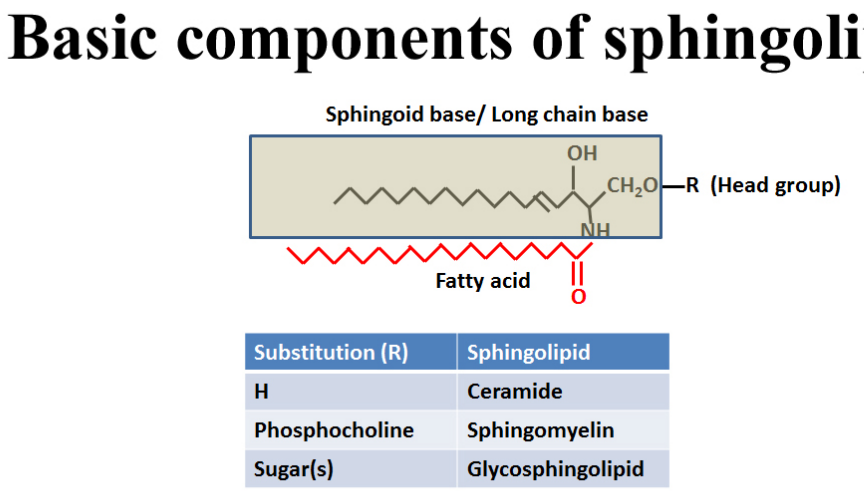
CHAPTER 1: Introduction

BACKGROUND:

Sphingolipids are an essential class of lipid molecules found in all eukaryotes and even in some prokaryotes. Their roles as structural components of eukaryotic membranes are well appreciated. A rapidly growing body of evidence has also revealed their critical roles in signaling. Sphingolipids represent a complex and diverse family of lipids with a sphingoid-base backbone, generally an 18 carbon containing (C18) amino alcohol, attached to a fatty acid through an amide linkage, and a polar head group (Figure 1.1).



Substitution (R)	Sphingolipid
H	Ceramide
Phosphocholine	Sphingomyelin
Sugar(s)	Glycosphingolipid

Figure 1.1. Basic composition of sphingolipids: a sphingoid base, attached in amide linkage to a fatty acid, and a polar head group represented as 'R'

SPHINGOLIPIDS:

The term 'sphingolipid' refers to lipids containing 'sphingosine', which were originally isolated and named by J.L.W Thudicum in 1884 (86). 'The 'sphingoid' or 'long chain base' (LCB) is now known to be the backbone of all complex sphingolipids. Biochemically these sphingoid bases are a long chain amino alcohol, formed by the serine palmitoyltransferase (SPT) catalyzed by the decarboxylative condensation of palmitoyl- CoA with serine to form 3-ketosphinganine (3-KDS). 3-KDS is reduced to form dihydrosphinganine (DHS), and the LCB can be further modified, most commonly by C4-hydroxylation to form phytosphingosine (PHS) or by introduction of a C4-5 double bond to form sphingosine. However, with the development of sensitive techniques to determine the structure of sphingoid bases, it has become clear that they are a diverse family of molecules that can differ in the number and positions of double bonds, hydroxyl groups, methyl groups, etc. (86). Structural diversity also extends to the chain length of the sphingoid bases. The majority of mammalian sphingoid bases are C18, although a small fraction of sphingoid bases ranging from C12 to C26 have been reported (86; 94). It is not clear however whether such variation comes from de novo synthesis in the host organism, from dietary LCBs, or from the microflora. Unusual chain lengths of sphingoid bases have been associated with infection. For example, C15 monomethylated branched chain sphingoid bases were found in infected dental tissues (79).

Such diversity is not limited to different tissues within mammalian systems, as there are numerous reports showing that organisms also have unique sphingoid base chain lengths. In insects, such as *Drosophila melanogaster*, the predominant chain lengths of the sphingoid bases are C14 and C16 (29; 31), whereas in

Schizosaccharomyces pombe, it is C20 (38; 89). Surprisingly, in the nematode *Caenorhabditis elegans* the sphingoid bases contain odd numbered monomethylated branched chains (13; 110; 111). These reports highlight the diversity of the sphingoid bases that exist among eukaryotes. The physiological relevance of these differences is unclear, but it has been hypothesized that the chain lengths of the sphingolipid backbones are important for maintaining optimal membrane fluidity. Interestingly, the *Sphingomonas*, gram negative bacteria, unlike the majority of other bacterial species, have sphingolipids in their membranes (55; 107).

STRUCTURAL ROLES OF SPHINGOLIPIDS:

Sphingolipids are one of the major components of eukaryotic membranes, which form the interface or the permeability barrier of the cell with its environment. Sphingolipids and sterol rich microdomains in the membrane, known as lipid rafts, harbor different receptors and transporters, which are essential for communication with the cells' extracellular milieu (101). In *Saccharomyces cerevisiae* (yeast) almost 7.4% of the total membrane mass is composed of sphingolipids (82) with the plasma membrane containing the highest amounts and other organelles such as the endoplasmic reticulum, mitochondria and vesicles reported to contain much less sphingolipid. The presence of sphingolipids has also been reported in the nuclear envelope, matrix and even in chromatin (66). The relatively high abundance of sphingolipids in the plasma membrane is consistent with their chemical and biophysical properties. Ceramides, the N-acylated long chain bases, form a monolayer in aqueous phase (91). Sphingolipids are amphipathic molecules; their long hydrocarbon chains are hydrophobic in nature, whereas the polar head groups interact with hydrophilic milieu. The physical properties of sphingolipids

change with hydration, heat and pH. For example, ceramides form stable bilayers over the range of 30 ºC – 64.2 ºC, but at higher temperatures, the bilayer transitions from the crystalline to the disordered phase (36). These observations show that the physical properties of sphingolipids are responsive to temperature and that membranes enriched in sphingolipids are relatively stable at high temperatures. Addition of C16 ceramides to dipalmitoylphosphatidylcholine has been reported to induce formation of clusters of gel and liquid crystalline phases (54). These observations suggest that the modulation of sphingolipid composition is required for the maintenance of appropriate membrane properties in eukaryotes. N-acyl chain length of membrane lipids plays a very important role in membrane properties (70). Reducing the chain length of saturated phospholipids induces mismatch and decreases melting temperature (70). It has also been shown that altering the sphingoid base chain length has an effect on raft stability and sterol content (73).

ROLES OF SPHINGOLIPIDS AND THEIR BIOSYNTHETIC INTERMEDIATES IN SIGNALING:

In addition to their roles as structural components of membranes, the sphingolipids serve as signaling molecules that control multiple cellular functions. Modulation of sphingolipid levels in response to heat shock in yeast is well studied (21; 22). Both in higher eukaryotes and in yeast, sphingolipid levels go up in response to heat shock (16). Different sphingolipid biosynthetic intermediates and their corresponding phosphates show transient but significant accumulation within 10 minutes after heat shock (20). However, their levels drop back to normal even when the cells are kept at elevated temperature, suggesting that the transient increase in sphingolipid intermediates is involved in signaling. Consistent with this, a yeast mutant which can bypass the

requirement for sphingolipids does not show trehalose accumulation (a hallmark of the heat shock response) upon heat shock, but the response is restored by addition of sphingolipid biosynthetic intermediates. Interestingly, even without the heat shock, addition of these biosynthetic intermediates can mimic the heat shock response of wild type yeast cells and induce accumulation of trehalose. It has also been reported that in response to heat shock there is a transient arrest of the cell cycle that is mediated by sphingolipid intermediates (26; 56). Such responses to heat shock are mediated by transcriptional activation of stress response elements, suggesting an intricate interplay between sphingolipids and signaling (21), and a dedicated pathway for heat shock response mediated by sphingolipids.

It has long been appreciated that membrane biogenesis is required during cell growth and division. Vesicle mediated membrane trafficking and membrane fusion are required for membrane growth when a mother cell divides into two daughter cells (39). There are two main pathways involved in vesicle mediated membrane trafficking. The endocytic pathway starts with the budding in of vesicles from the plasma membrane to form endosomes that can either be degraded by lysosomes, or recycled back to the membrane. The secretory pathway starts at the endoplasmic reticulum (ER) and ER-derived vesicles are transported to the Golgi for sorting, and then transported to the plasma membrane (72). This is consistent with the findings in plant cells, where during cytokinesis, an intricate structure of actin and microtubules are formed for proper and efficient vesicle transport (104). It has also been reported that in mammalian cells phosphatidylethanolamine is asymmetrically redistributed in the membrane during cytokinesis, and is required for the completion of cytokinesis (62). In fact, it has been

reported that blocking serine palmitoyltransferase, the first enzyme in de novo sphingolipid biosynthesis, disrupts cytokinesis and results in the formation of multinucleated mammalian cells. This phenotype can be chemically rescued by sphingosine, the most abundant mammalian LCB, suggesting that sphingolipids are required for the completion of cytokinesis (76). Surprisingly in yeast, unlike in mammalian cells, treatment with the SPT inhibitor myriocin blocks both nuclear division and cytokinesis, and thus only the DNA content of the cell increases (62). These observations are consistent with the recent finding that a *Drosophila melanogaster* mutant defective in male meiotic cytokinesis ('*Ghiberti*') has a mutation in a gene with low homology to the ssSPT subunits of human SPT (38; 43) and suggests that low SPT activity could be responsible for the cytokinesis defect. Indeed we have shown that this loss of function mutation is in a bona fide fly ssSPT. Multicopy suppressor analysis of yeast cells treated with myriocin identified the *YPK1* and *YPK2* genes as positive regulators of SPT. Very recently, the protein kinases encoded by the *YPK* genes were found to mediate de-repressive phosphorylation of the Orm proteins, negative regulators of SPT. Mammalian homologs of these kinases are involved in growth and differentiation and multiple downstream effectors are also conserved between yeast and mammals, suggesting that such kinase dependent signaling pathways may play a conserved role in growth and differentiation throughout eukaryotes, which is influenced by the levels of sphingolipids (20).

Sphingolipids play important roles in endocytosis. In yeast, a hypomorphic allele of *LCB1*, (encoding a catalytic subunit of serine palmitoyltransferase) was identified in a screen for temperature sensitive endocytic mutants (109). There is evidence showing that

yeast cells lacking components of the v-SNAREs, (required for fusion of vesicles to their target membranes) have defects in exocytosis that can be partially rescued by addition of phytosphingosine or dihydrosphingosine indicating that sphingolipids are important for vesicle fusion and thus modulating sphingolipid biosynthesis can alter the efficiency of vesicle mediated transport.

In mammalian cells exposed to stress, two major biosynthetic intermediates of the sphingolipid biosynthetic pathway, ceramides and sphingosine-1-phosphate, determine cellular fate. In response to a variety of stressful conditions, cells can either undergo apoptosis or can survive by autophagy (47; 103). Ceramide levels increase under stress, causing apoptosis, whereas, an increase in sphingosine-1-phosphate levels is associated with cell survival and proliferation (41). Elevation in ceramide levels upon heat shock is due to increased sphingomyelinase activity, as there is no evidence for increased synthesis of ceramide. Therefore, the balance between ceramides and sphingosine-1-phosphate dictates cell fate under stress, which is known as the 'sphingolipid rheostat' (Figure 1.2). It has been reported that ceramide induced apoptosis can be blocked by activation of sphingosine-1-kinase, indicating the sensitivity of the sphingolipid rheostat (17).

Intracellular ceramide concentrations can go up either by the removal of the head group from complex sphingolipids, or by de novo synthesis. In mammalian systems sphingosine formed from ceramides by the action of ceramidase, can be phosphorylated by sphingosine kinase to form sphingosine-1-phosphate. In yeast, phytosphingosine-1-phosphate and dihydrosphingosine-1-phosphate may have analogous functions to

sphingosine-1-phosphate in mammalian system, as it has been reported, that overexpression of sphingosine-1-kinase promotes cell proliferation (80). Autophagy, the process by which the cells eliminate damaged organelles by lysosomal degradation is believed to be a pro-survival mechanism. Surprisingly, increased levels of both sphingosine-1-phosphates and ceramides promote autophagy in yeast (103). Ceramides induce autophagy through inhibition of the PI3K/AKT (81) pathway whereas sphingosine-1-phosphate exerts its effect through the mTOR mediated pathway (98). Even in non-mammalian vertebrates, it has been shown that S1P and ceramides are involved in the regulation of apoptosis. Treatment with C2-ceramides mimicked apoptosis as it is induced by heat stress in Japanese flounder (103). From these observations it is evident that the effects of ceramide and sphingosine-1-phosphate on apoptosis are conserved throughout eukaryotes.

The recent identification of two proteins Orm1p and Orm2p in yeast (9), negative regulators of SPT, has significantly advanced understanding of sphingolipid homeostasis by suggesting a possible mechanism of how SPT might respond to different stimuli and how the enzymatic activity might be adjusted to maintain proper levels of sphingolipids and bioactive sphingolipid intermediates. Orm proteins have been reported to integrate signals, originally sensed by the TOR signaling pathway, to the SPT complex (40). All these proteins (Lcb1p, Lcb2p, Tsc3p and Orm1p/Orm2p) along with the phosphoinositide-4-phosphatase (Sac1p) form the "SPOTS" complex in yeast (4). This complex is capable of integrating signals such as nutrient availability, and sphingolipid levels and modulates SPT kinetics, in vivo. Studies show that the repressive effect of the Orm proteins on SPT is dictated by their phosphorylation status which is mediated by the

YPK kinases that are downstream of the TORC1 pathway, make it tempting to speculate that transient modulation of these sphingolipid intermediates (such as ceramides and sphingosine-1-phosphate) is mediated by the activity of SPT.

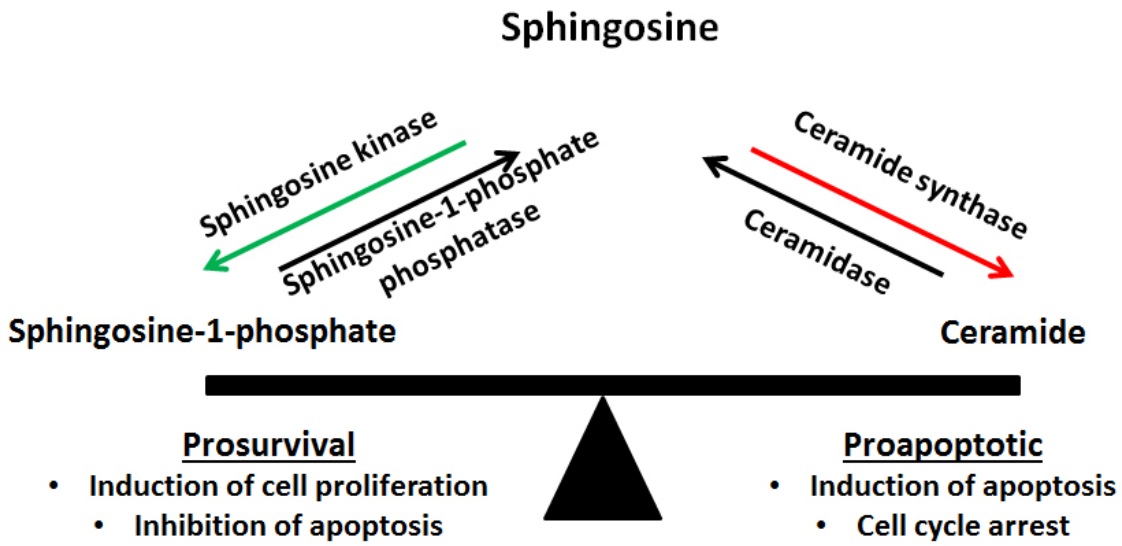


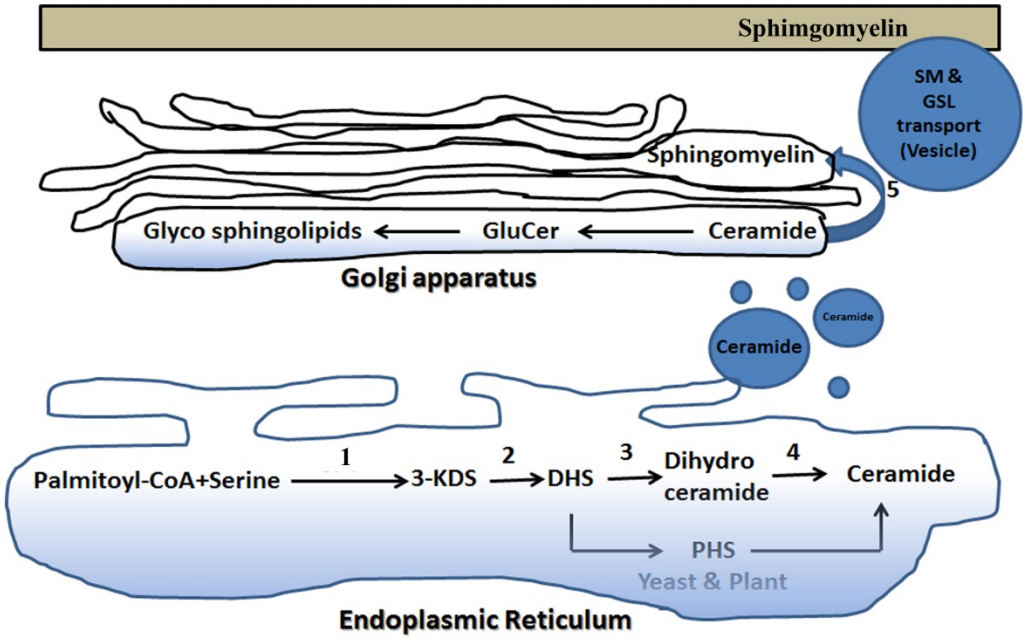
Figure 1.2. Sphingolipid rheostat: Ceramide and sphingosine-1-phosphate dictate the balance between pro and anti-apoptotic signals during stress.

SERINE PALMITOYLTRANSFERASE (SPT), SPHINGOLIPID METABOLISM AND REGULATION:

Sphingolipid biosynthesis starts with the decarboxylative condensation of an acyl-CoA, most often, palmitoyl-CoA with serine to form 3-ketosphinganine (3-KDS) in the endoplasmic reticulum. This rate limiting reaction is catalyzed by the enzyme serine palmitoyltransferase (Figure 1.3) (45). 3-KDS is then reduced to form dihydrosphinganine (DHS) by an NADPH-dependent reductase (6). In mammals, DHS is N-acylated to form dihydroceramide, by ceramide synthases (74), and the sphingoid base of dihydroceramide is then desaturated at C4,5 to form ceramide. In the plant and fungal systems, DHS is hydroxylated at C4 to form phytosphingosine and either DHS or PHS

can be N-acylated to form ceramides. Ceramides are transported from their site of synthesis in the endoplasmic reticulum to the Golgi complex, where they are further modified by addition of head groups to make the complex sphingolipids (30). In mammals, ceramides are converted to sphingomyelin by the addition of phosphocholine or to glycosphingolipids by the addition of sugars. In yeast, the complex sphingolipids all contain a phosphoinositol head group (and are thus called IPCs). Plants also have IPCs rather than sphingomyelin, as well as glycosphingolipids.

Broadly, the biosynthetic pathways can be differentiated into pathways found predominantly in fungal and plant species and the other in mammals, insects and nematodes. Interestingly a sphingolipid biosynthetic pathway also exists in some prokaryotes (*Sphingomonas multivorum*) (55) and even in a virus (*Coccolithovirus*) (42). Despite the divergence downstream of 3-KDS, SPT is highly conserved throughout evolution, and as this enzyme catalyzes the committed step, it is considered a likely target for regulation. Indeed, sphingolipid biosynthesis must be tightly regulated to maintain the balance of different bioactive metabolic intermediates such as sphingosine-1-phosphate, ceramides and ceramide-1-phosphates, involved in responses to different extra and intracellular stimuli. However, the mechanism of regulating sphingolipid homeostasis is poorly understood. The recent identification of two novel regulatory subunits of SPT, the ssSPTs and the Orms, and the appreciation of the influence of substrate availability, has now set the stage for potentially unravelling the mechanisms responsible for maintaining overall sphingolipid homeostasis.



1. Serine palmitoyltransferase
2. 3-ketodihydrosphingosine reductase
3. Ceramide synthase
4. Dihydroceramide desaturase
5. Sphingomyelin synthase

Figure 1.3: Adopted from: Sphingolipid metabolism: De novo sphingolipid biosynthesis and turnover in mammalian system. Nat Chem Biol. 2010 July; 6(7): 489–497

Figure 1. 3. Sphingolipid metabolism: De novo sphingolipid biosynthesis and turnover in mammalian system.

Substrate availability also appears to play a very important role in regulating sphingolipid biosynthesis. Acyl-CoA and serine, the two substrates that are condensed by SPT to form sphingoid bases, can be generated by de novo synthesis (Figure 1.3), but

they can also be supplied from exogenous sources (16). The main source of acyl-CoA is the cytosolic fatty acid synthase complex (FAS). Free fatty acids produced by FAS are esterified to Coenzyme A to form acyl-CoA prior to utilization by SPT. Upon heat shock, yeast sphingolipid levels go up, and consistent with this, treating the cells with cerulenin, a FAS inhibitor, completely inhibits the increase in sphingoid base synthesis (16). Moreover, blocking fatty acid import did not affect sphingolipid levels under either normal physiological conditions or during heat shock. Thus, de novo fatty acid synthesis appears to be the main source of acyl-CoA for sphingoid base synthesis (16) (Figure 1. 4). Interestingly, in the absence of serine in the medium, heat shock no longer induces increased sphingolipid synthesis (16), showing that an increase in serine uptake is required for the transient increase in sphingolipid synthesis upon heat shock.

The Lcb1p (LCB1) and Lcb2p (LCB2) subunits are highly conserved in eukaryotes, as are the ORM proteins. However, the functional orthologs of yeast Tsc3p (ssSPTs) were identified by a functional screen, as they share no sequence homology with Tsc3p. Once the human ssSPTs were identified, homologs (albeit in some cases weak) of the human ssSPTs were found in most eukaryotes, including those chosen for these studies, *A. thaliana*, *D. melanogaster*, *S. pombe*, and *C. elegans*. Each of these model organisms represents a distinct phylogenetic clade in eukaryotic evolution and each organism has a distinct sphingoid base chain length profile. Human ssSPTs can activate the yeast Lcb1-Lcb2p heterodimer indicating that although Tsc3p and the ssSPTs share no homology, the ssSPTs may have retained some key characteristics which are recognized by the yeast Lcb1-Lcb2p heterodimer. In contrast, yeast Tsc3p does not

activate the human LCB1-LCB2 heterodimer, suggesting that Tsc3p diverged so early during evolution that it is no longer recognized by the human LCB1-LCB2 heterodimer. Furthermore, heterologous expression of LCB1-LCB2 heterodimers from different species in a yeast *lcb1Δtsc3Δ* knockout consistently resulted in low microsomal SPT activity compared to microsomal SPT activity measured from the host. These observations suggested the likelihood that there are orthologs of the ssSPTs in most, if not all, eukaryotes. It is possible that different novel subunits or classes of novel subunits have evolved for optimal activity and regulation of SPT. Studies with human ssSPTs showed that these small proteins dictates acyl-CoA substrate preference of the heterodimer, which raises the possibility that these proteins can play important roles in response to different signals to produce different chain lengths of sphingoid bases or may have other physiological roles.

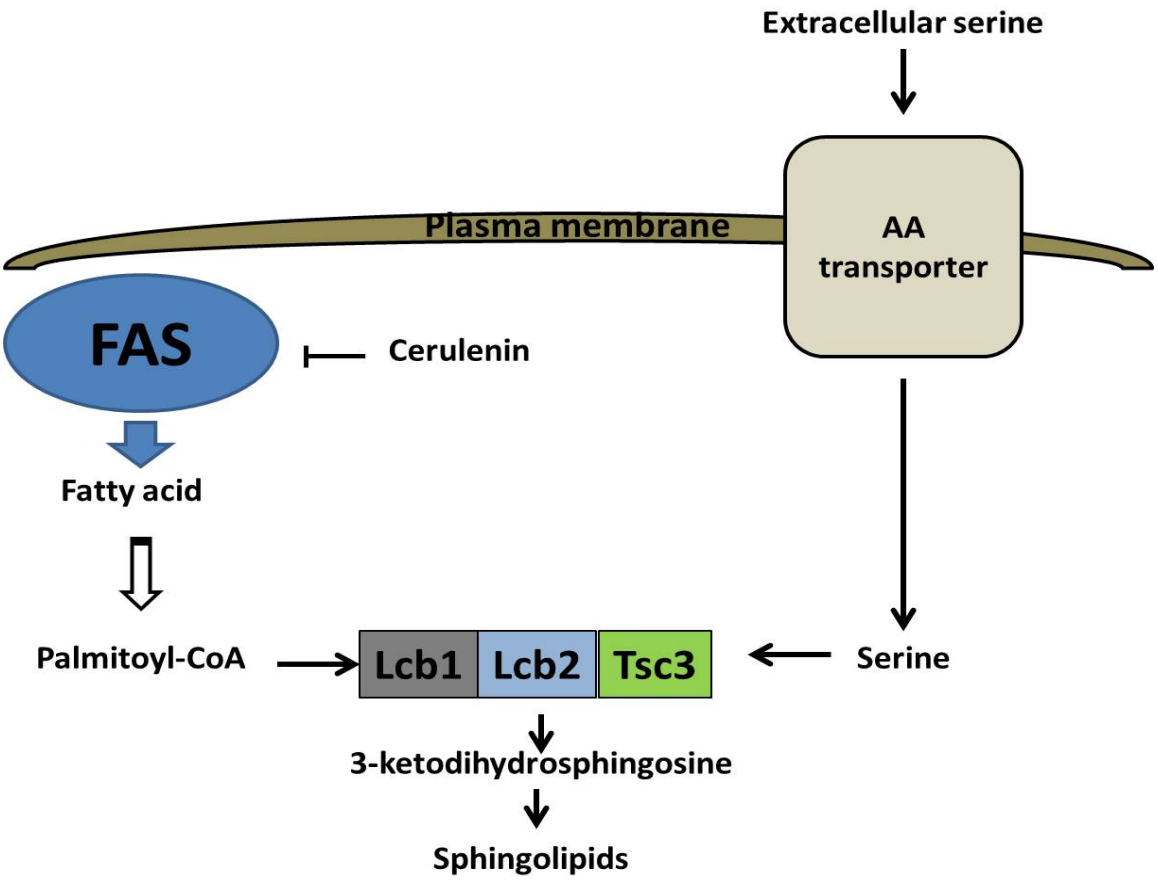


Figure 1. 4. Substrate supply for de novo sphingolipid synthesis.

CENTRAL HYPOTHESIS:

We hypothesized that small subunits of SPT (ssSPTs) exist in all eukaryotes and that despite their low homology, they activate their cognate SPT heterodimer(s) and also confer distinct acyl-CoA chain length substrate preference. Therefore, identification and characterization of such proteins from evolutionarily divergent species will provide a better understanding of their functions. Small subunit knockout mutants in model organisms will reveal the physiological roles of these proteins in different organisms.

Therefore we specifically proposed to:

1. Identify the candidate small subunits of SPT (ssSPTs) from evolutionary divergent species by bioinformatics approaches.
2. Biochemically characterize these candidate small subunits in yeast to determine their influence on their cognate heterodimers (activation and substrate selection).
3. Study their physiological roles in the host organisms.
4. Predict how these small subunits have evolved.

CHAPTER 2: ssSPTs in *Arabidopsis thaliana*

INTRODUCTION

In addition to their structural roles, sphingolipids are also involved in diverse cellular functions in plants. They are major components of endomembranes, including the plasma membrane, tonoplast and golgi. Along with sterols, they are enriched in detergent-resistant domains (lipid rafts) that are involved in trafficking and sorting of proteins required for critical processes at the cell surface, including cell wall deposition and auxin transport (8; 64; 93). In addition, LCB-Ps (long chain base phosphates) and ceramides have been implicated in cell signaling events including ABA-induced stomatal closure in response to drought (14; 15) and programmed cell death (65; 92).

The plant sphingolipidome reveals a large and structurally diverse family of molecules; in fact, nearly 168 different sphingolipids have been reported in *A. thaliana* (68). The predominant sphingolipids are the glucosylceramides, although inositolphosphorylceramides (24) are also present. The structural diversity reflects the presence of different chain lengths of fatty acids joined in amide linkage to the LCBs, the degree of unsaturation and hydroxylation of the LCB and amide- linked fatty acids, and the variety of different polar head groups. Despite their structural diversity, the chain length of the plant LCB backbone is exclusively C18 (59; 68); this is different from the situation in yeast and mammalian cells where, although the C18-LCBs are most abundant, C16 and C20-LCBs are also found. Since the genes involved in sphingolipid metabolism are conserved among eukaryotes, once the genome of *Arabidopsis thaliana* was sequenced, it became an extensively studied model for identifying and characterizing

the sphingolipid metabolic genes in plants. There is a single gene encoding an LCB1 homolog (AtLCB1) and two genes encoding LCB2 homologs (AtLCB2a and AtLCB2b), indicating that there are two different SPT isoforms (LCB1-LCB2a and LCB1-LCB2b). Modulating the expression of the AtLCB1 and AtLCB2 alleles showed that they are required for embryonic development (12; 96). Homozygous T-DNA knockout mutants lacking either *AtLCB1* or both *AtLCB2* genes were not recoverable, and partial suppression of *AtLCB1*results in small plants with altered leaf morphology (12; 23). Both heterodimeric isoforms are catalytically competent and, in contrast to the two different human LCB1/LCB2a/b heterodimers that show distinct acyl-CoA substrate preferences, both heterodimers have a very strong preference for palmitoyl-CoA. This is, of course, consistent with the presence of only C18 containing LCBs in Arabidopsis (7; 68).

As in higher eukaryotes, there are no homologs of Tsc3p in Arabidopsis or other plants. However, the discovery of the human ssSPTs (32; 43) led us to investigate the subunit composition of plant SPT further. Indeed homologs of the human ssSPTs can be found in the *A. thaliana* genome. These homologs share considerable homology with the human small subunits, and are almost identical to each other, thus raising the possibility that they might be functionally redundant. To address their potential roles as activators of Arabidopsis SPT heterodimer(s), whether they are functionally redundant and the consequences of altering their expression, we undertook a study with our long term collaborators, the laboratory of Dr. Edgar Cahoon at the University of Nebraska, Lincoln, Nebraska.

RESULTS

Identification of ssSPT homologs in *Arabidopsis thaliana*:

Two genes were identified as ssSPT candidates in the *A. thaliana* genome using Blast homology searches with human ssSPTa and ssSPTb as query sequences. These two candidate genes, designated as *AtssSPTa* and *AtssSPTb*, are encoded by At1g06515 and At2g30942 respectively. AtssSPTa and AtssSPTb share 25-30% homology with human ssSPTa and 18-25% with human ssSPTb, but less than 10% sequence homology with Tsc3p. These AtssSPT candidates are the smallest peptides (56 amino acids) of any of the ssSPTs identified to date (Figure 2.1). In contrast to the human ssSPTs, the candidate plant ssSPTs share 88% amino acid sequence identity with each other, and the differences are conservative substitutions, making it unlikely that they have different biochemical activities. Both AtssSPTa and AtssSPTb peptides are predicted to have a single transmembrane domain of 19-23 amino acids encompassing the central region, which is consistent with the presence of a single transmembrane domain in Tsc3p and human ssSPTs (32; 48).

Figure 2.1. Amino acid sequence alignment of ssSPTs from *Arabidopsis thaliana* (At) and *Homo sapiens* (human).

Sequences were aligned using ClustalW. The divergent amino acid shown to influence substrate specificity (M vs. V) of human SPT is indicated with an asterisk and the predicted transmembrane domain (TMD) is indicated with a bar.

Subunits of AtSPT are stably expressed and functional in *S. cerevisiae*:

S. cerevisiae lcb1Δtsc3Δ mutant cells lacking endogenous SPT were transformed with a plasmid expressing AtLCB1-Flag and Myc-AtLCB2a or Myc-AtLCB2b with or without a plasmid expressing either the HA-AtssSPTa or the HA-AtssSPTb candidate. Expression of each subunit was verified by immunoblotting using anti-Flag, Myc or HA antibodies. Microsomes prepared from the yeast SPT knockout mutants transformed with the empty vectors (grown on medium containing PHS) were used as a control. The results show that the subunits of the two heterodimeric isoforms (AtLCB1-LCB2a or AtLCB1-LCB2b) were expressed comparably with or without coexpression of HA-AtssSPTa or HA-AtssSPTb, and thus that the stability of the heterodimers was not influenced by the presence of the AtssSPTs. Surprisingly, for unknown reasons, AtssSPTa was consistently expressed at higher levels than AtssSPTb (Figure 2.2).

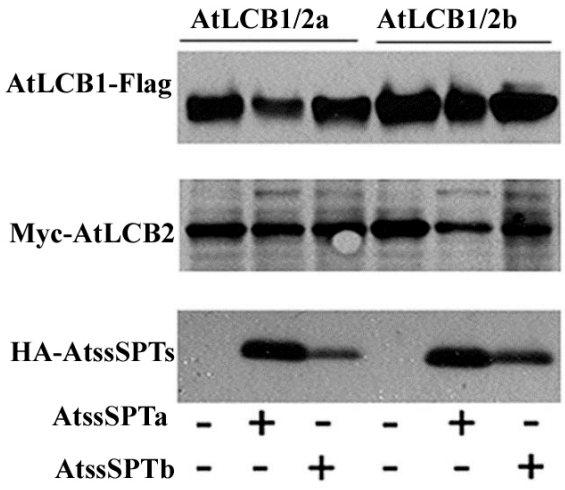


Figure 2.2. Immunoblot showing the expression levels of the different AtSPT subunits in yeast.

Two different heterodimeric isoforms of plant SPT (AtLCB1-Flag, Myc-AtLCB2a and AtLCB1-Flag, Myc-AtLCB2b) were heterologously expressed in yeast lacking endogenous SPT with and without candidate the AtssSPTs. The subunits of *A. thaliana*

SPT were detected using anti-Flag, Myc and HA antibody to detect, AtLCB1, AtLCB2 and AtssSPTa, AtssSPTb respectively.

To address the functionality of the AtssSPTs as bona fide small subunits of SPT, their ability to stimulate activity of the AtLCB1-LCB2a and AtLCB1-LCB2b heterodimers was investigated. Expression of the AtLCB1-LCB2a or AtLCB1-LCB2b heterodimers alone failed to complement the PHS requirement of the yeast *lcb1Δtsc3Δ* mutant indicating insufficient SPT activity to fulfill the essential functions of sphingolipids (Figure 2.3). It should be noted that we showed in an earlier study that expression of AtLCB1 and AtLCB2a or AtLCB2b alone supports weak growth of the yeast mutant, but this was dependent on high level expression of the AtLCB1 and AtLCB2a or AtLCB2b subunits (23). In these studies constructs designed for relatively low expression of the AtLCB1 and AtLCB2 subunits were used for the purpose of assessing whether the AtssSPTs activate the heterodimers. Indeed, coexpression of either candidate AtssSPT along with AtLCB1 and AtLCB2a or AtLCB2b resulted in robust PHS-independent growth, even at 37 ºC, where the requirement for sphingolipids is relatively high. These results clearly show that both AtssSPTs significantly enhance the activity of the Arabidopsis AtLCB1/LCB2a/b heterodimers and are therefore bona fide small activating subunits of SPT (Figure 2.3).

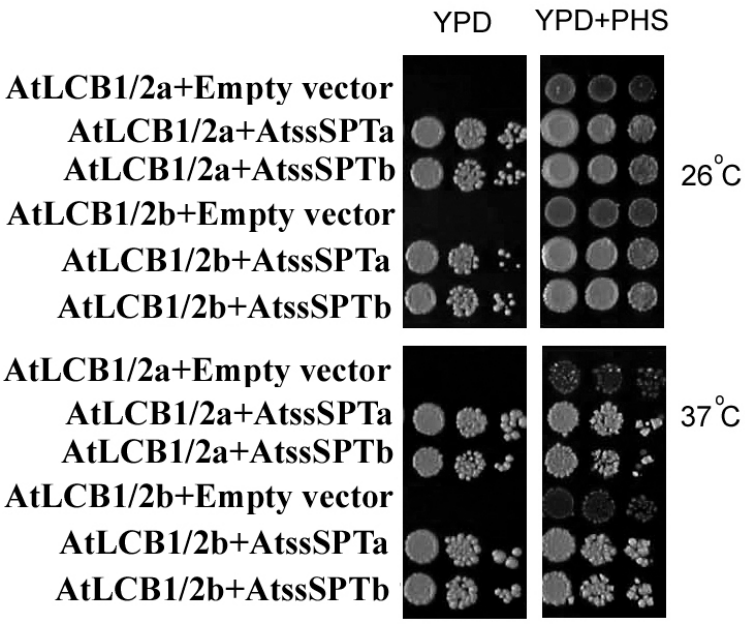


Figure 2.3. Complementation, of the AtLCB1-LCB2a/LCB2b heterodimers by AtssSPTa and AtssSPTb.

Co-expression of AtssSPTa and AtssSPTb with the Arabidopsis SPT subunits AtLCB1-Flag/Myc-LCB2a or AtLCB1-Flag/Myc-LCB2b complements cell inviability of yeast lacking endogenous SPT by activating the core SPT heterodimer. Yeast lacking endogenous SPT activity and expressing either the Arabidopsis LCB1/LCB2a or LCB1/LCB2b are viable only when provided with the long-chain base phytosphingosine (t18:0). The long-chain base auxotrophy of these cells is rescued at 26 °C and 37 °C when the Arabidopsis SPT heterodimers are co-expresssed with either AtssSPTa or AtssSPTb. Though not shown, the AtssSPT subunits alone do not complement.

AtssSPTs coimmunoprecipitate with plant SPT heterodimer:

To further investigate the interaction of the AtssSPTs with the plant LCB1-LCB2a heterodimer, immunoprecipitation experiments were conducted using solubilized microsomes from yeast cells heterologously expressing AtLCB1-Flag, Myc-AtLCB2a and HA-AtssSPTa or HA-AtssSPTb (Figure 2.4 and Figure 2.5).

As expected immunoprecipitation of Flag-tagged AtLCB1 subunits resulted in coimmunoprecipitation of Myc-AtLCB2a subunit. Moreover, when solubilized microsomes from cells coexpressing AtssSPTa or AtssSPTb were used for the Flag-IPs, HA-AtssSPTa or HA-AtssSPTb co-purified with the AtLCB1-Flag and Myc-AtLCB2a. The absence of Elo3p (a component of the ER-localized elongase complex that does not interact with SPT) in the bound fraction shows that the interaction of the AtssSPTs with the heterodimer is specific. When the same experiment was repeated with untagged AtLCB1 (for which we have no antibodies), neither AtLCB2a nor AtssSPTa coimmunopurified, validating the conclusion that coimmunopurification of AtssSPTs with the heterodimer reflects its specific association with the AtLCB1/LCB2a heterodimer.

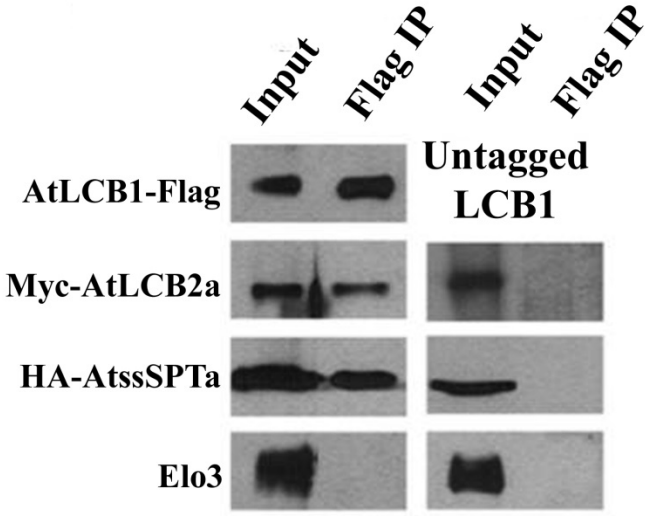


Figure 2.4. Immunoblot showing the interaction of AtssSPTa with the AtLCB1-LCB2a heterodimer in yeast.

Solubilized microsomes from cells expressing AtLCB1-FLAG, Myc-AtLCB2, and HA-ssSPTa (left panel), or untagged AtLCB1, Myc-AtLCB2, and HA-ssSPTa (right panel), were incubated with anti-FLAG beads, the beads were washed, and eluted with FLAG

peptide. Aliquots of the solubilized microsomes (input) and eluent (Flag IP) were resolved by SDS-PAGE and the Arabidopsis SPT subunits were detected by immunoblotting with anti-Flag, anti-Myc, anti-HA, and anti-Elo3p (negative control) antibodies.

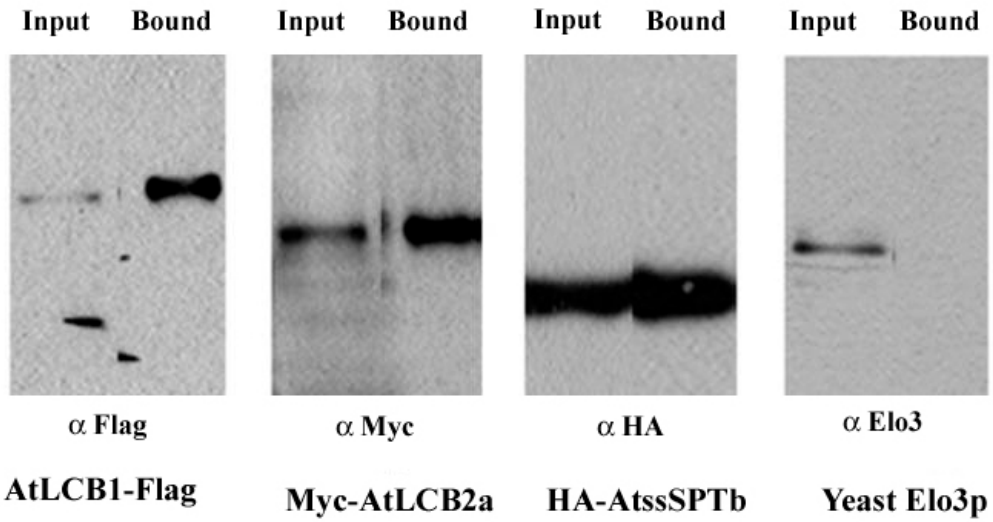


Figure 2.5. Immunoblot showing the interaction of AtssSPTb with the AtLCB1-LCB2a heterodimer in yeast.

Microsomes were prepared from yeast cells heterologously expressing AtLCB1-Flag, Myc-AtLCB2a and HA-AtssSPTb. The SPT complex was pulled down with anti-Flag beads and then probed with anti-Flag, Myc and HA antibodies. Elo3p was used as a control.

AtssSPTs are integral membrane proteins with a single transmembrane domain:

As mentioned above, the AtssSPTs are the smallest ssSPTs identified thus far and the hydropathy analysis predicts the presence of a single transmembrane domain (TMD). This is in contrast to the human and other ssSPTs, which are predicted to have two TMDs. It was therefore of interest to experimentally investigate the membrane topology of the AtssSPTs. We first investigated the nature of the membrane association of the AtssSPTa and AtssSPTb using different salts and detergents. The results show that

the AtssSPTs, when coexpressed with the AtLCB1 and AtLCB2 subunits, are solubilized only with detergents and not by salt or bicarbonate, and thus behave like an integral membrane proteins (Figure 2.6).

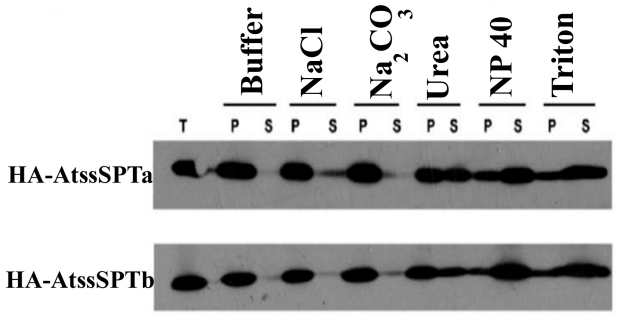


Figure 2.6. Immunoblot showing that the AtssSPTa and AtssSPTb are integral membrane proteins.

Microsomes from yeast expressing HA-AtssSPTa or HA-AtssSPTb, along with AtLCB1and AtLCB2a, were extracted on ice with an equal volume of IP buffer or buffer containing 1 M NaCl, 0.2 M Na_2CO_3, 5 M urea, 0.4% Nonidet P-40 or 2% Triton X-100 for 60 min. The samples were subjected to centrifugation at 100,000 x g for 30 min and equal proportions of the supernatants and pellets were resolved by SDS-PAGE. HA-AtssSPTa and HA-AtssSPTb were detected by immunoblotting with anti-HA antibody.

To address the number and orientation of the transmembrane domain(s), a glycosylation cassette (GC) was appended to the C-terminus of HA-AtssSPTb and the tagged protein was expressed with the AtLCB1-LCB2a heterodimer in yeast. The GC is a 56-amino acid domain from invertase that contains 3 potential glycosylation sites. If this domain resides in the lumen of the ER, these sites will be glycosylated and the mobility of the protein will decrease; treatment with EndoH, which removes the glycans, will result in increased mobility. As shown in (Figure 2.7), treatment of microsomes

prepared from yeast expressing AtssSPTb-GC with EndoH resulted in increased electrophoretic mobility of the AtssSPTb-GC thereby demonstrating that the C-terminus is glycosylated and thus is in the lumen of the ER (Figure 2.7). These results indicate that the AtssSPTs are integral membrane proteins with a single TMD, a result that is consistent with recent studies on the topology of the human ssSPTs (48).

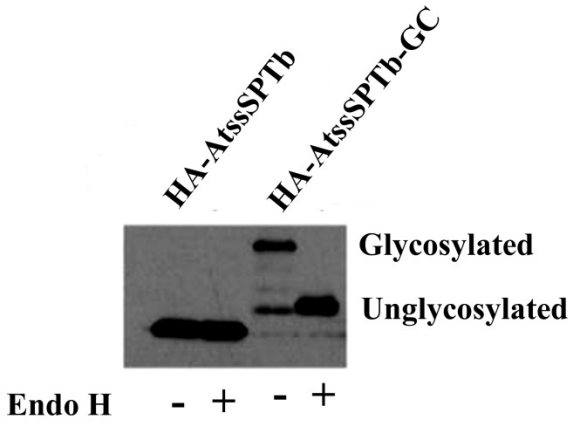


Figure 2.7. Immunoblot showing that the C-terminus of AtssSPTb is in the ER lumen.

HA-AtssSPTb and HA-AtssSPTb-GC were expressed in yeast along with AtLCB1and AtLCB2a. Microsomal protein (20 μg, with or without EndoH treatment) was resolved by SDS-PAGE and HA-AtssSPTb was detected by immunoblotting as described in Kimberlin et al (59).

AtssSPTs show strong activation of the heterodimer and confer strict preference for C16-CoA as substrate:

To investigate the extent of activation of the SPT heterodimer by the AtssSPTs and their influence on the substrate utilization, microsomal SPT activities of

the yeast SPT knockout mutants heterologously expressing the At-LCB1-LCB2a/LCB2b heterodimers with or without HA-AtssSPTa or HA-AtssSPTb was measured. Acyl-CoA of different chain lengths (C14, C16 and C18) were used in the assay to determine the acyl-CoA substrate preferences of the heterodimeric SPTs. Consistent with the complementation data (Figure 2.2), coexpression of both AtssSPTs resulted in robust activation of the heterodimers. Irrespective of the AtLCB2 or AtssSPT subunits, all Arabidopsis SPT heterotrimers showed a strong preference for palmitoyl-CoA (C16) as a substrate. This result is entirely consistent with the exclusive presence of C18- LCBs reported in *Arabidopsis thaliana* (69) (Figure 2.8).

The AtLCB1-LCB2b heterodimer showed relatively weak activation by the AtssSPTs compared to AtLCB1-LCB2a heterodimer, despite their comparable expression (Figure 2.2), suggesting that the enzymatic activity of the LCB1-LCB2b isoform, at least when expressed in yeast, is intrinsically low. Both heterodimers showed higher activation by AtssSPTa than by AtssSPTb, but this is most likely due to the higher expression level of AtssSPTa compared to AtssSPTb (Figure 2.2.)

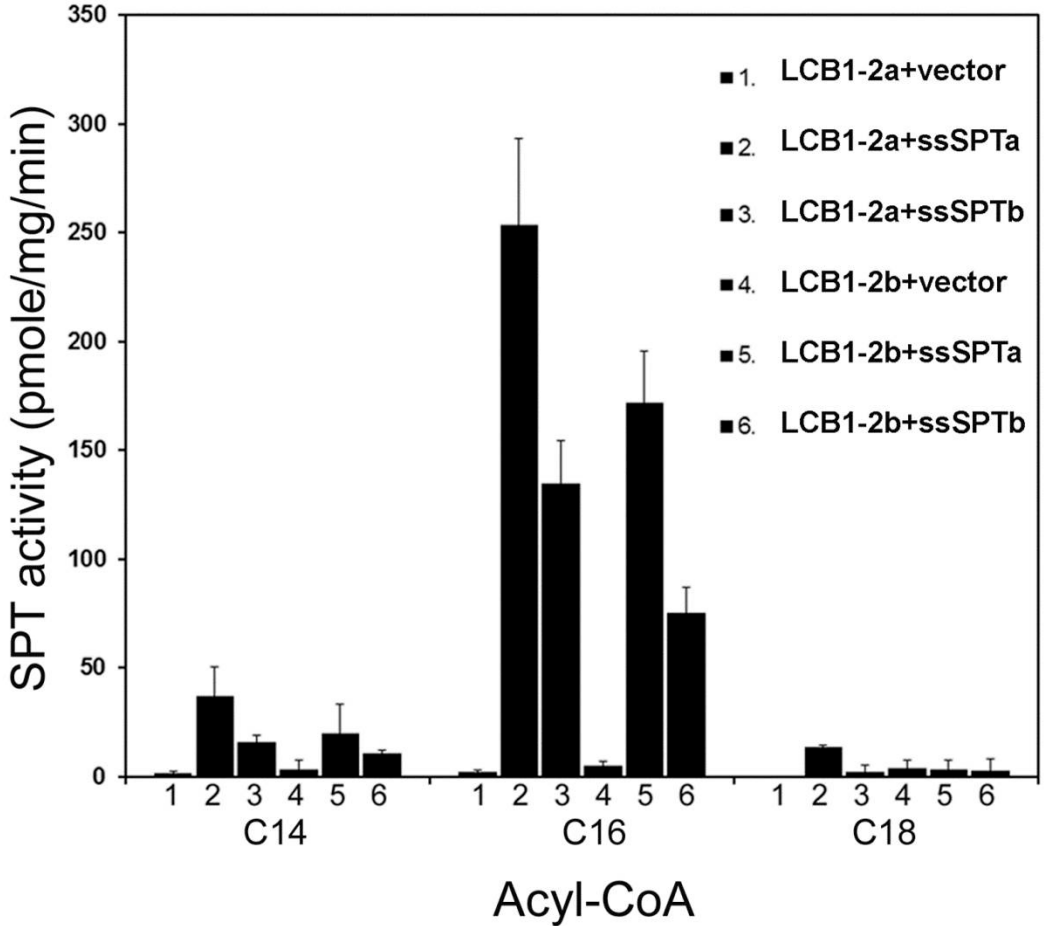


Figure 2.8. Comparison of Arabidopsis SPT activity +/- AtssSPTa or b with myristoyl (14:0)-, palmitoyl (16:0)-, and stearoyl (18:0)-CoA.

SPT activity was measured in yeast microsomes expressing AtLCB1-Flag+Myc-AtLCB2a or AtLCB1-Flag+Myc-AtLCB2b with or without HA-AtssSPTa or HA-AtssSPTb to assess their ability to enhance AtSPT activity. SPT activity was measured using [^{3}H] serine (2 mM final), 50 µM of C14, C16 or C18-CoA and 200 µg of protein. Values shown are the average of three independent assays ± SD.

Strict preference for C16-CoA of the heterodimers dictated by the AtssSPTs was verified with the total LCB profiles of the yeast cells heterologously expressing all four different isoforms of AtSPT heterotrimers. The results show that the only LCB species detected are the C18-CoA (C18-PHS and C18-DHS) species (Figure 2.9).

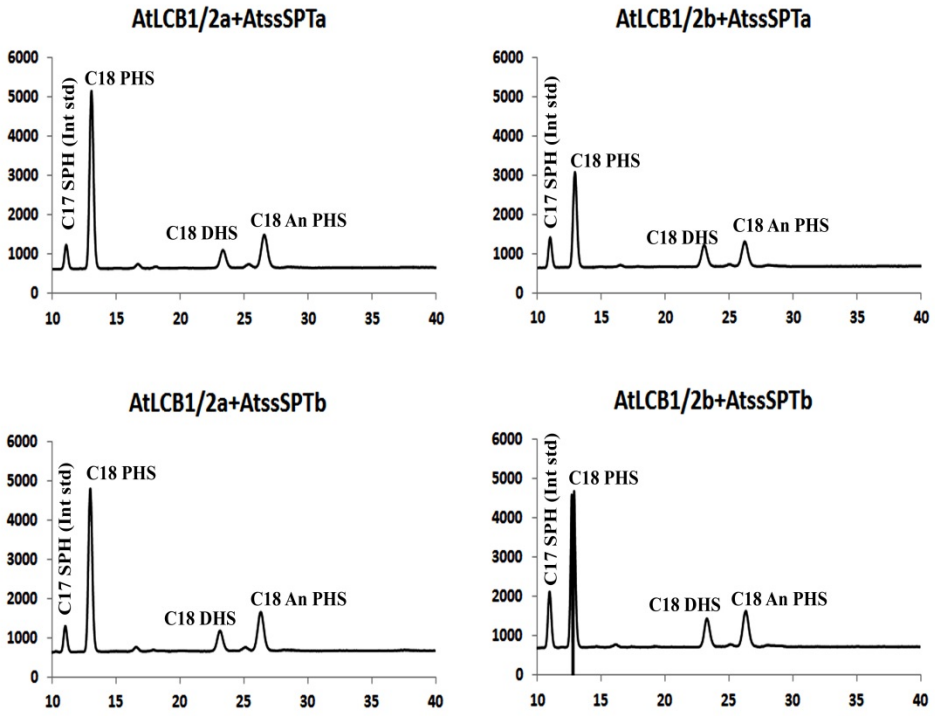


Figure 2.9. HPLC analysis of total LCBs from yeast cells expressing AtssSPTa or AtssSPTb show predominant accumulation of C18-LCB species irrespective of the AtLCB1-LCB2a/LCB2b isoforms.

Residue responsible for substrate preference:

From mutational studies with the human ssSPTs, the difference in substrate preference between the C16-CoA preferring ssSPTa and the C18-CoA preferring ssSPTb (C18-CoA) has been shown to be dictated by a single residue, Met-25 in ssSPTa and Val-25 in ssSPTb (48). Amino acid sequence alignments of the human ssSPTs with the AtssSPTs (Figure 2.1) revealed that the corresponding residue is a Met (Met-19) in both AtssSPTa and AtssSPTb. This is consistent with the selective C16-CoA preference of human ssSPTa and the AtssSPTs. To investigate if Met-19 in AtssSPTb dictates substrate selection, it was mutated to Val (M19V). Long chain base analysis from yeast cells heterologously expressing AtLCB1-LCB2a with wild type AtssSPTb or mutant AtssSPTb (M19V) showed significant accumulation of C20-LCB species with the mutant, but not with the wild type AtssSPTb (Figure 2.10). This result shows that, as for the human ssSPTs, the methionine at position 19 is critical for the acyl-CoA substrate selectivity conferred by the AtssSPTs.

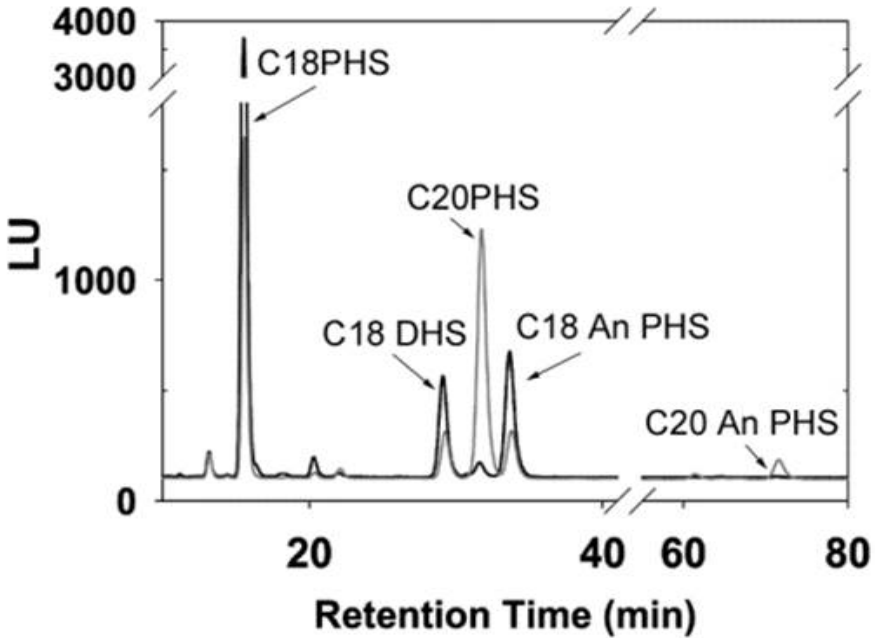


Figure 2.10. The AtssSPTb M19V mutant has altered SPT acyl-CoA preference.

Yeast expressing wild-type AtssSPTb along with AtLCB1 and AtLCB2a accumulated exclusively C18-LCBs (dark line), while those expressing the AtssSPTb M19V mutant accumulated high levels of C20-LCBs reflecting condensation of serine with stearoyl-CoA (light line).

Discussion:

Here we have shown the identification of two weak but significant homologs of human ssSPTs in *Arabidopsis thaliana*. In contrast to the human ssSPTs, these two 56 amino acid proteins are almost identical to each other, suggesting they might be functionally redundant (Figure 2.1). Coexpression of these candidate genes rescued the PHS auxotrophy of the yeast cells heterologously expressing the AtLCB1-LCB2a/b heterodimers, by activating the heterodimers >100 fold (Figure 2.2, Figure 2.3 and Figure 2.8). Comparison of *in vitro* enzymatic activities measured in yeast microsomes expressing all four heterotrimeric SPT isoforms show that both AtssSPTa and AtssSPTb

not only activate the heterodimers but also confer a strict palmitoyl-CoA (C16) preference. Activation of the AtLCB1-LCB2a heterodimer by the small subunits is more efficient than the activation of AtLCB1-LCB2b heterodimer. Strict C16-CoA substrate preference observed in *in vitro* enzymatic activity of all four Arabidopsis SPT heterotrimers is consistent with the product formation (C18-LCBs) in yeast cells heterologously expressing each different AtSPT isoforms.

The single residue that has been reported to dictate C16-CoA and C18-CoA substrate preferences of human ssSPTa and human ssSPTb are Met-25 and Val-25 respectively. The residue equivalent to that position in both the AtssSPTs is Met-19 and both of these small subunits confer a C16-CoA preference. To test the importance of Met-19 in AtssSPTs, we mutated that residue to Val. Coexpression of AtssSPTb M19V with the AtLCB1-LCB2a heterodimer in yeast showed significant accumulation of C20-LCBs, suggesting that indeed this residue is responsible for substrate selection. Moreover, Kimberlin et al have shown that overexpression of this mutant AtssSPTb M19V shows significant accumulation of C20-LCBs in plant (59).

Similar to what has been reported about humans ssSPTs and yeast Tsc3p; AtssSPTs are also integral membrane proteins, with their C-termini in the ER lumen. This result is consistent with the confocal microscopy of transiently expressed AtssSPTs fused to a florescent protein (YFP) in *N. benthamiana* (tobacco cells) showing that they colocalize in the ER (59). A previous report showed that the AtLCB1 (12) and AtLCB2 (96) subunits of the heterodimer are also ER proteins , and now we confirm that AtssSPTs show a positive physical interaction with the AtLCB1, AtLCB2 heterodimer in yeast, suggesting that they belong to the same complex in plant.

Kimberlin et al have shown that transcripts of AtssSPTa and AtssSPTb are present in different plant organs (59), suggesting they are ubiquitously expressed. This finding is consistent with the previous reports showing that AtLCB1 and AtLCB2 subunits are also ubiquitously expressed (12). Surprisingly, quantification of *AtssSPTa* and *AtssSPTb* transcript levels from different plant tissues showed that the transcript levels of *AtssSPTa* are consistently higher than *AtssSPTb* in all the tissues. The maximum level of transcript in pollen (~400 fold) may explain the observed pollen lethality in homozygous *atssspta* knockout mutants (59). In fact, it has also been reported that the LCB2 subunit is required for male gametophyte development (23), which has similarity with the phenotype observed with homozygous *atssspta* knockouts. Surprisingly homozygous *atsssptb* knockout mutant plant lines did not show any defects compared to wild type. This may be explained by the fact that AtssSPTa and AtssSPTb are functionally redundant, and that AtssSPTb transcripts are lower in all tissues studied. Genomic complementation of homozygous AtssSPTa knockout mutants with AtssSPTb under the control of the AtssSPTa promoter resulted in normal plants indistinguishable from the wild type (59). This suggests that pollen lethality shown by the complete loss of AtssSPTa is due to its high expression levels which in turn may contribute to higher sphingolipid levels. Expression of AtssSPTa under the control of a pollen and ovule specific promoter (At-DMC1) in a homozygous *atssspta* knockout background showed that gametophyte carrying the mutant allele can undergo pollen development but no transgenic plants (atssspta-/-::*pAtDMC-AtssSPTa*) were recovered. This indicate that AtssSPTa is also required for vegetative growth (59).

Interestingly, microsomal SPT activity measured in plant lines with overexpressed AtssSPTa resulted in increased in vitro enzymatic activity compared to wild type, suggesting that under normal physiological conditions AtssSPTs are limiting (59). This is consistent with the fact that AtssSPTa over expressing lines show increased sensitivity to Fumonisin B1, whereas AtssSPTa knockdown lines show increased resistance compared to wild type (59). In addition to that, over expression of AtssSPTb M19V showed significant accumulation of C20-LCBs in plant, which also indicate that indeed both the AtssSPTs are limiting in plant. This is the first evidence which indicate that controlled expression of the small subunit is required for maintaining sphingolipid homeostasis.

CHAPTER 3: ssSPTs in *Schizosaccharomyces pombe*

INTRODUCTION:

Schizosaccharomyces pombe forms a major clade of the 'Ascomycete fungi' but it is believed to have branched off early in evolution (27). *S. pombe* and *S. cerevisiae* show a high degree of divergence between themselves, but neither of them are more closely related to humans than the other. Interestingly, the majority of *S. pombe* biological processes share more similarity with the metazoans (87) than with *Saccharomyces cerevisiae* (budding yeast). These differences between the two fungal species have been exploited to provide a better understanding of complex biological phenotypes in terms of phylogenetic divergence and for the identification of proteins which share common functions despite a lack of sequence homology.

The sphingoid base composition of *S. pombe* (fission yeast) is unique in that the predominant chain length of the LCB backbone is C20 compared to C18 in budding yeast, plants (33) and mammals. The candidate *lcb1* (SPAC29A3) and *lcb2* (SPAC2C4.02) subunits of *S. pombe* SPT were easily identified by their homology to the Lcb1p and Lcb2p subunits of *S. cerevisiae* SPT, and the Lcb2p subunit has been reported to be essential for *S. pombe* viability (58).

Since SPT catalyzes the first and committed step in sphingolipid biosynthetic pathway, it has been considered a potential regulatory point of sphingolipid metabolism in all organisms. From the studies in *S. cerevisiae*, plants and humans, it has become clear that SPT has a more complex subunit composition than a simple Lcb1/Lcb2p heterodimer. As discussed above, in *S. cerevisiae* the Lcb1-Lcb2p heterodimer requires Tsc3p (32) for its optimal activity, and these subunits exist in a complex with the

Ormp(s) (9; 44), which negatively regulate SPT activity. Similarly in humans, there are two functional orthologs of yeast Tsc3p (ssSPTa and ssSPTb) (43) and three homologs of Orms (ORMDL1-3) (9; 51; 90) (Dunn's lab, Sita Gupta unpublished data), which also physically associate with SPT and have been implicated in the regulation.

Arabidopsis also has two ssSPTs (see chapter 2) as well as two ORMs (AtORM1 and AtORM2), which have been recently been shown to regulate plant SPT (Kimberlin, A and Han, G unpublished data).

When expressed in *S. cerevisiae*, the *S. pombe* Lcb1p/Lcb2p heterodimer has negligible SPT activity, suggesting it too must have one or more small activating subunits. Interestingly, although evolutionary *S. pombe* is close to *S. cerevisiae*, there is no Tsc3p homolog in *S. pombe* genome, but there is a candidate ssSPT. If this candidate ssSPT is the activating subunit of *S. pombe* SPT heterodimer, this would represent the first ssSPT to be reported from a single cell organism. In addition, the presence of C20-LCBs in *S. pombe* raises the possibility that this ssSPT would confer a novel acyl-CoA chain preference.

The *S. pombe* ORM is also interesting as it lacks the well-defined N-terminal extension, phosphorylations of which regulate the *S. cerevisiae* ORMs. Surprisingly, in contrast to the human or plant ORMs, the candidate ORM in *S. pombe* does have a small N-terminal extension. This raises question about how this ORM might be regulated. Preliminary observations showed that human ORMs, which completely lacks the N-terminal extension failed to regulate human SPT in yeast. It would be interesting to investigate if the *S. pombe* ORM with its predicted small N-terminal extension will show regulation of *S. pombe* SPT, when all the subunits are heterologously expressed in *S.*

cerevisiae. Therefore *S. pombe* might provide a good genetic system for elucidating regulation by these ORMs. Identification and characterization of these novel candidate subunits of *S. pombe* SPT would be an important first step toward exploiting this lower eukaryote as a model system for better understanding of SPT regulation.

RESULTS:

LCB profile and SPT activity from wild type *Schizosaccharamoyces pombe*:

A previous report indicated that *S. pombe* has exclusively C20-LCBs (33). To verify this, total LCBs were isolated from wild type *Schizosaccharomyces pombe* (*S. pombe 972*) and analyzed by HPLC; showed that the vast majority of LCBs are C20. It is also interesting to note the presence of C20 sphingosine, which is produced by Δ^4 – desaturase (SDCB3b8.07c) (Figure 3.1) (33).

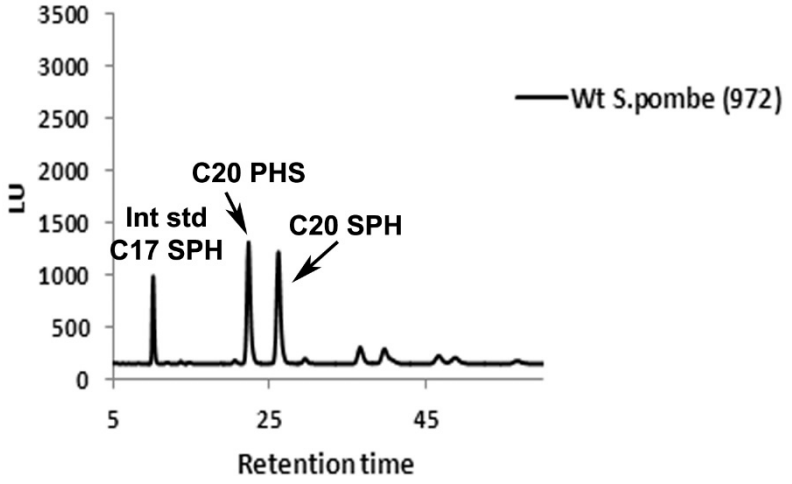


Figure 3.1. Total long chain bases (LCBs) were extracted from wild type *S. pombe* cells (972), and then resolved by HPLC.

Since the LCB chain length is dictated by the acyl-CoA species that is condensed with serine by SPT, it appears that *S. pombe* SPT has a preference for C18-CoA as substrate, thereby synthesizing C20-LCBs. To investigate this further, in vitro SPT activity was measured from wild type *S. pombe* microsomes with different chain lengths of acyl-CoAs. While the *S. pombe* microsomal SPT activity does have a clear preference for C18-CoA as substrate. Surprisingly there is also a robust activity with C16-CoA.

Thus the lack of C18-LCBs *in vivo* is unexpected. As it will be discussed later, there is not always a strong correlation between in vitro acyl-CoA utilization and the species of LCBs present *in vivo*, raising questions as to whether there is a dedicated acyl-CoA pool that is accessible to SPT *in vivo*.

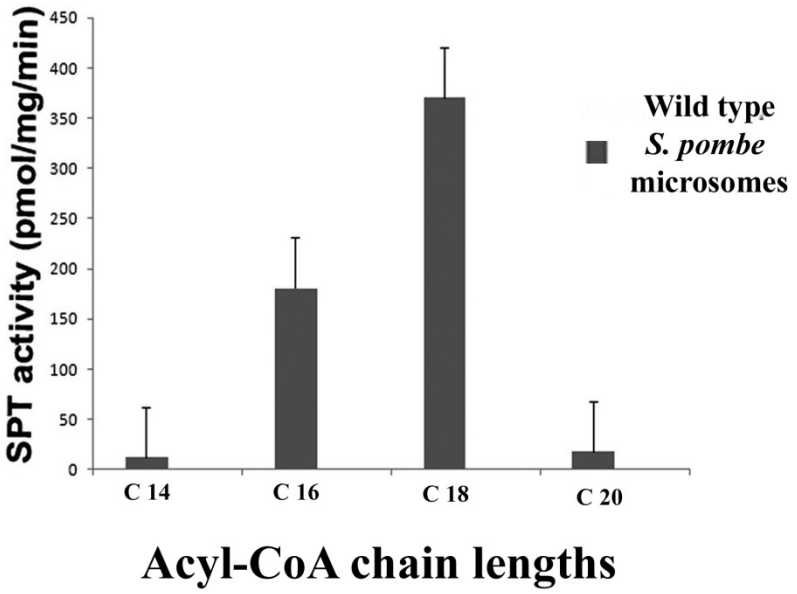


Figure 3.2. Wild type *S. pombe* microsomes have high SPT activity and a preference for C18-CoA.

Wild type *S. pombe* microsomes showed high SPT activity, with C18-CoA being the preferred substrate. Microsomes prepared from a yeast SPT knockout mutant expressing *S. pombe* Lcb1p and Lcb2p SPT heterodimer had undetecable SPT activity. SPT activities were measured using 200 µg of membrane proteins with 50 µM acyl-CoAs and 2 mM [^{3}H] serine.

Strikingly, microsomes prepared from *S. cerevisiae* cells heterologously expressing the *S. pombe* SPT heterodimer (PLcb1p and PLcb2p) had undetectable SPT activity. The lack of enzymatic activity associated with the heterologously expressed *S. pombe* Lcb1/Lcb2p heterodimer, taken together with the fact that human (43), plant (59) and yeast (*S. cerevisiae*) (32) all have their ssSPTs, motivated a search for candidate ssSPTs from *S. pombe*.

Identification of ssSPT homolog in *S. pombe*:

While there are no Tsc3p homologs, a candidate ssSPT was identified in the *S. pombe* genome by a Blast homology search using human ssSPTa as a query sequence (Figure 3. 3).

This weak ssSPT homolog encoded by SPAC23A1.05 (hereafter PssSPT) is 32.16% identical to human ssSPTa. Nonetheless this 11.65 kd protein (101 amino acid) is predicted to have a single transmembrane domain (amino acid 50-73) similar to human and plant ssSPTs. In contrast to the Arabidopsis ssSPTs (AtssSPTs), even when comparing the minimal functional (35 amino acid) domain of human ssSPT, there are only few residues in PssSPT that are conserved in the comparable region (Fig 3.3 bottom panel).

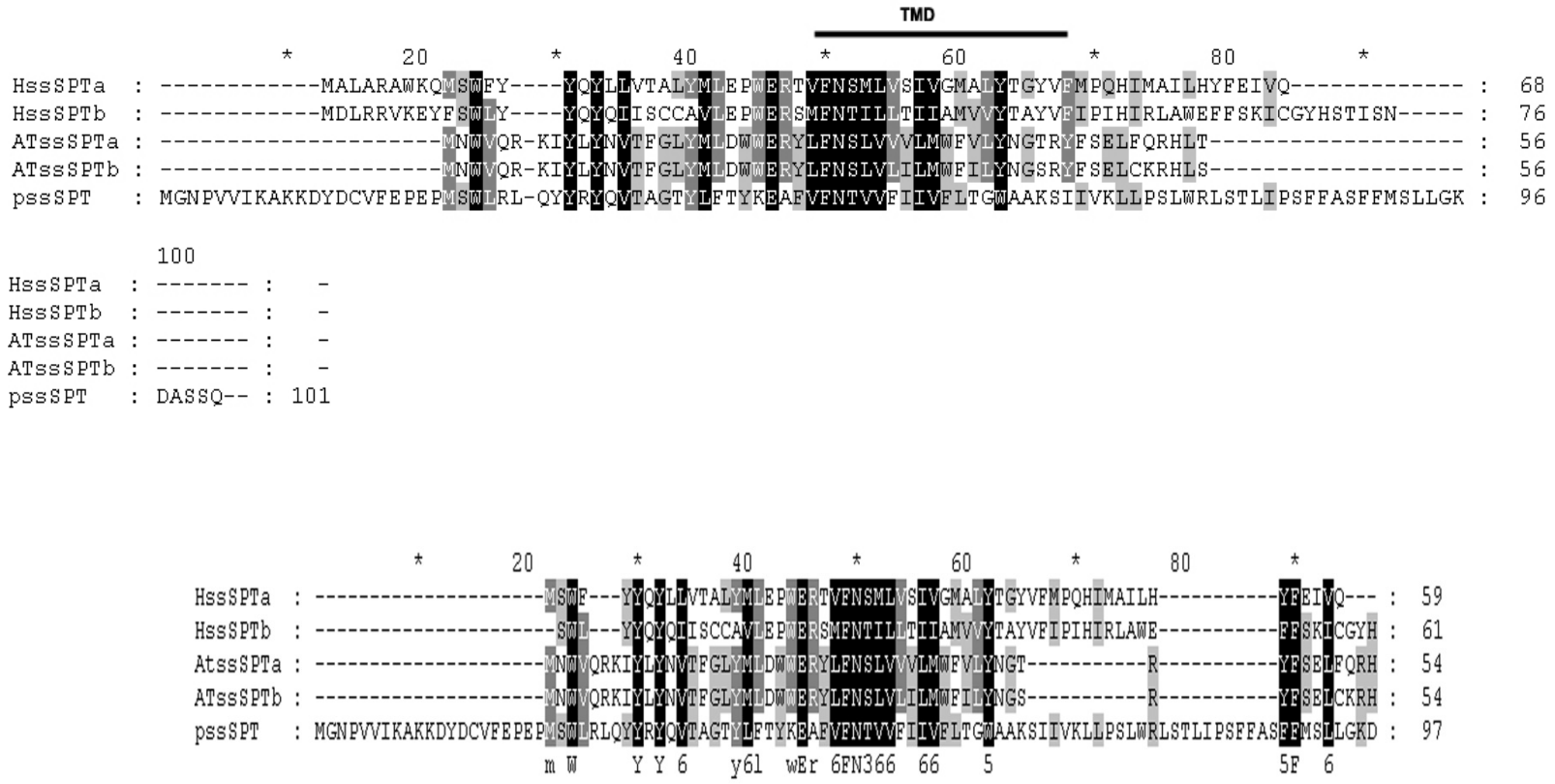


Figure 3.3. Sequence alignment of PssSPTp from *S. pombe* with human and plant small subunits. The solid black line represents the transmembrane domain designated as 'TMD' (Top panel). Sequence alignment of PssSPTp with the 'core' human ssSPTs and full length AtssSPTs.

PssSPTp is stably expressed and functional in *S. cerevisiae*:

S. cerevisiae lcb1Δtsc3Δ mutant cells lacking endogenous SPT were transformed with a plasmid expressing the epitope-tagged SPT subunits of *S. pombe* SPT (Lcb1p-Flag and Lcb2p-Myc with or without a plasmid expressing HA-tagged pombe ssSPTp [HA-PssSPT] candidate). As verified by immunoblotting, all of the subunits were expressed well, and expression of PssSPTp did not affect stability of the Lcb1p or Lcb2p subunit.

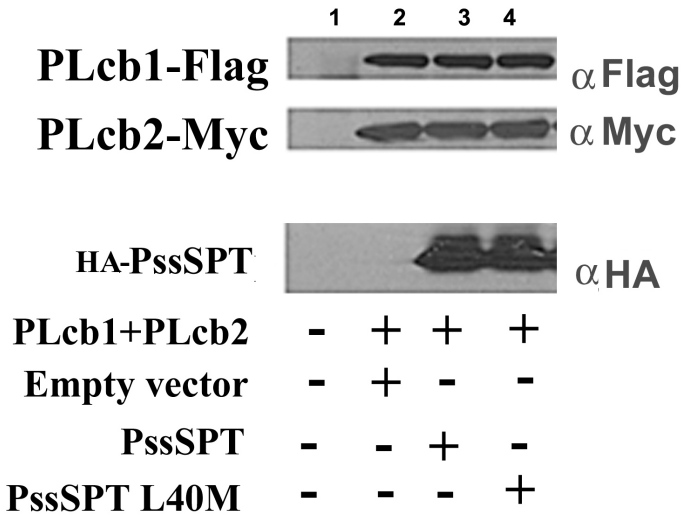


Figure 3.4. Expression of *S. pombe* SPT subunits.

The *S. pombe* SPT heterodimer Lcb1p-Lcb2p was heterologously expressed in yeast lacking endogenous SPT. The subunits of *S. pombe* SPT were detected using anti-Flag, Myc and HA to detect, PLcb1p, PLcb2p, PssSPTp and PssSPT L40M respectively.

The ability of the PssSPTp to activate the *S. pombe* Lcb1p/Lcb2p heterodimer was investigated. *S. pombe* Lcb1p/Lcb2p heterodimer, was unable to support growth of an *S. cerevisiae lcb1Δtsc3Δ* mutant without PHS, coexpression of the PssSPTp complemented the PHS auxotrophy, not only at 26 ºC, but even at 37 ºC, where the

requirement for SPT activity is higher. Thus, the PssSPTp is indeed a bona fide activating subunit of the pombe (Lcb1p-Lcb2p) heterodimer (Figure 3.5).

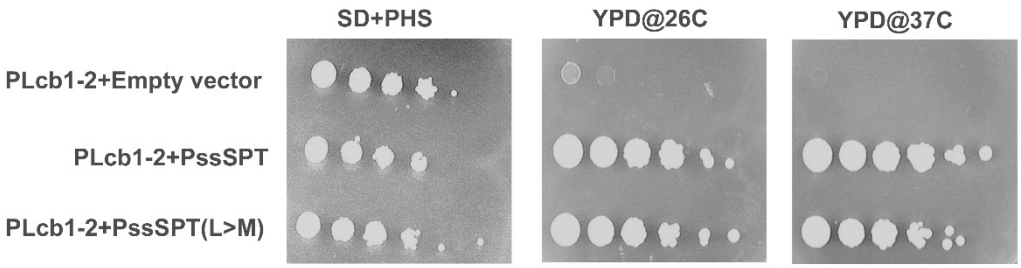


Figure 3.5. PssSPTp activates the *S. pombe* SPT heterodimer in yeast.

> Yeast SPT knockout cells heterologously expressing PLcb1p-Flag, PLcb2p-Myc with empty vector or with wild type or mutant HA-tagged PssSPTp were tested for complementation. Results indicate that both the wild type and the L40M mutant can complement the *S. pombe* SPT heterodimer.

PssSPTp coimmunoprecipitates with its cognate SPT heterodimer:

To confirm that PssSPTp binds directly to *S. pombe* Lcb1p/Lcb2p heterodimer, immunoprecipitation experiments were conducted. Immunoprecipitation of the pombe Lcb1-Flag subunit resulted in coimmunoprecipation of the pombe Lcb2p-Myc subunit. Moreover, when microsomes from cells coexpressing the candidate HA-PssSPTp with the heterodimer were used for the Flag-IPs, HA-tagged PssSPTp specifically co-purified with Lcb1p-Flag and Lcb2p-Myc (Figure 3.6). These data show that PssSPTp interacts

with the heterodimer, and taken together with the data presented above, show that SPAC23A1.05 encodes an ssSPT in *S. pombe*.

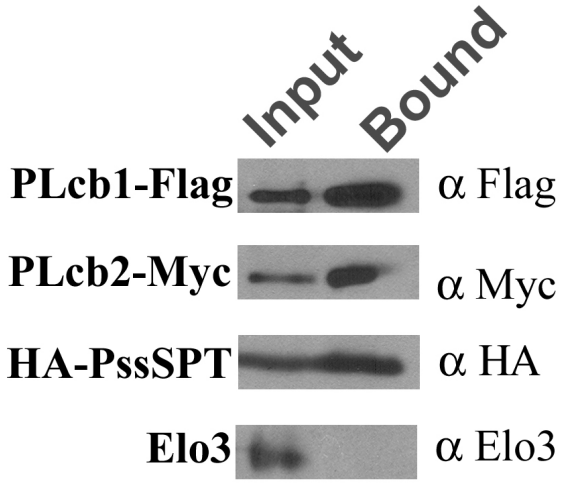


Figure 3.6. PssSPTp coimmunoprecipitates with the *S. pombe* SPT heterodimer.

Microsomes were prepared from yeast SPT knockout mutants heterologously expressing PLcb1-Flag/Myc-PLcb2p heterodimer with PssSPTp. Cells were grown on minimal media overnight. Microsomes were then solubilized with 1% Digitonin (~1:30 hr) and incubated with anti-Flag beads. The immunoprecipitate was resolved by SDS-PAGE and the indicated proteins were detected by immunoblotting.

PssSPTp shows strong activation of the heterodimer and confers a preference for C18-CoA as substrate

To investigate the extent of activation of the SPT heterodimer by PssSPTp and its influence on substrate utilization, we measured SPT activities of wild type (972) *S. pombe* microsomes and compared them with *S. cerevisiae* microsomes expressing the PLcb1p-PLcb2p heterodimer with or without HA-PssSPTp using different chain lengths of acyl-CoA (C14, C16, C18 and C20) as substrates (Figure 3.7).

Coexpression of PssSPTp resulted in robust activation of the SPT (PLcb1p-Lcb2p) heterodimer with all four acyl-CoAs. Enzymatic activity was highest with C18-CoA, but also robust with C16-CoA. The level of activity of wild type *S. pombe* microsomes with C18-CoA is similar to that of microsomal SPT activity of *S. cerevisiae* with C16-CoA (typically about 400 pmol/mg/min). Although not shown, SPT activity of PLcb1p/Lcb2p heterodimer is undetectable. This is consistent with the complementation data (Figure 3.5), where coexpression of PssSPTp showed strong rescue of PHS auxotrophy, and LCB analysis from wild type *S. pombe* where C20 sphingoid bases are predominant. This is also consistent with total long chain base analysis from yeast expressing *S. pombe* SPT heterotrimer with PssSPTp which showed significant accumulation of C20 sphingoid base products (Figure 3.8).

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Surprisingly, in wild type *S. pombe* microsomes, enzymatic activity with C18-CoA was much higher than in *S. cerevisiae* microsomes expressing the *S.pombe* SPT heterotrimer. It seems unlikely that this is due to lower expression, because activity with C14- and C20-CoA was much higher in the *S. cerevisiae* microsomes. In addition to that, lower expression of the PssSPTp must result in lower SPT activity but should not alter the substrate utilizations. This raises the possibility that there are other regulatory components that contribute to SPT activity in *S. pombe*.

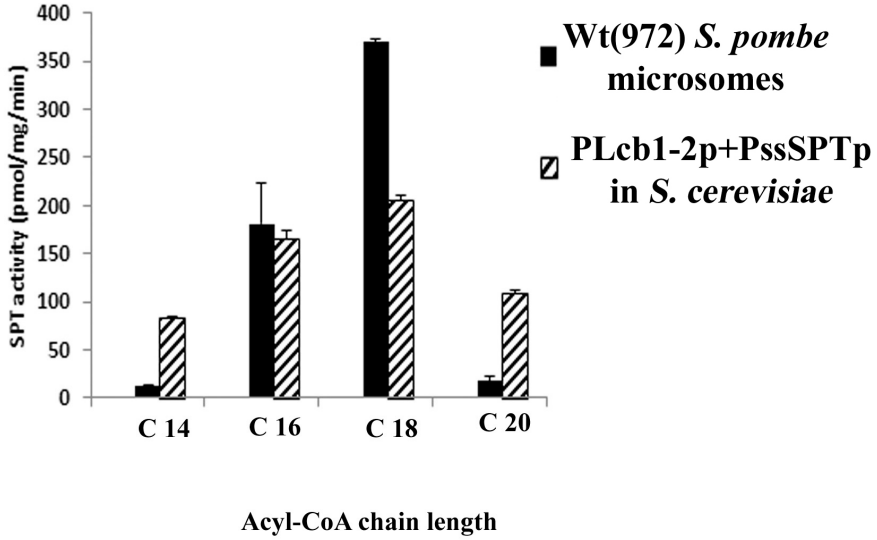

Figure 3.7. PssSPTp activates the SPT heterodimer *in vitro*.

Microsomal SPT activities were compared between wild type *S. pombe* microsomes and microsomes prepared from *S. cerevisiae* cells heterologously expressing *S. pombe* SPT heterodimer and PssSPTp. Microsomal SPT activity of yeast microsomes expressing the *S. pombe* heterodimer with an empty vector was undetectable. SPT activities were measured using different chain lengths of acyl-CoA substrates. Results indicate that coexpression of PssSPTp restores the activity almost to the levels of wild type *S. pombe* microsomes.

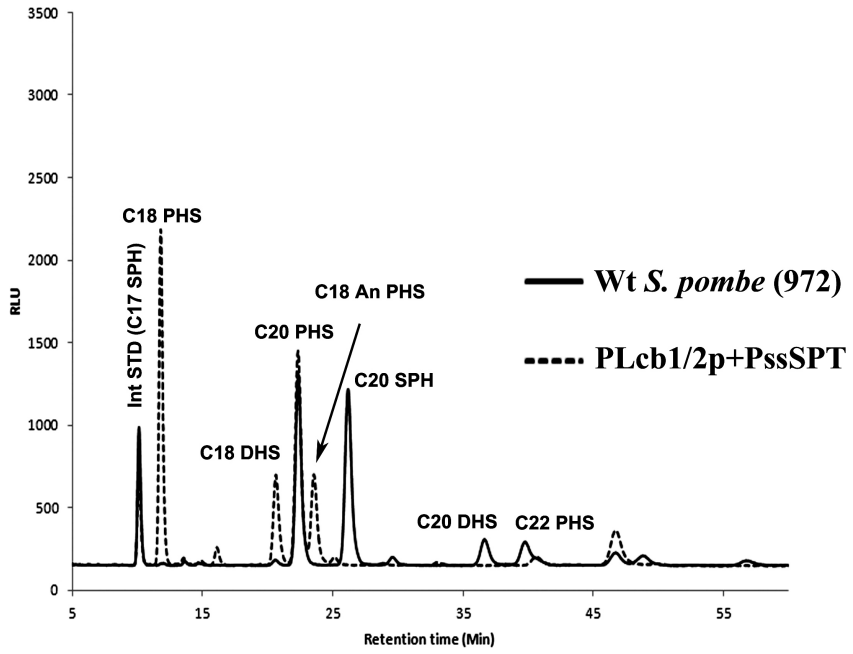

Figure 3.8. Comparison of LCB profiles of yeast cells expressing *S. pombe* SPT heterotrimer (PLcb1/Lcb2p+PssSPTp) and LCB profiles of wild type *S. pombe* strain.

5 ODs of cells were harvested, and the total long chain base profiles were compared between wild type *S. pombe* strain and yeast cells heterologously expressing *S. pombe* SPT trimers. In both strains, total LCB profiles showed that significant accumulation of C20-LCBs, suggesting C18 is the preferred substrate.

Residue responsible for substrate preference:

A single residue has been found to be responsible for dictating substrate preference in human and in plant ssSPTs (48; 59). In human ssSPTa, Met-25 and the corresponding residue in plant ssSPTs (Met-19) confers C16 preference to their cognate SPT heterodimers. In contrast, the corresponding residue in human ssSPTb (Val-25) confers a preference for C18-CoA.

To address if the corresponding residue (Leucine 40) in PssSPTp is responsible for substrate selection, Leu 40 was mutated to Met, and tested for altered substrate preference (L40M). Expression of this mutant PssSPTp was comparable to the wild type PssSPTp (Fig 3.4) and resulted in similar complementation profile to that of the wild type PssSPTp (Fig 3.5). *S. cerevisiae* cells heterologously expressing the wild type PssSPTp showed accumulation of C20 long chain bases, whereas the L40M mutant PssSPTp showed a significant decrease in C20 sphingoid bases and a corresponding increase in C18 products (Figure 3.9).

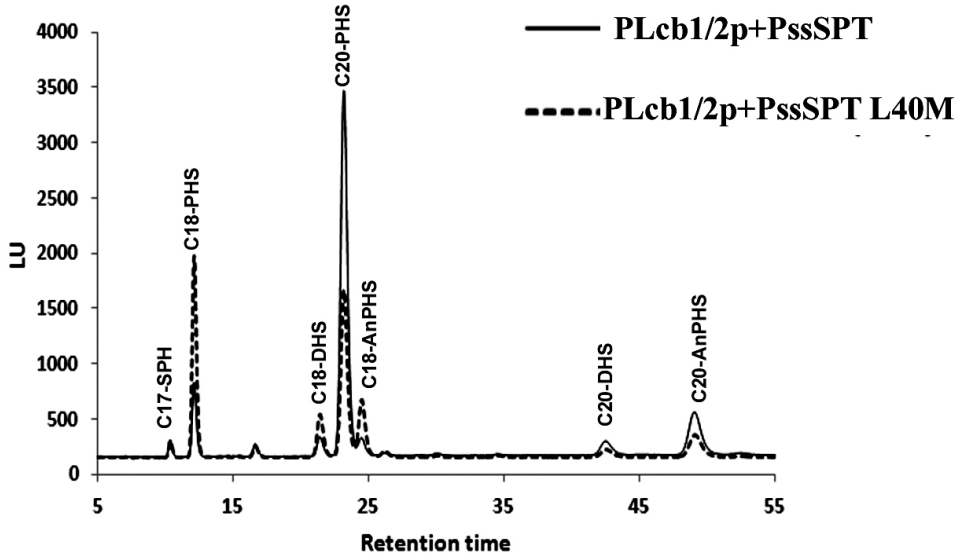

Figure 3.9. L40M mutation in PssSPTp showes altered substrate preference.

HPLC analysis of total long chain bases (LCBs) were compared between yeast cells heterologously expressing the *S. pombe* Lcb1-2p heterodimer either with the wild type (solid line) or the with PssSPTp L40M mutant (broken line). The

PssSPT L40M mutant activated the PLcb1p/PLcb2p but altered the acyl-CoA preference from C18 to C16.

Activation of a broad range of higher eukaryotic SPT heterodimers:

It was shown by Han et al that the human ssSPTs rescue the PHS auxotrophy of a yeast *tsc3Δ* single mutant, indicating that the lack of homology between the human ssSPTs and Tsc3p both are recognized by the *S. cerevisiae* Lcb1p/Lcb2p heterodimer (43). In contrast, Tsc3p fails to activate higher eukaryotic heterodimers in *S. cerevisiae* suggesting that Tsc3p is not recognized by higher eukaryotic SPT heterodimers. It should be pointed out that Tsc3p is unstable without coexpression of the *S. cerevisiae* Lcb1p-Lcb2p, and thus it has not been possible to determine whether Tsc3p can bind the higher eukaryotic heterodimers. Both *S. cerevisiae* and *S. pombe* belong to the same Ascomycota phylum, but there is no homology between yeast Tsc3p and PssSPTp. As an ssSPT that presumably arose early in evolution, it was of interest to determine whether PssSPTp is able to activate higher eukaryotic SPT heterodimers.

Accordingly, PssSPTp was coexpressed with the plant, human or drosophila SPT heterodimers (Figure 3.10, Figure 3.11, and Figure 3.12) respectively in an *S. cerevisiae lcb1Δtsc3Δ* mutant and growth without PHS at 26 ºC, 32 ºC and 37 ºC was measured.

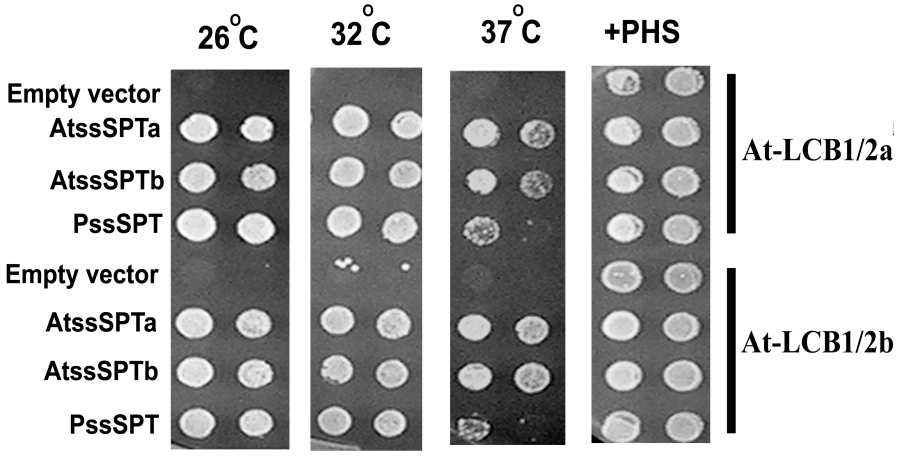

Figure 3.10. PssSPTp activates the plant (*A. thaliana*) SPT heterodimers (AtLCB1-LCB2a and AtLCB1-LCB2b) in yeast.

PssSPTp was expressed along with AtLCB1-AtLCB2a or AtLCB1-AtLCB2b in an *S. cerevisiae lcb1Δtsc3Δ* mutant. Cells were serially transferred to YPD or SD+PHS and incubated at the indicated temperatures for 4 days.

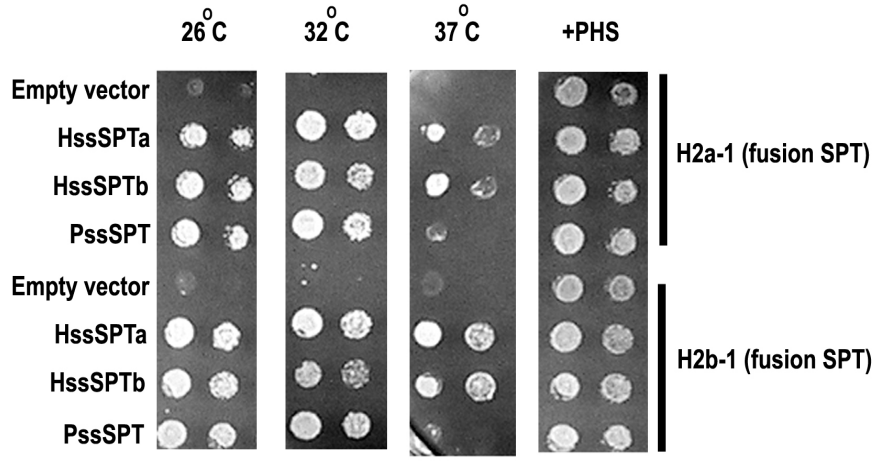

Figure 3.11. PssSPTp activates the human SPT heterodimers (HLCB2a-LCB1 and HLCB2b-LCB1 (fusion protein lacking first transmembrane domain of Lcb1p) in yeast.

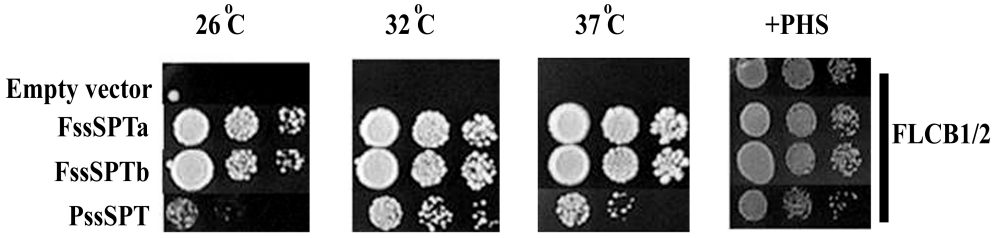

Figure 3.12. PssSPTp weakly activates the fly LCB1-LCB2 heterodimer.

An *S. cerevisiae lcb1Δtsc3Δ* mutant heterologously expressing the fly SPT heterodimer (FLCB1-LCB2) along with FssSPTa or FssSPTb (discussed in chapter 4) or with PssSPTp, was tested for growth on YPD or SD+PHS at 26 °C, 32 °C or 37 °C. PssSPTp rescued the PHS auxotrophy of the fly SPT heterodimer and allowed growth at elevated temperatures. However, compared to the cognate fly ssSPTs, activation by PssSPTp is weak.

Coexpression of PssSPTp in *S. cerevisiae* with heterologously expressing the plant or human SPT heterodimers provided sufficient SPT activity to support growth at

26 ºC or 32 ºC, but not at 37 ºC., Activation of the fly SPT heterodimer by PssSPTp was weaker, but nonetheless evident.

Activation of the higher eukaryotic SPT heterodimers, by PssSPTp, *in vitro* SPT activity of yeast microsomes heterologously expressing the plant (AtLCB1-AtLCB2) heterodimer with an empty vector or with AtssSPTs or PssSPTp was measured (Figure 3.13).

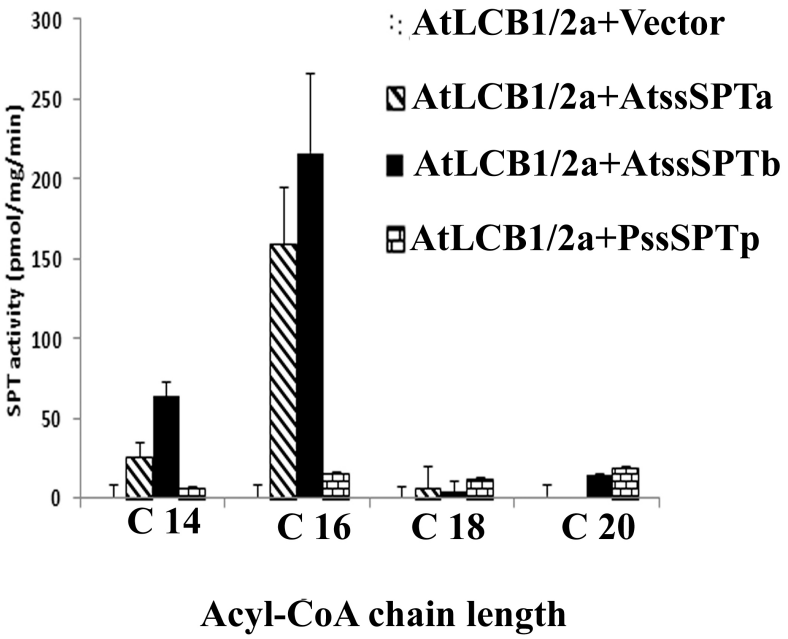

Figure 3.13. PssSPTp weakly activates the AtLCB1-AtLCB2a heterodimer *in vitro*.

Microsomes were prepared from yeast (*S. cerevisiae*) cells heterologously expressing the AtSPT heterodimer with empty vector, the AtssSPTs or PssSPTp. SPT activity was measured with the indicated chain lengths of CoAs as substrate. 200 µg of microsomal protein and 50 µM CoA was used per assay.

Indeed PssSPTp showed weak activation of the plant heterodimer. As discussed later (See chapter 6), a screen for suppressors of *tsc3Δ* identified gain of function mutations in Lcb2p that increased the basal SPT activity. From these studies, it is clear that very low SPT activity (10-20 pmol/mg/min) is adequate to support growth of *S. cerevisiae* at 37 ºC. Thus it is not surprising that this low level of activation is sufficient to complement the PHS auxotrophy at 26 ºC and 32 ºC. When total long chain bases were compared between *S. cerevisiae* cells expressing plant or human SPT heterodimers either with their cognate small subunits or with PssSPTp. The results showed that PssSPTp exerted its C18 preference on the plant and human SPT heterodimers expressed in *S. cerevisiae* (Figure 3.14, Figure 3.15).

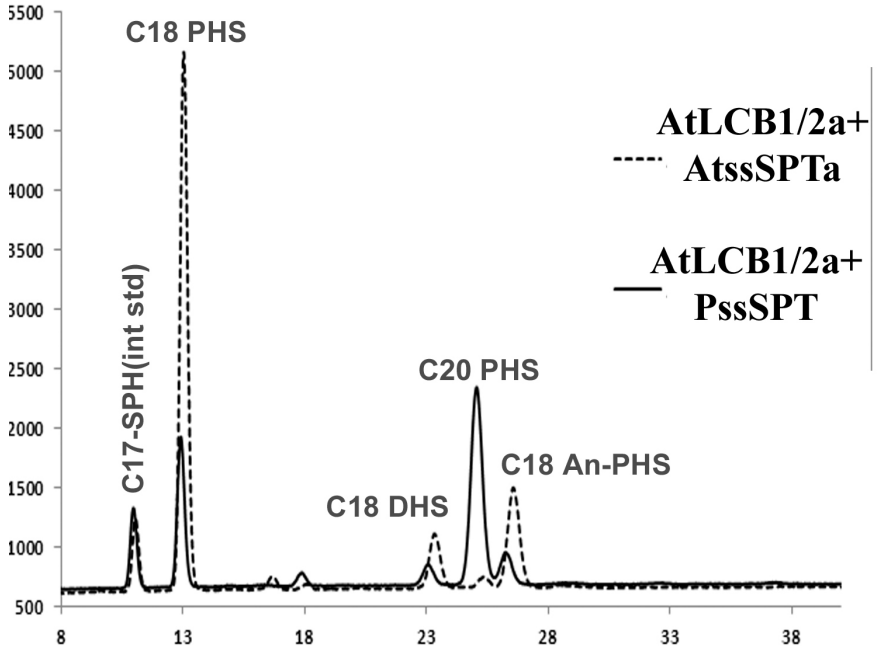

Figure 3.14. Consistent with its in vitro enzymatic activity, the PssSPTp-activated AtLCB1-AtLCB2a heterodimer generates significant amounts of C20-LCBs in *S. cerevisiae*.

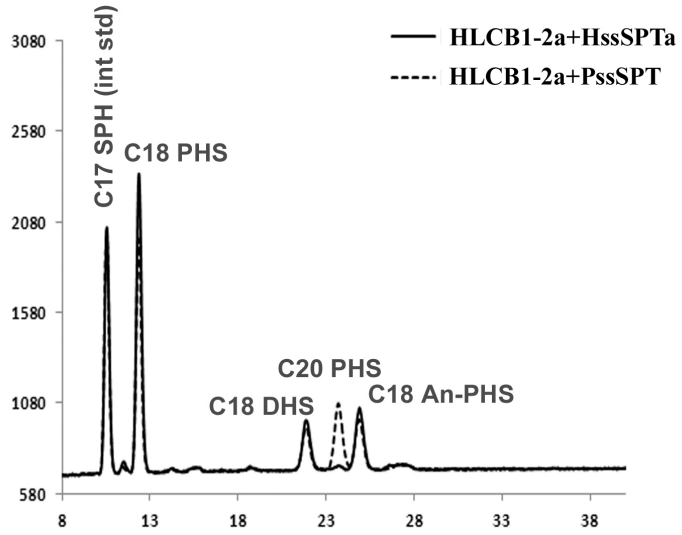

Figure 3.15. PssSPTp also influences the acyl-CoA preference of the human SPT heterodimer (human LCB2a-LCB1 fusion protein lacking the first transmembrane domain of LCB1) resulting in significant accumulation of C20-LCBs.

'Orm' proteins in *S. pombe*:

The recent discovery of the Orm proteins in *S. cerevisiae* (9; 44), and their role in integration of different physiological and environmental cues to maintain sphingolipid homeostasis is an active area of research. In *S. cerevisiae*, two Orm proteins (Orm1p and Orm2p) interact with the *S. cerevisiae* (Lcb1p, Lcb2p and Tsc3p) SPT complex to down regulate sphingolipid biosynthesis. The extent to which they inhibit SPT is controlled by the phosphorylation state of their N-terminal domains (9).

Identification of Orm proteins in *S. pombe*:

An homology search with yeast Orm2p as query identified a single homolog in *S. pombe* (POrmp) with 52.15% identity, annotated as a 'predicted ORMDL family protein'. This 186 amino acid protein is a transcript of SPBC119.09c, with four predicted transmembrane domains consistent with other members of the Orm family of proteins (Figure 3.16). In contrast to the *S. cerevisiae* ORMs, but similar to the human ORMDLs, the *S. pombe* ORM lacks the N-terminal extension that is important in the regulation by the *S. cerevisiae* ORMs.

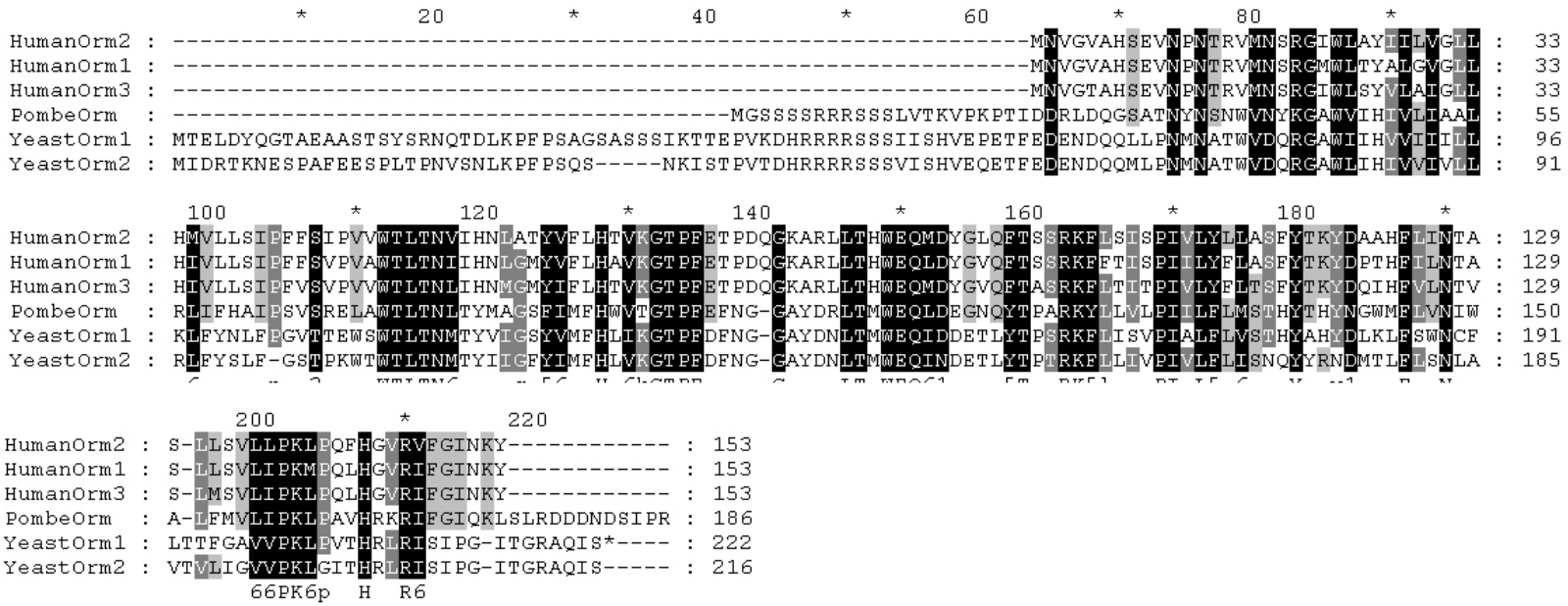

Figure 3.16 Amino acid sequence alignment of *orm* candidate gene from *S. pombe* (POrmp) with human, and yeast Orm proteins.

Functionality of Porm in yeast:

To test whether the POrm binds and regulates *S. pombe* SPT, HA-POrmp was expressed in a *S. cerevisiae* homolog, POrmp was expressed in a yeast *orm* knockout strain (*lcb1Δorm1Δorm2Δ*) along with PLcb1-Flag, PLcb2p-Myc, and HA tagged PssSPTp. Expression of the subunits was confirmed by immunoblitting with anti-Flag, Myc and HA antibodies. The results indicate that POrmp is stably expressed with *S. pombe* SPT heterotrimer (Figure 3.17).

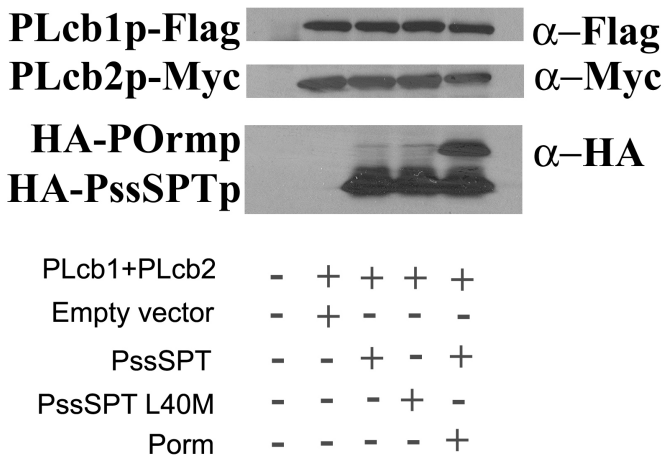

Figure 3.17. *S. pombe* Orm protein (POrmp) is stably expressed in yeast along with Lcb1p, Lcb2p, and PssSPTp.

Microsomes were prepared from yeast cells expressing the *S. pombe* SPT heterodimer (PLcb1p-Flag, PLcb2-Myc) along with empty vector (pADH)/HA-tagged PssSPTp/ HA-tagged PssSPT (L40M) and HA-tagged POrmp. 45 µg of membrane proteins were resolved by SDS-PAGE and the proteins were detected by immunoblotting.

It has been shown the Orm proteins down regulate long chain base levels in *S. cerevisiae* (9; 44). The *orm1Δorm2Δ* mutant has highly elevated LCBs, which cause a cold-sensitive phenotype. The *orm1Δorm2Δ* mutant is also sensitive to tunicamycin because, tunicamycin amplifies the cytotoxic effects of high free long chain bases, resulting in ER stress. Expression of POrmp weakly rescued the tunicamycin sensitivity of *S. cerevisiae* expressing the S. pombe SPT heterotrimer (Figure 3.18), indicating that this protein might be functional in *S. cerevisiae*.

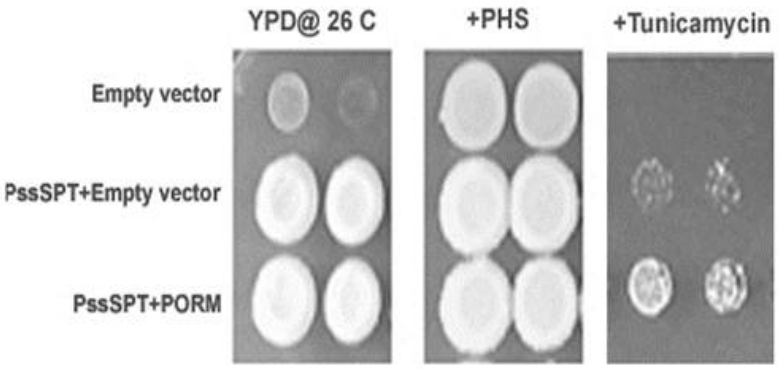

Figure 3.18. The *S. pombe* Orm (Pormp) weakly rescues the tunicamycin (1 mg/ml) sensitivity of yeast cells heterologously expressing PLcb1p-PLcb2p/ PssSPTp.

S. cerevisiae endogenous SPT knockout mutants heterologously expressing the *S. pombe* SPT heterotrimer (PLcb1p-PLcb2p and PssSPTp) with or without the POrm subunit. Coexpression of POrmp showed weak rescue of the tunicamycin (1mg/ml) sensitivity associated with expression of *S. pombe* SPT.

Pormp barely interacts with the *S. pombe* SPT heterotrimer and does not alter the total long chain base levels:

To further investigate whether POrmp down regulates *S. pombe* SPT we quantified the total long chain base levels of *S. cerevisiae* expressing *S. pombe* SPT with or without POrmp (Figure 3.19). The results showed that expression of POrmp had no effect on the sphingolipid levels. This raises the possibility that although it is expressed (Fig 3.17) POrmp might not interact with the *S. pombe* SPT heterotrimer expressed in S. cerevisiae.

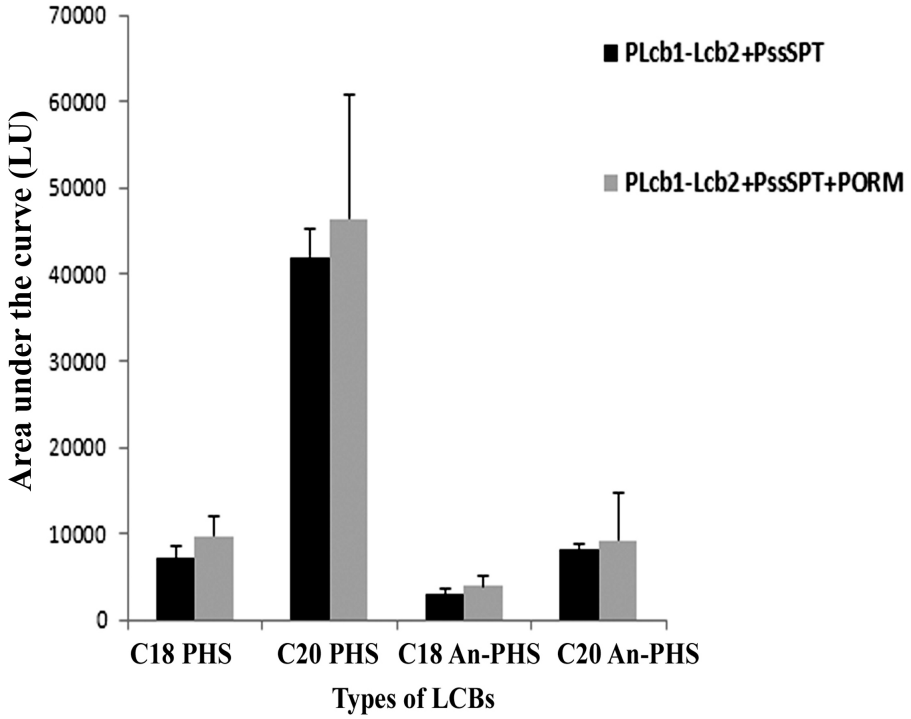

Figure 3.19. Pormp does not alter LCB levels of *S. cerevisiae* expressing the *S. pombe* SPT heterotrimer.

Total long chain bases were extracted from yeast cells expresssing the *S. pombe* SPT heterotrimer with or without Pormp. Three individual transformants were

grown on YPD media. 15 ODs of cells were harvested, LCBs were extracted and 5 ODs were used for the analysis by HPLC.

Immunoprecipitation of PLcb1p-Flag co-precipitated PLcb2p-Myc, HA-PssSPTp, but HA-POrmp was barely associated with the complex (Figure 3.20). This weak interaction of POrmp with the core SPT complex is consistent with the total LCB analysis. Further studies are needed to determine the POrmp binds *S. pombe* SPT in the host organism (*S. pombe*).

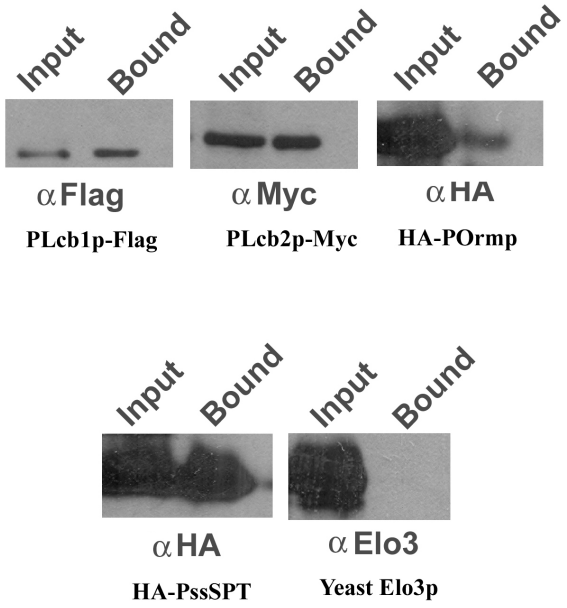

Figure 3.20. Pormp is weakly bound to the *S. pombe* SPT heterotrimer when the proteins are expressed in *S. cerevisiae*.

Solubilized microsomes were prepared from *S. cerevisiae* heterologously expressing *S. pombe* Lcb1-Flag, PLcb2-Myc, HA- PssSPTp and HA- Pormp.

PLcb1p was pulled down using Flag resin, and the immunoprecipitate was resolved by SDS PAGE. Yeast Elo3p was used as a negative control.

POrmp does not regulate *S. cerevisiae* SPT:

To test the ability of POrmp to regulate the *S. cerevisiae* SPT heterotrimer, HA-tagged POrmp was expressed in an *S. cerevisiae orm1Δorm2Δ* mutant and tested for its ability to rescue tunicamycin (1 mg/ml) and cold sensitivity (growth at 17 ºC). The results show that, as expected, expression of either *S. cerevisiae* Orms (Orm1p or Orm2p) rescued both phenotypes. However expression of POrmp failed to do so, showing that POrmp does not regulate yeast SPT (Figure 3.21).

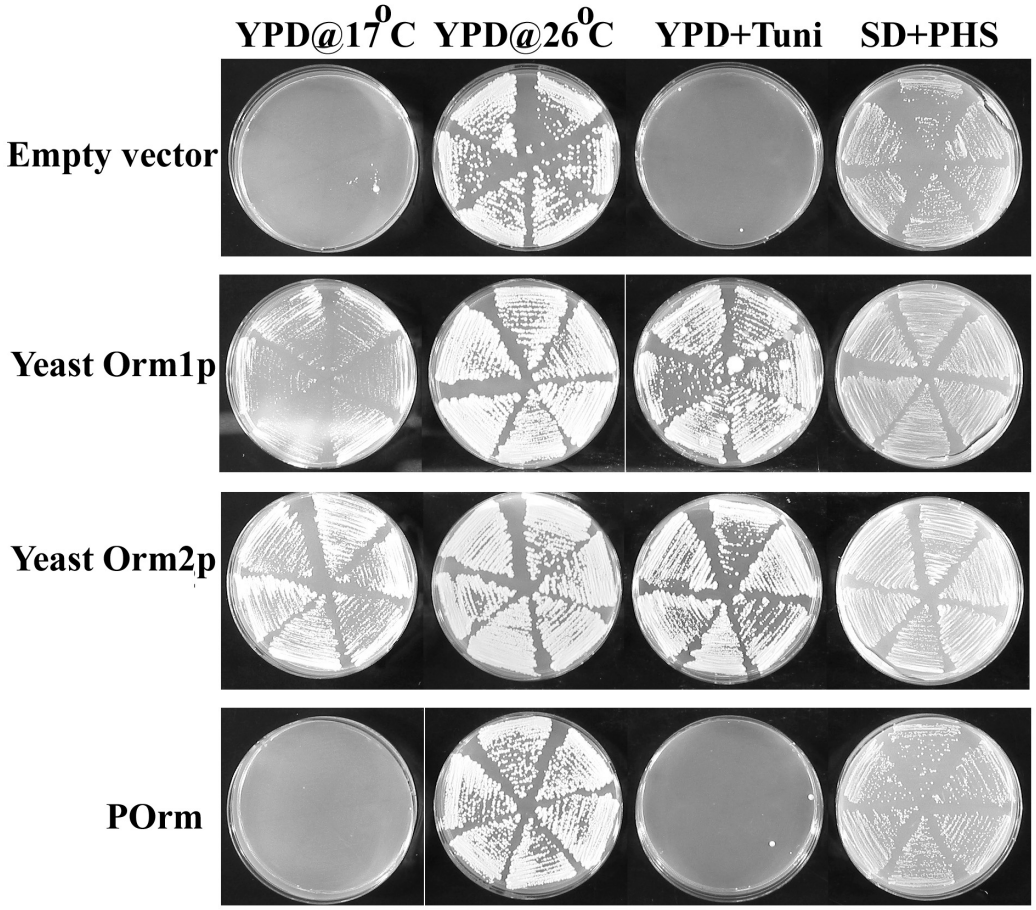

Figure 3.21. POrmp does not regulate the *S. cerevisiae* SPT.

Plasmids expressing the *S. cerevisiae* and the *S. pombe* Ormp (POrmp) were transformed into an *S. cerevisiae orm1Δorm2Δ* knockout mutant. 6 transformants were picked and tested for growth at 17 °C or on tunicamycin (1 mg/ml). *S. cerevisiae* Orm1p and Orm2p rescued sensitivity of the *orm1Δorm2Δ* mutant to cold and tunicamycin sensitivity, whereas Pormp failed to do so.

DISCUSSION:

A single 101 amino acid protein was identified as a 'small subunit of serine palmitoyltransferase' in *S. pombe*. Similar to Tsc3p, and the human and plant ssSPTs, this protein also interacts with and activates its cognate SPT heterodimer. Despite its size, this protein is predicted to have a single predicted transmembrane domain, consistent with small subunits from other species.

Although the basal activities of other eukaryotic heterodimers, such as yeast, plant and human, are very low, they are detectable. Surprisingly, expression of the *S. pombe* SPT heterodimer in *S. cerevisiae* resulted in undetectable enzymatic activity, raising the possibility that, unlike other eukaryotes, the *S. pombe* SPT heterodimer is completely dependent on PssSPTp for activity. In this regard, a whole genome wide knockout study reported that PssSPTp is essential (58).

A comparison of *in vitro* SPT activities revealed that the specificity for C18-CoA utilization is much higher in the wild type *S. pombe* microsomes than in microsomes prepared from *S. cerevisiae* cells heterologously expressing the *S. pombe* SPT heterotrimer (Figure 3.7). This is consistent with the LCB profiles of wild type *S. pombe* and *S. cerevisiae* cells heterologously expressing *S. pombe* SPT trimer (PLcb1p-PLcb2p+PssSPTp) (Figure 3.8), because unlike wild type, *S. cerevisiae* expressing *S. pombe* SPT (PLcb1p, PLcb2p and PssSPTp) showed significant accumulation of C18 sphingoid bases. This finding raises the possibility that other factors, such as selective substrate availability may be responsible for this observation. It is also possible that there are other proteins that orthologs which confer a strict C18-CoA preference either by modulating SPT or by perpetually degrading shorter LCBs. It is surprising that the insight

obtained from the human and plant small subunits regarding the residue responsible for acyl-CoA selectivity can be successfully translated to PssSPTp, despite the low homology (Figure 3.9).

Here we have shown that unlike Tsc3p, PssSPTp has the ability to activate a broad range of higher eukaryotic SPT heterodimers. This is consistent with the fact that PssSPTp shares homology with higher eukaryotic ssSPTs, but not with *S. cerevisiae* Tsc3p. Major clustering of the conserved residues among different higher eukaryotic ssSPTs is in the central domain, encompassing the predicted transmembrane domain. The residue that dictates the substrate preference of the heterodimer is within this region as well. However we failed to predict any such 'core region' in yeast Tsc3p because deletion of even a few residues from either the N or C-terminus renders Tsc3p unstable. Whether a single residue of Tsc3p that is responsible for shifting the acyl-CoA preference of *S. cerevisiae* Lcb1p-Lcb2p from C14-CoA to C16-CoA is not known.These suggest that functionally higher eukaryotic ssSPTs including PssSPTp belong to a different class than yeast Tsc3p.

Only one reported *S. cerevisiae* Ormp homolog exists in *S. pombe*. Orm family members show a relatively high degree of homology between different species, compared to the small subunits. Coexpression of this protein weakly rescued the tunicamycin sensitivity exhibited by the yeast cells expressing *S. pombe* SPT heterotrimer. However, coexpression of POrmp did not show any influence on the total long chain base levels, suggesting that weak rescue of tunicamycin sensitivity is not because of decreased long chain base levels. Despite good expression of POrmp, it interacted weakly with the heterotrimeric SPT complex, explaining its lack of function in yeast.

Homology alignments of eukaryotic Orms show that the higher eukaryotic Orms lack the amino-terminal extension that contains the sites of phosphorylation that is reported to be responsible for modulating the inhibitory influence of yeast Ormp(s) on yeast SPT (Figure 3.16). Specifically when the *S. cerevisiae* Ormps are phosphorylated, they no longer inhibit SPT although they may still bind. POrmp also lacks the well defined N-terminal extension like Orm1p, Orm2p, but it does contain a short stretch of 'Ser/Thr' in its N-terminus which may be important for POrmp regulation. It is possible that in the *S. cerevisiae* background POrmp post translational modifications required for binding.

In contrast to POrmp, heterologously expressed is PssSPTp is competent to activate the *S. pombe* heterodimer. This is consistent with the fact that influence of PssSPTp is not only shown in the in vitro enzyme kinetics, but it also showed its ability to activate broad range of higher eukaryotic SPT heterodimers. Our characterization of the candidate Pormp indicates that it might influence the SPT complex indirectly. It is possible that Pormp requires other factors for its activity. Identification of ssSPT homolog in *S. pombe* and the candidate Orm homolog will help us to understand the physiological roles of these novel subunits and underlying mechanism of SPT regulation in *S. pombe*.

CHAPTER 4: ssSPTs in *Drosophila melanogaster*

INTRODUCTION:

Sphingolipid metabolism in *Drosophila melanogaster* (fly) is an active area of research. It is a good model system to study the roles of sphingolipids in growth, survival and development (3; 5). By analogy to other eukaryotic SPTs, fly SPT is believed to be a heterodimer composed of LCB1 and LCB2 (also called Lace) (2; 84) and there is one gene encoding each of these highly conserved subunits in the drosophila genome. LCB1 (468 amino acid) is encoded by the Spt-1/CG4016 gene and LCB2 or Lace (597 amino acids) by the Lace/CG4162 gene. Flies that are homozygous for the LCB2 null allele die during the first instar larval stage. However, LCB2 hypomorphic mutants survive to adulthood but display distinct external organ deformities (2), which can be rescued by elevated activity of Dsor1, a MAP Kinase (2), indicating a complex interaction between sphingolipid biosynthesis and signaling pathways. Sphingosine-1-phosphate (S1P), a byproduct of the sphingolipid biosynthetic pathway, plays important roles as a signaling molecule in Drosophila. Aberrant S1P levels have been associated with different morphological defects in flies, which can be rescued by the introduction of a hypomorphic LCB2 gene (50). These observations suggest that fly SPT is not only required for maintaining proper sphingolipid levels, but that tight regulation of this enzyme is required for insuring proper homeostasis of metabolic intermediates that act as essential signaling molecules.

Drosophila has a unique sphingolipid composition of C14 and C16 containing sphingoid bases, whereas in mammals LCBs are composed mainly of C18 and C20 (1;

31; 38). It has been proposed that the shorter chain lengths may be maintaining membrane fluidity at their preferred body temperature of ~25ºC (52; 63). The presence of the C14, C16-sphingoid bases suggests that fly SPT should use C12- and C14 acyl-CoAs as substrate. However, this had not been experimentally verified and in fact, it has been reported that the SPT activity is barely detectable in fly microsomes (2).

The recent discovery of human and plant 'small subunits of serine palmitoyltransferase (ssSPTs) (43; 59) raised an interesting possibility that such homologs of ssSPTs exist in fly. Not only do the ssSPTs activate their cognate LCB1/LCB2 heterodimers, but, they also dictate the acyl-CoA chain length preference of the heterodimer, and thus we hypothesized that such homologs of ssSPTs from fly would activate the heterodimer and confer C12- or C14-acyl-CoA substrate preference. Identification and biochemical characterization of these subunits will help to understand the underlying mechanism of sphingoid base diversity in flies.

It has been reported that lipids play a very important role in fly sperm development. (105). A functional screen to identify genes involved in fly spermatogenesis revealed 19 genes (34), mutations in which altered spermatogenesis. Interestingly, one of the genes identified in this screen, known as 'Frodo" or alternatively as 'ghiberti', encodes a protein that has 37% homology to the human ssSPTa. Flies carrying this mutation show male meiotic cytokinesis defects, where the furrow regresses, a phenotype that is also found in other mutants with defects in membrane trafficking (10; 38). Guan et al mapped one of the Ghiberti mutant alleles and showed that the mutation converts the stop codon to a tyrosine codon that results in a 44-amino acid C-terminal extension. Consistent with a possible role in regulating SPT activity, the

Ghiberti mutant flies have higher levels of C16-sphingoid bases as compared to wild type. To further investigate the subunit composition of drosophila SPT, an exhaustive homology search was carried out and revealed, in addition to the LCB1, LCB2 and Ghiberti/ssSPT, two additional candidate ssSPTs exist. The cDNAs corresponding to the fly LCB1, LCB2 and ssSPTs were cloned and tested for expression and biochemical characterization in yeast. An increasing body of evidence suggests a distinct shift in sphingolipidomic profiles during fly development. SPT, being the first and rate limiting enzyme in sphingolipid biosynthesis, has the potential to dictate the levels and diversity of the different long chain base species. A better understanding of this enzyme will help us to understand sphingolipid homeostasis under normal physiological conditions. It would also allow us to possibly translate different human neurological and metabolic disorders which are caused by aberrant sphingolipid metabolism, to the fly model (18; 28; 35).

RESULTS:

Identification of ssSPT homologs in *Drosophila melanogaster:*

Three genes have been identified as candidates of small subunits of serine palmitoyltransferase in *D. melanogaster* (fly) by a homology search, using the human ssSPT sequences as query. These three genes are designated here as FssSPTa (CG34194), FssSPTb (CG34293) and FssSPTc (CG32038). All these candidate genes share weak homology with the human ssSPTs, and vary in size. FssSPTa is predicted to be 104 amino acids long whereas FssSPTb and FssSPTc are 84 and 81 amino acids respectively (Fig 4.1). Despite their differences in size, they are all predicted to have a single transmembrane domain with the N-terminus in the cytosol and the C-terminus in the lumen. These topological predictions are consistent with the experimentally determined topologies of the human and plant ssSPTs (48; 59). To address the functionality of these candidate genes as ssSPT subunits, we tested their ability to activate the fly LCB1/LCB2 heterodimer and their influence on the substrate selection of the fly heterodimer.

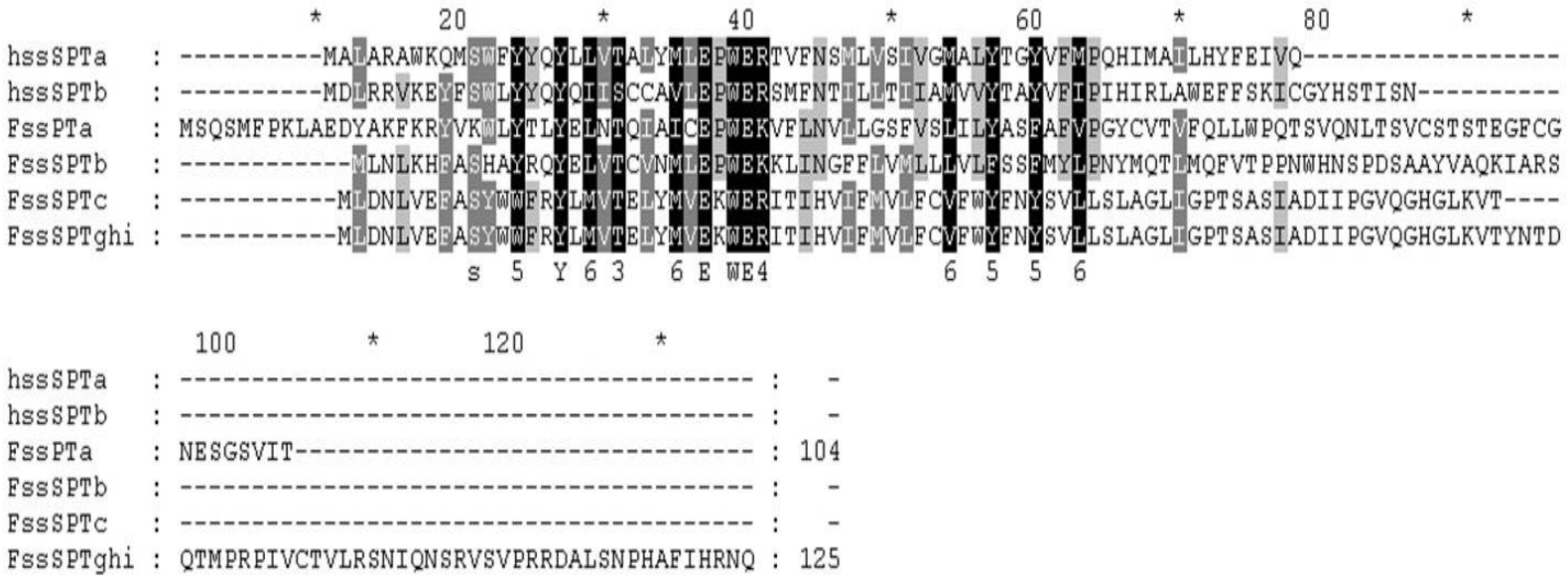

Figure 4.1. Amino acid sequence alignment of FssSPTs with human small subunit (HssSPTa, HssSPTb).

Cloning and expression of different small subunits and the 'ghiberti' mutant from Drosophila

N-terminally HA-tagged FssSPTa, FssSPTb, FssSPTc and the 'ghiberti' mutant, FssSPTc with the C-terminal 44-amino acid extension (FssSPTghi) were expressed along with N-terminally flag-tagged fly LCB1 (Flag-FLcb1) and C-terminally Myc-tagged fly LCB2 (FLcb2-Myc) in a yeast mutant lacking endogenous SPT *(lcb1Δtsc3Δ)*. Expression of the SPT subunits were detected by immunoblot using anti Flag, Myc and HA antibodies. The fly ssSPT subunits had the expected electrophoretic mobilities with FssSPTb (84 amino acids) and FssSPTc (81 amino acids) migrating faster than FssSPTa (104 amino acids) and FssSPTghi (125 amino acids) migrating the slowest (Fig 4.2). Expression of FLCB2 was comparable in all the samples whereas FLCB1 expression was variable.

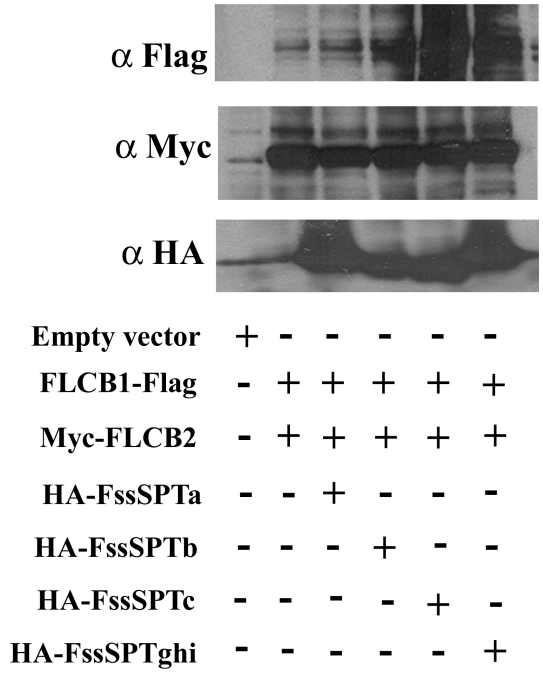

Figure 4.2. Heterologous expression of fly SPT subunits in yeast.

Microsomes were prepared from yeast cells heterologously expressing the *D. melanogaster* SPT heterodimer with different FssSPT subunits. 45 μg of membrane protein were loaded per lane, resolved by SDS-PAGE and analyzed by immunoblotting to detect FLCB1-Flag, Myc-FLCB2-Myc and the HA-FssSPTs using the indicated antibodies. Microsomes prepared from yeast cells with empty vectors were used as a negative control.
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Activation of the heterodimer and substrate utilization:

To address the functionality of these candidate ssSPTs, growth was compared between the yeast cells heterologously expressing the fly LCB1/LCB2 heterodimer with and without coexpression of the individual small subunits including the FssSPTghi mutant. The results showed that the expression of the SPT heterodimer (FLCB1, FLCB2)

failed to rescue the long chain base auxotrophy of yeast cells lacking endogenous SPT. In contrast, coexpression of any FssSPT candidate genes including FssSPTghi rescued that phenotype (Fig 4.3). Despite the comparable expression levels of different small subunits (Fig 4.2), FssSPTa and FssSPTb showed a robust rescue of long chain base auxotrophy even at elevated temperature where the sphingolipid requirement is high, indicating a strong activation of the SPT heterodimer. Coexpression of FssSPTc showed weaker growth and the FssSPTghi barely rescued growth at elevated temperature. These results indicate that the FssSPTghi protein, elongated by 44 amino acids, is likely a loss of function mutation.

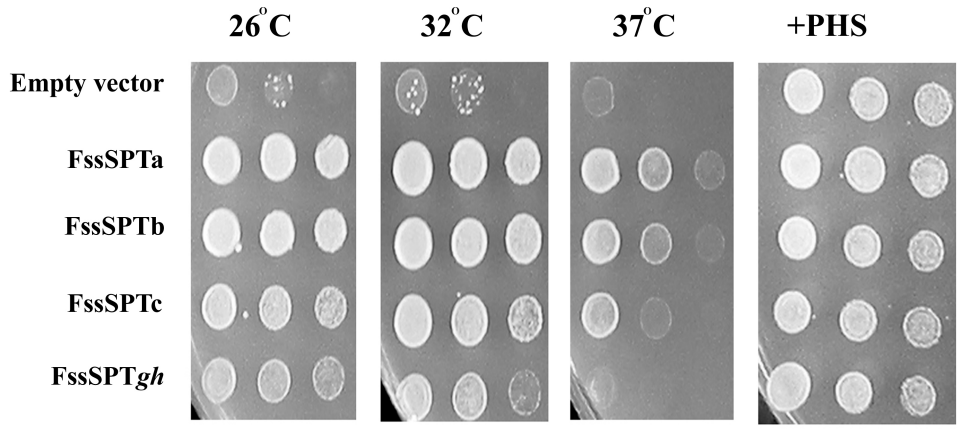

Figure 4.3. FssSPTs activate fly SPT heterodimer in yeast.

Yeast *lcb1*Δ*tsc3*Δ cells heterologously expressing FLCB1 and FLCB2 with the empty vector (pADH) barely grow without PHS. Coexpression of the candidate FssSPTs significantly improved growth in the absence of PHS at even at 37 °C where the sphingolipid requirement is high. Complementation by the FssSPTghi allele of FssSPTc was observed at 26 °C and 32 °C , but not at 37 °C.

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Total LCB analysis in *S. cerevisiae* and substrate availability:

Total LCB analysis of wild type *S. cerevisiae* showed that the predominant LCBs in yeast are C18-PHS and C18-DHS. There are barely detectable levels of C16-LCB species and undetectable levels of any other LCB species with shorter chain lengths (Fig 4.4). This raised the possibility that the differences in complementation by the different fly SPT isoforms might actually reflect a requirement for C18-LCBs for growth rather than a lack of SPT activity. Alternatively, even if C14- or C16-LCBs were able to fulfill the sphingoid base requirements of yeast, a restrictive availability of the C12 and C14 acyl-CoA substrate pools in yeast might prevent the fly SPT isozymes from generating enough products to support growth. However, unlike wild type yeast, total LCB analysis of yeast cells heterologously expressing the different heterotrimeric SPT isozymes, showed a significant accumulation of C16-LCB species (Fig 4.5, Fig 4.6 and Fig 4.7), indicating the availability of C14-CoA and showing that all three SPT isozymes have a preference for the shorter chain acyl-CoAs. LCB profiles from cells expressing the FssSPTc- and FssSPTghi- containing heterotrimers showed that accumulation of total long chain bases in the yeast cells expressing FssSPTcghi is lower compared to wild type FssSPTc (Fig 4.7), which is consistent with the complementation data. These results suggest that that the phenotypes associated with the FssSPTghi mutation reflect reduced activity of the FssSPTc and indicate that FssSPTc plays a unique role in male meiosis that cannot be fulfilled by either FssSPTa of FssSPTb. It is also possible that tissue specific expression levels of different FssSPTs may be responsible for such phenotype.

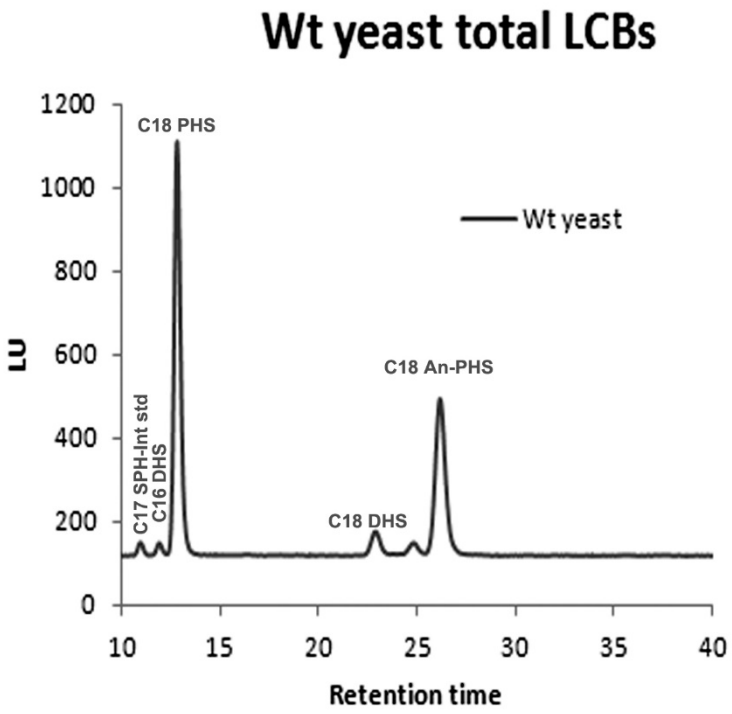

Figure 4.4. HPLC analysis of wild type yeast (*S. cerevisiae*) has very low levels of C16-LCBs

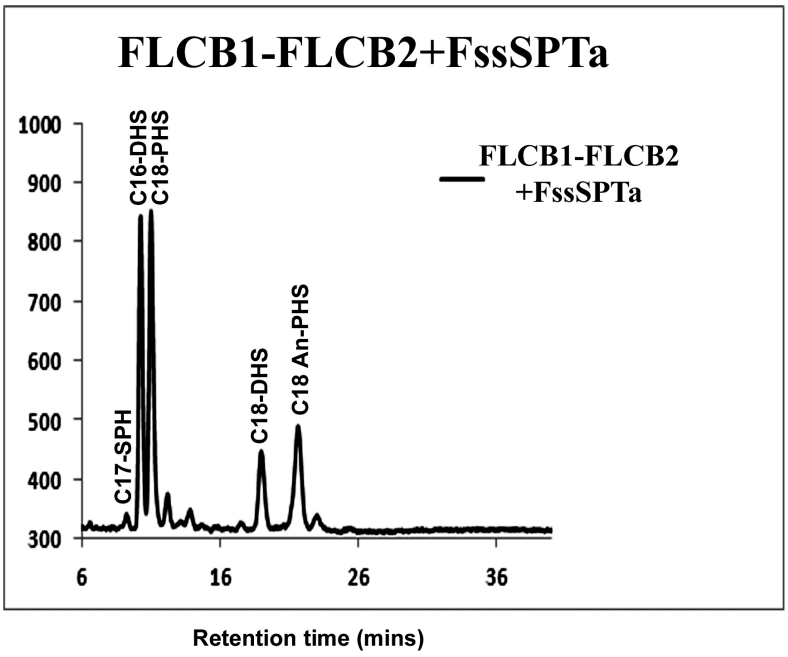

Figure 4.5. HPLC analysis of yeast SPT knockout mutant cells showing that the coexpression of FssSPTa, FLCB1 and FLCB2 results in accumulation of substantial levels of C16-LCBs in *S. cerevisiae*.

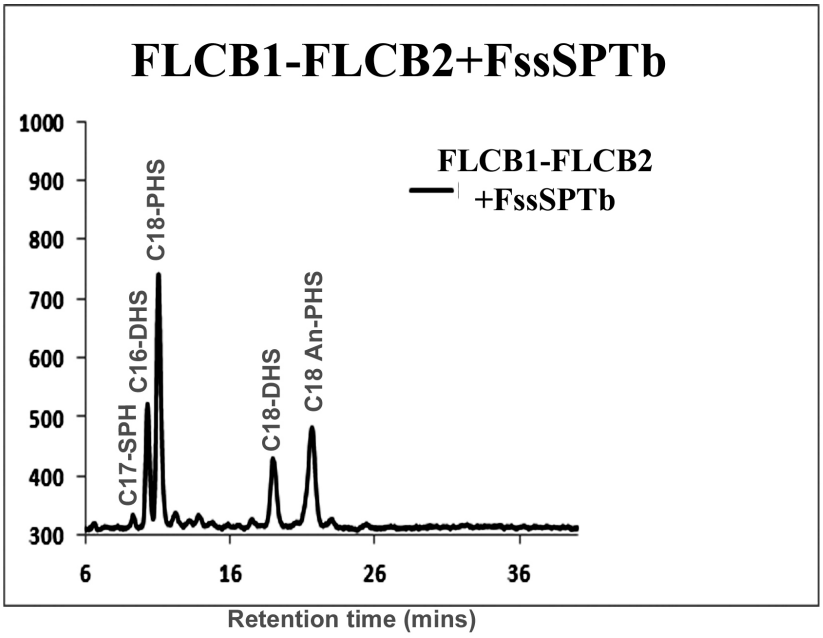

Figure 4.6. HPLC analysis of yeast cells showing that coexpression of FssSPTb confers shorter chain length CoA preference on the fly SPT heterodimer.

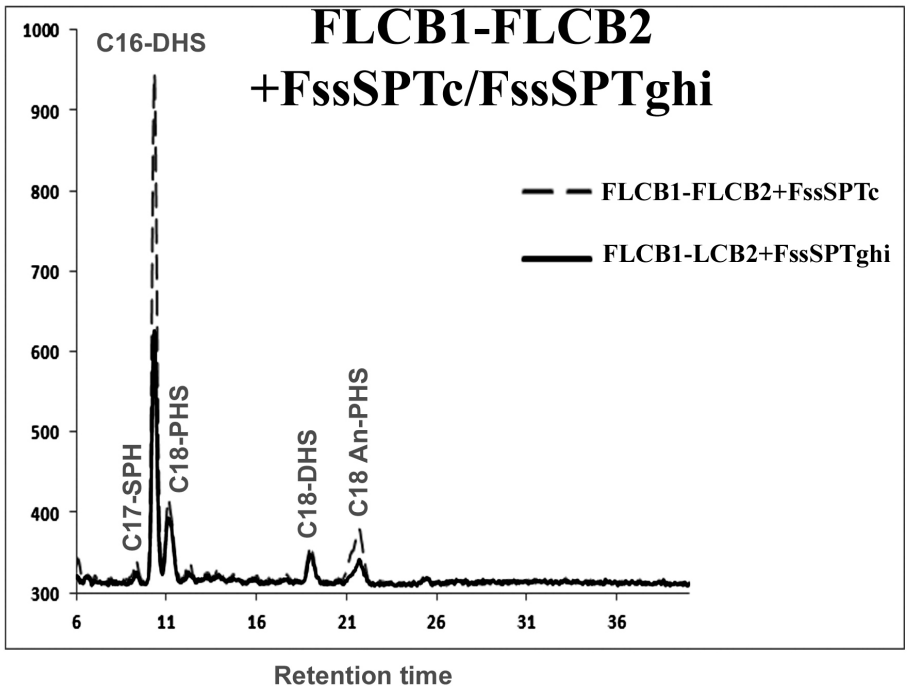

Figure 4.7. Comparison of LCB profiles of FssSPTc with FssSPTcghi in yeast.

LCB profiles of yeast cells heterologously expressing FssSPTc or FssSPTcghi with the fly LCB1-LCB2 heterodimer were compared. FssSPTc confers a C14-CoA preference on the heterodimer, and FssSPTcghi shows less LCB accumulation, suggesting it is a loss of function mutation of the wild type FssSPTc gene.

FssSPTs can activate the fly SPT heterodimer in yeast:

To address the level of activation of the fly LCB1/LCB2 heterodimer by the small subunits and the acyl-CoA preferences of the heterotrimers, in *vitro* SPT activities were measured using microsomes prepared from yeast heterologously expressing the FLCB1/FLCB2 heterodimer alone or the heterodimer with each of the small subunits (FssSPTs). Since the predominant sphingoid bases in drosophila have chain lengths of 14

or 16 carbons (29; 31), and the small subunits are predicted to dictate the acyl-CoA chain length preferences of the heterotrimers, C12-, C14-, and C16-CoAs were used for these assays. As expected, the fly LCB1/LCB2 heterodimer alone (empty vector) showed barely detectable enzymatic activity with any of the acyl-CoA substrates. Coexpression of the fly ssSPTs activated the heterodimer several fold. Activation by the FssSPTa subunit was >100 fold with C14-CoA as the substrate and the FssSPTa containing heterotrimer also showed significant activity with C12-CoA as substrate, though it was only about 33% the activity observed with C14-CoA(Fig 4.8). The FLcb1-FLcb2+FssSPTb heterotrimer also showed a preference for C14-CoA as substrate, but the activity was more than 20-fold lower than that of FLCB1-FLCB2+FssSPTa with all three acyl-CoA substrates. This was surprising because the FssSPTb/FLcb1/FLcb2 heterotrimer showed a very robust rescue of long chain base auxotrophy of the yeast cells (Fig 4.3). It is worth noting that very small increases in the activity of the yeast heterodimer (as a result of single amino acid substitutions in the Lcb2p subunit (78), comparable to those observed with the FssSPTb-activated fly heterodimer, are sufficient to support growth of the yeast *tsc3Δ* mutant at 37 ºC. However, this raises questions as to why the FssSPTc/FLCB1/FLCB2 and the FssSPTcghi/LCB1/FLCB2 heterotrimers, both of which have higher *in vitro* activity with all three acyl-CoA substrates than the FssSPTb/FLCB1/FLCB2 heterotrimer, fail to complement well. One possible explanation for the inconsistency between the complementation and the *in vitro* enzymatic activity is that the rescue of the yeast cell is dependent on the preferred C18-LCBs, since yeast expressing FssSPTa and FssSPTb show higher accumulation of C18-

LCBs than yeast expressing either the wild type or mutant FssSPTc (FssSPTghi), but the lack of correlation between the *in vivo* an *in vitro* activities is not understood.

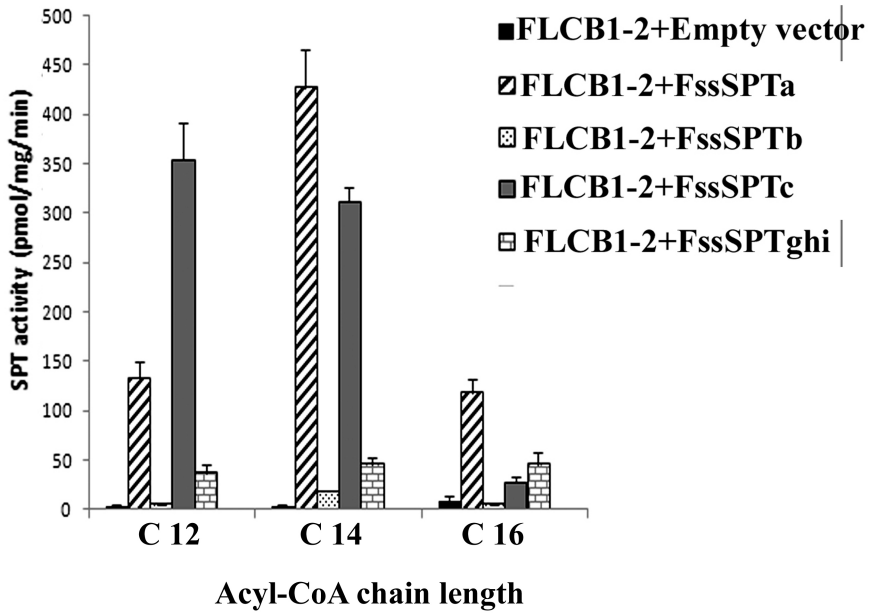

Figure 4.8. *In vitro* SPT activities of fly SPT heterodimers activated by different FssSPTs.

Microsomes were prepared from yeast cells heterologously expressing the Fly SPT heterodimer with empty vector or with different FssSPT subunits. SPT activity was measured with 200 µg of yeast microsomal membrane protein expressing different Fly SPT isoforms. 2 mM final serine and 50 µM CoA were used in the presence of BSA at 37 ^{o}C for 10 mins.

Varying C14-CoA concentration did not restore the activity of the SPT heterodimer activated by FssSPTb

It has been reported that *in vitro* SPT activity increases sigmoidally with increasing acyl-CoA concentration until it reaches a plateau and that even higher acyl-CoA concentrations actually inhibit SPT activity (32). Since fly heterotrimeric SPT containing FssSPTb appears to have high *in vivo,* but low *in vitro* SPT activity, the possibility that the acyl-CoA concentration used in the standard *in vitro* SPT assay (50 μM CoA) was inhibitory to this SPT isozyme was investigated. Accordingly, SPT activities of the FssSPTa- and FssSPTb-containing heterotrimers were compared over a wide range of myristoyl-CoA (5-200 μM) concentrations. Both SPT isozymes showed similar sigmoidal activities at low acyl-CoA and reached maximal activity at 50 μM myristoyl-CoA. However, at any given substrate concentration, activity of the FssSPTa-LCB1-LCB2 heterotrimer was much higher than that the FssSPTb-LCB1-LCB2 heterotrimer (Fig 4.9). These results show that FssSPTb is a weaker activator of the heterodimer than FssSPTa and FssSPTc, at least *in vitro*.

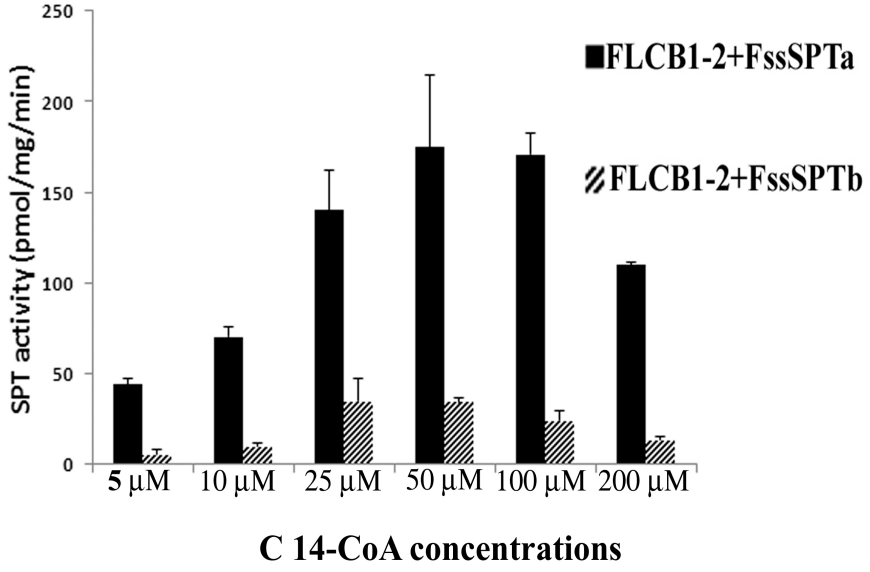

Figure 4.9. Comparison of the activities of FssSPTa- and FssSPTb- containing fly SPT heterotrimers with increasing acyl-CoA concentration.

Microsomes were prepared from yeast cells heterologously expressing the FssSPTa/FLCB1/FLCB2 or FssSPTb/FLCB1/FLCB2 heterotrimers. SPT activity in 200 µg of microsomal protein was measured using the indicated concentrations of C14-CoA and 2 mM serine as substrates. BSA was included and the assays were done at 37 ^{o}C for 10 min as described in Materials and Methods.

Optimization of Drosophila microsomal SPT activity:

It has been reported by Yamada et al that SPT activity in microsomes prepared from wild type Drosophila is undetectable (2). This was surprising both because flies have significant levels of sphingolipids in their membranes and because of the robust activity that was seen in the microsomes prepared from yeast expressing the different fly SPT isozymes (Figs. 4.8 and 4,9 and (43; 75). Therefore, we decided to investigate SPT activity in microsomes from adult flies. The results indicate that wild type adult

Drosophila microsomes show modest SPT activity over a range of myristoyl-CoA concentrations (Fig 4.10). Importantly, SPT activity showed a sharp substrate inhibition compared to the substrate inhibition observed in yeast microsomes (Fig 4.9), possibly explaining the reported inability to detect significant SPT activity in Drosophila microsomes. Indeed, addition of BSA (20 μM), which binds to and buffers the acyl-CoA, resulted in significantly increased SPT activity.

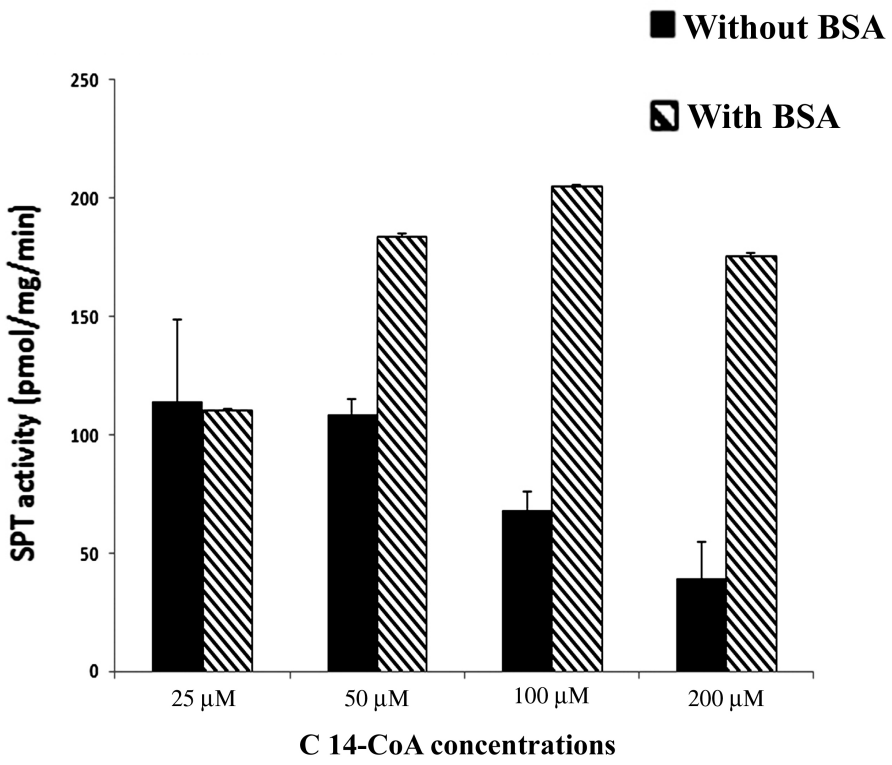

Figure 4.10. Optimization of the assay conditions for fly microsomal SPT activity.

Microsomes were prepared from wild type (y/w) *D. melanogaster* (fly). 400 µg of membrane protein and 10 mM serine, were used, either in presence or in absence

of 20 μM of BSA, along with the indicated C14-CoA concentrations. Assays were done for 10 min at 37 ^{o}C.

Developmental changes in SPT activity:

Sphingolipid metabolites such as ceramides and sphingosine-1 phosphate play a variety of important roles in physiology and development (3; 63). Different metabolites of the sphingolipid biosynthetic pathways have been reported to show gender and development dependent speciation. For example, at early pupal stage membrane bound sphingolipids, such as ceramides, mono and di hexosyl ceramide concentrations increase (38). This raises the interesting possibility that the regulation of the activity of SPT, as the first and rate limiting enzyme in sphingolipid biosynthesis, is important for normal development. Since the fly small subunits activate the SPT heterodimer several fold, regulation of expression of these small subunits could be important during development. Indeed, the developmental expression profiles of the three FssSPTs show interesting differences according to data in 'FlyBase' (Table 4.1). FssSPTa and FssSPTc are reported to be highly expressed in early developmental stages, whereas FssSPTb (which showed relatively weak activation of the heterodimer) is maximally expressed in the late larval stage. This is consistent with the expectation that sphingolipid requirement should be high during rapid development. To investigate this further, *in vitro* SPT activities were compared between microsomes prepared either from adult or larval stages (Fig 4.11). The results indicate that SPT activity is indeed significantly higher during early development, although there were no changes in acyl-CoA substrate preference between the two stages.

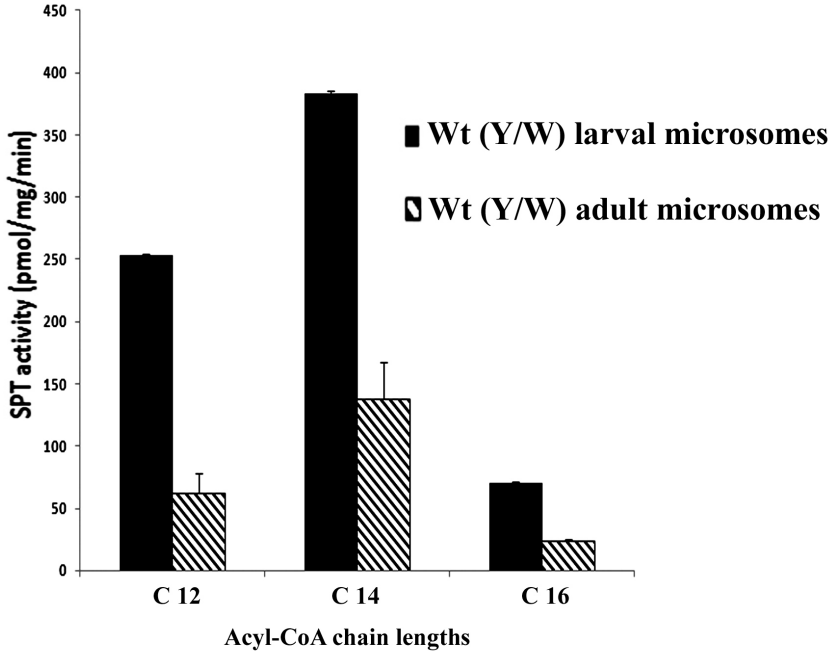

Figure 4.11. Wild type larval microsomes have higher SPT activity than adult microsomes.

Microsomes were prepared from wild type (y/w) adult and larval *D. melanogaster* (fly). 400 µg of membrane protein, 10 mM serine, 20 µM BSA and the indicated acyl-CoAs (50 µM) were used and the assays were conducted for 10 min at 37 ^{o}C.

DISCUSSION:

Here we report the identification of three small subunits of serine palmitoyltransferase from *Drosophila melanogaster*. This is the first report of an organism with more than two ssSPT subunits (32; 43). It is interesting that, unlike other higher eukaryotes such as mammals and plants, fly has a single LCB2 (Lace) isoform. Perhaps the presence of three different small subunits is required to provide the diversity of sphingolipid species at the appropriate levels during fly development. Coexpression of each of the three small subunits, with the cognate fly SPT heterodimer resulted in a significant increase in enzymatic activity and the fly SPT heterotrimers all showed a preference for C12- and C14-CoAs as substrate, which is consistent with the reported sphingoid base analysis from fly. Surprisingly, although it is reported to cause elevated levels of C16-LCBs (38), the 'gheberti' mutation appears to reduce its capacity to activate the heterodimer as well as to abolish its ability to confer a preference for C12 and C14-CoAs as substrates; unlike the wild type FssSPTc, FssSPTghi confers an equal preference for C12, C14 and C16-CoAs. Guan et al have shown that in the 'ghiberti' mutant fly, the C16/14-LCB ratio of sphingolipids goes up. It is not clear if there is an increased production of C16-LCBs, a decreased production of C14-LCBs, or whether these changes arise from compensatory changes in the expression of other genes involved in sphingolipid metabolism.

It has been reported that a single residue dictates the acyl-CoA substrate chain length preference in human ssSPTs. That insight has been successfully translated to identify the residue responsible for substrate selection in the plant and *S. pombe* small subunits. However, the analogous position in fly small subunits has an isoleucine in FssSPTa, and a methionine in FssSPTb and FssSPTc, yet they prefer shorter chain CoAs.

This raises the possibility that either the position of a substrate-conferring residue in fly ssSPTs is not conserved or that this residue is a part of an interface created by the heterodimer and the small subunits that dictates substrate preference. This idea is consistent with the fact that the human ssSPTb subunit predominantly confers a C18-CoA preference to the hLCB1/LCB2a heterodimer, but a wider range of acyl-CoA preferences when expressed with the hLCB1/LCB2b heterodimer (43).

This is also the first evidence which suggests that the substrate selection property of the small subunits can be affected by modifications in the luminal domain of the small subunit. The C-terminal deletion mutants of human ssSPTa had no effect on its substrate preference (48) raising the possibility that this phenomenon is unique for the FssSPTc/ghi gene. It is hard to reconcile the fact that FssSPTghi showed no substrate preference, when lipidomics analyses from 'ghiberti' mutants shows drastic change in C14/C16 ratio (38), but as mentioned above, this may reflect compensatory changes in the expression of other sphingolipid metabolic genes. For example, there is an increasing body of evidence supporting the idea that sphingolipid homeostasis is tightly regulated. In yeast, SPT belongs to a bigger complex known as the SPOTS complex. Thus, it is possible that other factors are contributing to the properties of 'ghiberti' mutant phenotype.

These findings lay the base line for a better understanding of SPT composition and function which plays essential roles in maintaining sphingolipid homeostasis, growth and development. New evidence from *S. cerevisiae* (9; 44; 88) and human, show that the Orm proteins are a part of SPT complex and are required for maintaining sphingolipid homeostasis. It will be interesting to address the native subunit composition of fly SPT

from different tissues and developmental stages, and to correlate that with sphingolipidomic profiles.

CHAPTER 5: ssSPT in *Caenorhabditis elegans*

INTRODUCTION:

Caenorhabditis elegans (*C.elegans*) is an emerging model system for studying lipid metabolism in eukaryotes. In contrast to most other higher eukaryotes, this model organism has an unusual fatty acid (13; 25; 60) and sphingolipid composition (110). In addition to the typical straight chain fatty acids, this organism also has monomethylated branched chain fatty acids, known as ISO fatty acids, usually C15ISO and C17ISO, where a single methyl group is appended next to the terminal carbon. Synthesis of mono methylated branched chain fatty acids starts with an iso-branched primer, leucine, isoleucine or valine, and, then follows the canonical fatty acid biosynthetic pathway (60).

This suggests that *C.elegans* has evolved and is equipped to maintain both straight and branched chain fatty acid pools.

Ceramides are composed of a sphingoid base (LCBs) attached to a long chain fatty acid (47). Interestingly, in *C.elegans* the sphingoid bases are predominantly C15 and C17ISOs whereas the long chain fatty acid component of the ceramides are strictly C22-C26 straight chain saturated fatty acids (111) (Figure 5.1).

Serine palmitoyltransferase catalyzes the first and rate limiting reaction in sphingolipid biosynthesis which is the condensation of a serine with a particular chain length of acyl-CoA to form the sphingoid base. It has been reported there are two small subunits of serine palmitoyltransferase in human (ssSPTa, ssSPTb) (43) and two in

A.thaliana (59) that dictate the preference for a particular acyl-CoA chain length (48). This suggests that the preference for branched chain fatty acid over straight chain to make the sphingoid base in *C.elegans* may be dictated by *C.elegans* SPT, possibly mediated by ssSPT homolog(s).

Sphingolipids are involved in diverse biological functions. They play both structural and functional roles in the growth and development of the organism. Perturbation of sphingolipid biosynthesis leads to severe phenotypes including larval arrest (13) as well as defects in growth and development. Ectopic expression of components of SPT showed similar phenotypes (110). Knockdown of Sptl2 gene, a homolog of human LCB2 subunit, resulted in loss of apico-basal membrane polarity during tubulogenesis.

Therefore identification and characterization of ssSPT homolog(s) will help to understand the subunit composition of *C.elegans* SPT complex. The candidate ssSPT homolog is predicted to increase heterodimeric SPT activity and influence the heterodimer to utilize C13 and C15ISOs as substrates. If the *C. elegans* SPT is indeed highly specific for branched chain acyl-CoAs, this would explain the C15 and C17ISOs found predominantly in sphingolipid analysis of this organism, despite the presence of both branched and straight-chain fatty acids in *C. elegans*.

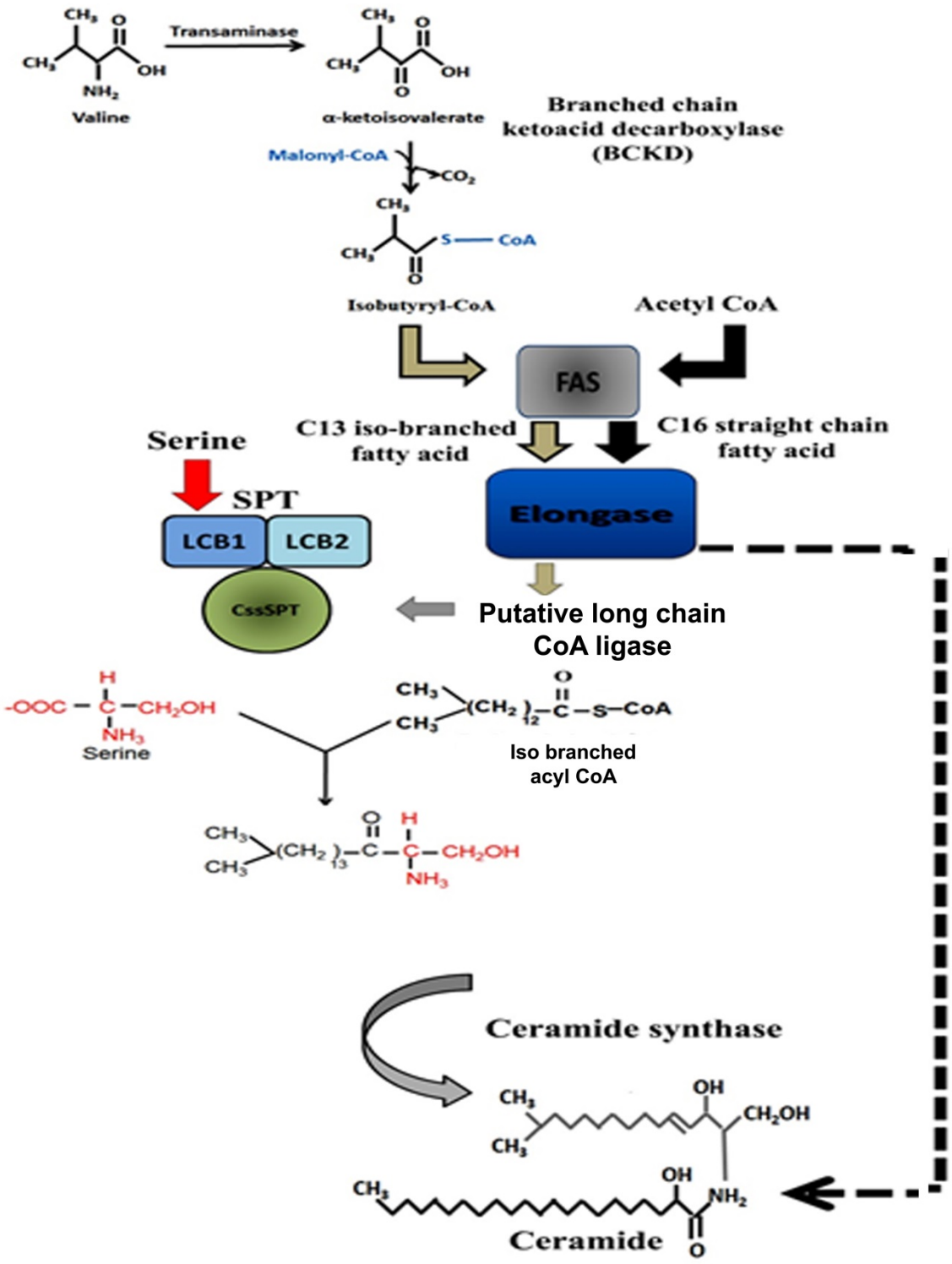

Figure 5.1: Sphingoid bases in *C. elegans* are predominantly C15/17 mono-methylated branched chain synthesized from a ISO branched precursor, whereas the fatty acids in ceramides are generally saturated even numbered.

RESULTS:

Identification of ssSPT candidate gene(s) in *Caenorhabditis elegans:*

A *C. elegans* homolog of HssSPTa was identified by a homology search against the *C. elegans* genome database, using human ssSPTa as the query sequence. Blast analysis yielded one questionable homolog with 37% identity (WB Gene: 00044784/ F13H10.8A) (Fig: 5.2). This candidate homolog is an 81 amino acid long polypeptide with two predicted transmembrane domains. ssSPTs that have been characterized to date, all are small (50-110 amino acids) peptides with at least one predicted transmembrane domain. Despite the limited homology, this was the best candidate ssSPT from *C. elegans* and it was therefore tested for its ability to activate the *C.elegans* serine palmitoyltransferase heterodimer.

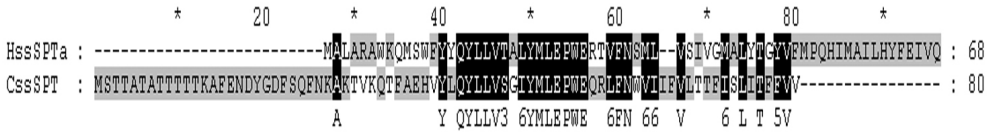

Figure 5.2. Amino acid sequence alignment of human ssSPTa with candidate ssSPT in *C. elegans* (CssSPT).

CssSPT can activate a *C.elegans* SPT heterodimer in yeast:

The core catalytic subunits of *C.elegans* SPT, LCB1 and LCB2 were identified by Blast homology searches, with human LCB1 and LCB2 as the query sequences. There are two LCB2 (Sptl-2/WB Gene: 00018398 and Sptl-3/WB Gene: 00011932) isoforms present in 'WormBase' database. The characterization of the *C. elegans* small subunit was done with Sptl-1 and Sptl-3 heterodimer (CLCB1-CLCB2). Sptl-1 has been designated as CLCB1 and Sptl-3 as CLCB2. Sptl-2 protein has significant homology with the LCB2 subunits, although it does not have a canonical 'PATP' domain in its C-terminus, instead it has 'PATH' domain, whereas Sptl-3 (CLCB2) has the canonical 'PATP' domain. This 'PATP' domain is highly conserved throughout eukaryotes because it is directly involved in the catalysis. Moreover expression of CLCB1-Sptl2 heterodimer resulted in robust complementation of yeast lacking endogenous SPT rendering it unsuitable for screening of ssSPTs by complementation.

The identified candidate genes were then PCR amplified, cloned into the pAL2-TRP plasmid, and expressed in a yeast SPT knockout mutant (*lcb1Δtsc3Δ*). The heterodimer alone (CLCB1-Flag) and untagged CLCB2) failed to support growth of the yeast cells even at the permissive temperature of 26 ºC, as the yeast SPT mutant retained a strict PHS dependency for growth. However, coexpression of HA-CssSPT along with the LCB1 and LCB2 subunits from *C. elegans* rescued the yeast SPT mutant, supporting growth at 26 ºC, but not at the more restrictive temperature of 37 ºC (Fig:5.3). This result suggests that the candidate gene is indeed an activator of *C.elegans* SPT heterodimer.

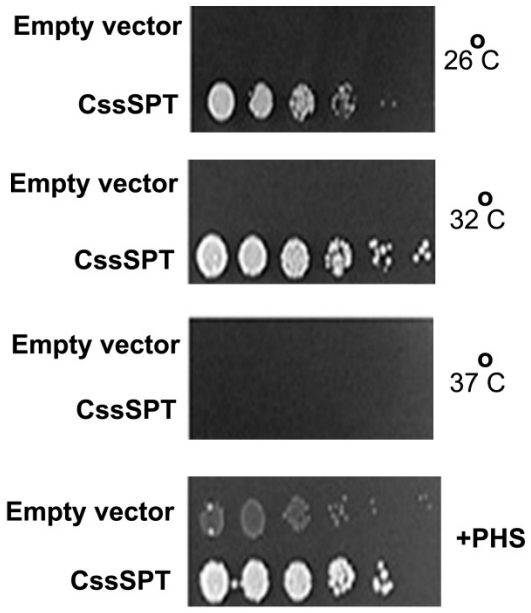

Figure 5.3. CssSPT rescues PHS auxotrophy of the yeast cells expressing *C. elegans* SPT heterodimer (CLCB1-CLCB2).

The expression of the *C. elegans* SPT subunits was verified by immunoblotting. Microsomes were prepared from yeast cells heterologously expressing C-terminally Flag-tagged CLCB1 (CLCB1-Flag), untagged LCB2 and N-terminally HA-tagged CssSPT (HA-CssSPT). 45 μg of microsomal protein were resolved by SDS-PAGE, transferred to nitrocellulose and the epitope-tagged proteins were detected using αFlag, and αHA antibodies (Figure 5.4).

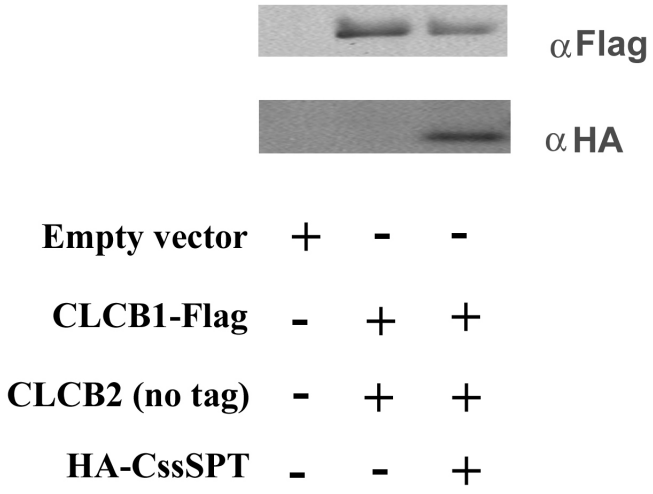

Figure 5.4. Immunodetection of *C. elegans* SPT subunits expressed in yeast.

Microsomes were prepared from yeast cells heterologously expressing CLCB1-Flag, untagged CLCB2 and HA- CssSPT. 45µg of membrane proteins were loaded in each lane and resolved by SDS-PAGE and detected by anti-Flag, and HA antibodies.

Substrate utilization and level of activation of *C.elegans* SPT heterodimer by CssSPT:

To test the level of activation of the CLCB1 and CLCB2 by the CssSPT, microsomal SPT activities were measured using C12-C16 saturated straight chain-CoA as a substrate. As discussed above, it has been reported that the predominant sphingoid bases found in *C.elegans* are C15 and C17 mono-methylated branched chain (110). Since the *C.elegans* SPT complex supports growth of the yeast SPT knockout mutant, and the fact that yeast has undetectable amount of mono-methylated branched chain fatty acids, we hypothesized that *C.elegans* SPT can condense straight chain fatty acids with serine.

Indeed, the results indicate that the *C.elegans* SPT trimer (LCB1-2/CssSPT) recognizes straight chain saturated-CoAs of varying chain lengths (C12-16). As predicted from the complementation studies (Figure 5.3), the heterodimer alone has undetectable SPT activity. The preferred substrate for the heterotrimer is C14-CoA. Activities with C12 and C16-CoAs are much reduced compared to C14. Therefore C14 is the preferred saturated straight chain-CoA substrate (Figure 5.5).

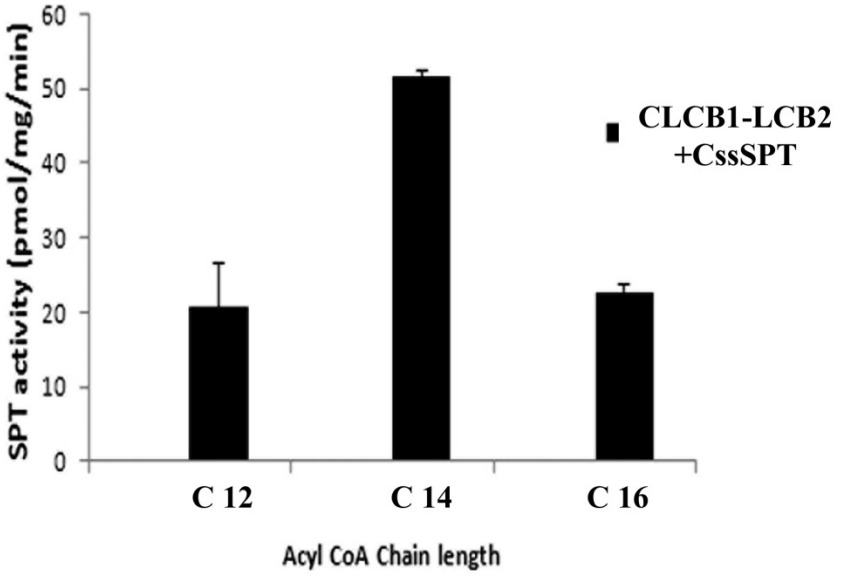

Figure 5.5. In vitro SPT activity of the C. elegans SPT heterotrimer.

Microsomes were prepared from yeast SPT knockout mutants (*lcb1Δtsc3Δ)* heterologously expressing CLCB1-Flag, untagged CLCB2 and HA- CssSPT. SPT activities were measured using C12, C14 and C16 CoA (150 µM) as substrate. Assays were done using 600 µg of microsomal protein for 10 mins at 37^{o}C.

To further validate the *in vitro* substrate utilization with ex vivo product formation, total long chain base was analyzed from yeast cells heterologously expressing CLCB1, CLCB2 and CssSPT. The results show predominant accumulation of C18-LCB species suggesting in yeast system CSPT heterotrimer can utilize C16-CoA to make C18 sphingoid bases (Figure 5.5).

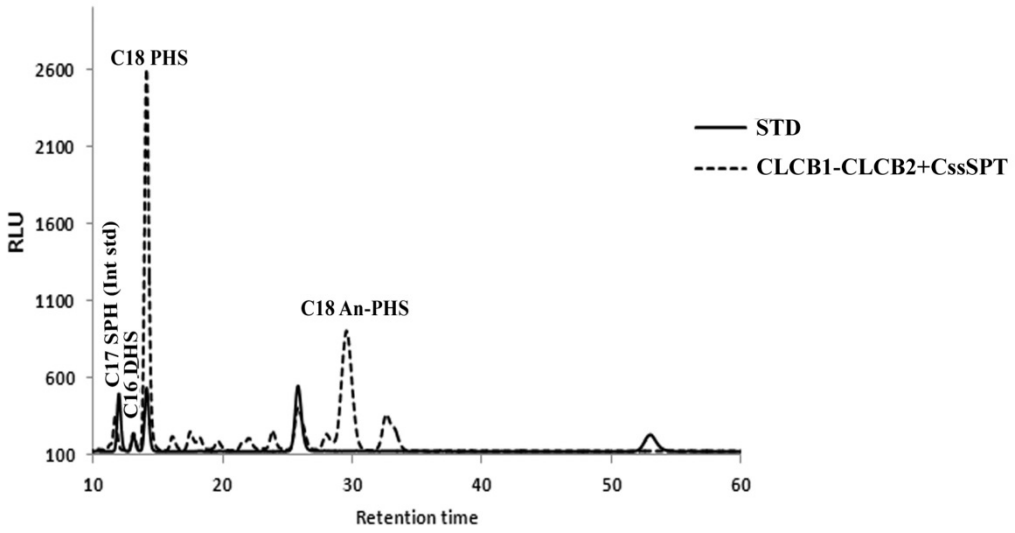

Figure 5.6. HPLC analysis of total long chain bases from yeast cells expressing *C. elegans* SPT heterotrimer showed C18-LCBs.

DISCUSSION:

Here we report the identification of a 'small activating subunit of serine palmitoyltransferase' in *Caenorhabditis elegans* (*C.elegans*). It is surprising to find only one *C.elegans* ssSPT candidate gene since the other higher eukaryotic organisms, including human, plant and fly, all have multiple ssSPT subunits.

This 81-amino acid polypeptide activates the *C.elegans* SPT heterodimer (CLCB1-2) in a yeast SPT knockout background. Overall SPT activity of the *C.elegans* SPT heterotrimer is low compared to human, plant, yeast or *S.pombe* SPT activities measured in yeast. This is not surprising because it has been reported that predominant sphingoid bases found in *C.elegans* are iso-branched chain C15/C17(13) species. Since SPT catalyzes the decarboxylation condensation of CoA with serine, it was predicted that in yeast background the lack of iso-branched chain-CoA substrate pool would limit *C.elegans* SPT activity. It is therefore surprising to find that expression of *C.elegans* SPT heterotrimer can rescue the PHS auxotrophy of the yeast SPT knockout mutant cells (Figure 5.3). It has also been reported that the ssSPTs (human, plant and pombe) confer the acyl-CoA substrate preference of the heterodimer (48; 59). This raises the interesting possibility that in the host organism, CssSPT not only activates the SPT heterodimer it also preferentially influence the heterodimer to select iso-branched-CoA as a substrate over straight chain-CoAs. The ability of the *C. elegans* SPT complex (LCB1, LCB2 and CssSPT) to utilize straight chain-CoA as a substrate is not surprising because it has been reported that although human ssSPTs show preference for a particular chain length of CoA, they can utilize a wide range of CoA chain lengths (43). It is possible that *C.elegans* SPT heterotrimer has a strong preference for C13 and C15ISOs and only a

minor fraction of straight chain sphingoid bases are produced, or are subject to high turnover rate. Alternatively, the straight chained and ISO branched CoAs may be differentially compartmentalized such that the only available substrate for the SPT is ISO branched-CoAs. These models do not exclude the possibility that there are other factors contributing to the substrate selection of *C.elegans* SPT.

CHAPTER 6: Conclusions

Subunit composition of SPT: bacteria to higher eukaryotes:

S. cerevisiae serine palmitoyltransferase (SPT) is one of the most well characterized enzymes among eukaryotic SPTs. Functional rescue of long chain base auxotrophy of yeast cells identified two proteins, Lcb1p and Lcb2p which constitute the catalytically active SPT enzyme, and localize in the endoplasmic reticulum (11; 85; 100). Homologs of the yeast Lcb1p and Lcb2p subunits can be found in almost all eukaryotes (96). Both of these subunits share ~40% homology with their mammalian homologs (45). The relative homology of these two subunits among the higher eukaryotes is much higher. In fact based on such homology predictions, the LCB1 gene has been successfully identified and characterized in Chinese hamster (CHO), mouse (46) and other eukaryotes. Interestingly, the earliest ancestors of these subunits can be found in the sphingolipid producing bacteria, *Sphingomonas* (55). In contrast to the yeast SPT, this bacterial SPT is cytosolic, and homodimeric (107). The bacterial SPT shows low but comparable homologies with both the LCB1 and LCB2 subunits of eukaryotic SPT. This raises the possibility that the subunits of eukaryotic SPT heterodimers may have originated from the bacterial enzyme or they may have arisen independently from a common ancestor based on the physiological requirements of the organism.

Whereas all eukaryotic organisms have a single LCB1 subunit, multiple isoforms of LCB2 are found in many higher eukaryotes including plants (96) and mammals (53). Therefore heterodimeric SPT can be present in different isoforms in higher eukaryotes. Identification of the ssSPTs added another layer of complexity to the subunit composition

of eukaryotic SPTs. The homodimeric bacterial SPT shows robust catalytic activity (55), whereas the eukaryotic SPT heterodimers require the ssSPTs for their optimal enzyme activity. Interestingly, no ssSPT-like homolog can be found in *Sphingomonas*. Physiologically, the absence of any ssSPT-like protein in this bacterium is consistent with the high enzymatic activity of the homodimer.

Unlike the subunits of the core SPT heterodimer (Lcb1 and Lcb2p), the ssSPTs are highly divergent among eukaryotes. In fact, no yeast Tsc3p homolog can be found in higher eukaryotes. Yeast Tsc3p orthologs (human ssSPTa and ssSPTb) were identified by a functional screen (43). Here we have shown that homologs of the human ssSPTs exist in several evolutionarily divergent species *(Arabidopsis thaliana, Drosophila melanogaster, Caenorhabditis elegans, and Schizosaccharomyces pombe)*. In fact multiple homologs have been identified in plant, human, and fly, thus increasing the number of catalytically active SPT complexes that can exist in an organism.

Topological similarities of eukaryotic SPT subunits:

Very little is known about the structure of eukaryotic SPTs, because, unlike the prokaryotic enzyme, they are comprised of multiple integral membrane subunits, and the stability of some of subunits is dependent on the presence of other subunit(s). For example, the stability of yeast Lcb2p is dependent on the presence of Lcb1p. Membrane association studies of the yeast, human and plant LCB1 and LCB2 subunits showed that they are integral ER membrane proteins, and detailed topological studies of the yeast, mammalian, and plant LCB1 and LCB2 subunits showed they have similar membrane topologies (32; 48; 108). In particular, LCB1 has three membrane spanning domains with the N-terminus in the lumen and the C-terminus in the cytosol. The LCB2 subunits have

two transmembrane domains, with both the N and C-termini in the cytosol. It has also been shown that catalytic site of mammalian SPT is cytosolic (67). These studies indicate that the topological properties of the two major subunits that form the catalytically active core of SPT are conserved throughout eukaryotes.

Interestingly, identification of a viral (*Coccolithovirus*) SPT that infects the marine microalgae *(Emiliana huxleyi)* revealed a unique topological conformation in which this enzyme can exist (42). Unlike eukaryotic SPT which is a heterodimer, or prokaryotic SPT which is a soluble homodimer, this gene is encoded by a single open reading frame. The product of this transcript is a single peptide with the N-terminal domain resembling yeast Lcb2p and the C-terminal domain resembling Lcb1p. This fusion SPT, when heterologously expressed in yeast lacking endogenous SPT, rescued the long chain base auxotrophy and had detectable *in vitro* SPT activity. This insight, that SPT can exist as a fusion protein, has been successfully translated to make functional yeast and human single-chain fusion SPTs. These findings indicate that topologically, eukaryotic SPTs are similar but flexible.

Similar to the heterodimeric subunits, the ssSPTs are ER membrane associated proteins. The majority of these proteins are predicted by hydropathy analyses to have a single membrane spanning domain, but the mammalian ssSPTs are predicted to have two transmembrane domains. The AtssSPTs are the smallest eukaryotic ssSPTs characterized to date; as they are only 56 amino acids long, it seemed unlikely that they have more than one transmembrane domain. Indeed, they were experimentally shown to have a single TMD and to be oriented with their C-terminus in the lumen of the ER (59). This finding motivated an investigation of the number of transmembrane domains in the human

ssSPTs and led to the discovery that the human ssSPTs also have a single membrane spanning domain with the N-terminus in the cytosol and the C-terminus in the ER lumen (48). Furthermore, deletional studies of the human ssSPTs showed that their central core, which is homologous to the AtssSPTs, is sufficient to direct membrane insertion and specification of acyl-CoA preference to the human LCB1-LCB2 heterodimers (48). Yeast Tsc3p is not stable without its heterodimer, whereas the human ssSPTs do not depend on coexpression of their cognate LCB1/LCB2 heterodimers for stability. For example, the human ssSPTs alone can be stably expressed in yeast. AtssSPTa and AtssSPTb are almost identical to each other, but when they are heterologously expressed in yeast with AtLCB1-LCB2 heterodimer, the expression level of AtssSPTa was found to be consistently higher than AtssSPTb. PssSPTp has always been found to be robustly expressed. It is not clear whether such differences in the relative expression of ssSPTs reflect an intrinsic property of the proteins, or whether it is related to translational efficiency upon heterologous expression in yeast.

Catalytic activation of the heterodimer by ssSPTs:

The eukaryotic SPT heterodimers all have low but detectable enzymatic activities (32; 43; 75). Since the heterodimer is capable of forming the catalytic core, and they have similar topologies it can be assumed that the eukaryotic SPT heterodimers are mechanistically similar. This is consistent with the fact that several dominant suppressor mutations in yeast Lcb2p, isolated in a screen for suppressors of the *tsc3Δ* mutant phenotype (78), increase the heterodimeric SPT activity ~2-3 fold (78). Mutations in human *LCB1* gene (S331F) increase the basal SPT activity of the human LCB1-LCB2 heterodimer several fold. Curiously, this "gain-of-function" mutation in human LCB1, as

well as several mutations in human LCB2a that are analogous (same amino acid substitutions in the same relative positions) to the gain-of-function mutations in yeast Lcb2p, has been identified as HSAN1-disease causing mutations. It has been shown that these mutations make SPT more promiscuous for use of alanine as substrate, thereby leading to the accumulation of 1-deoxy-LCBs that apparently underlie the neuronal pathology associated with this disease. Based on these observations, it is interesting to speculate that the evolution of an enzyme that is highly selective for serine resulted in an enzyme with low catalytic activity, thereby necessitating the coevolution of ssSPTs. It is worth noting that all of the mutant SPT heterodimers are fully responsive to their respective small subunits (48). The ability of both yeast Tsc3p and the human ssSPTs to activate the gain-of-function mutants in the yeast SPT heterodimer indicate that although there are differences between yeast and human SPT subunits the small subunits may activate the heterodimers by a similar mechanism. That inhibitors of the eukaryotic SPTs also inhibit the bacterial enzyme further argues for a conserved catalytic mechanism of the SPTs (77; 95).

S. cerevisiae and *S. pombe* represent two distinct clades in 'Ascomycete fungi'. In yeast, Tsc3p is required for growth at elevated temperature (32), where the sphingolipid requirement is high. Other higher eukaryotic SPT heterodimers, such as human, Arabidopsis and Drosophila also showed detectable enzymatic activities, suggesting that the majority of these SPT heterodimers are catalytically competent. Surprisingly, the PssSPT deletion mutant has been reported to be lethal (58), which is consistent with the fact that the *S. pombe* SPT heterodimer, when expressed in *S. cerevisiae*, showed undetectable SPT activity over a wide range of acyl-CoA chain lengths. This raises the

possibility that the *S. pombe* SPT heterodimer is strictly dependent on PssSPTp for its catalytic activity, but further work is needed to resolve whether the heterodimer alone has activity under different experimental conditions or when assayed using microsomes from the *S. pombe* PssSPT knockout mutant. The level of activation of the AtSPT heterodimers by AtssSPTa and AtssSPTb is very similar, which is consistent with the high degree of homology between them. It appears that activation by AtssSPTa is higher than AtssSPTb, but this undoubtedly reflects the higher expression levels of AtssSPTa.

Expression levels of the FssSPTs were comparable in yeast, but surprisingly FssSPTb showed weak *in vitro* activation of the heterodimer over a wide range of acyl-CoA concentrations. This suggests that unlike FssSPTa or FssSPTc, this small subunit is intrinsically weak in activating the fly SPT heterodimer. Such properties of the FssSPTb may have physiological relevance. It will be interesting to determine whether a low level of sphingolipid synthesis is needed in fully developed adults where it is maximally expressed, i.e., in the late pupal and early adult stages (see Table 4.1.). As discussed above, N and C terminal deletion mutants of human ssSPTa and ssSPTb showed that the central region surrounding the transmembrane is sufficient for enzymatic activation (48). As this is the region of maximum homology between the different ssSPTs, it is likely that the ssSPTs are all influencing the heterodimer in a similar way.

ssSPTs and Substrate utilization:

Palmitoyl-CoA is the best substrate for mammalian SPT (45; 75) and hence the name. It has been proposed that in eukaryotes, because of abundant availability of palmitoyl-CoA as a substrate, the predominant LCB species are C18, but these studies reveal a role for the ssSPTs in determining what LCBs are present. All the ssSPTs that

have been characterized in this study (*A. thaliana, S. pombe, D. melanogaster and C. elegans*) are representative of different clades of the phylogenetic tree with a unique sphingoid base composition in terms of chain lengths. The yeast Lcb1p/Lcb2p heterodimer prefers C14-CoA as substrate, but Tsc3p dictates C16-CoA as the preferred substrate, consistent with the predominance of C18-LCBs found in this organism (32), whereas in human ssSPTa confers C16-CoA and ssSPTb confers C18-CoA preference (48) and the predominant LCB species found in mammals are the C18- and C20-LCBs. Similarly the predominant LCB species found in *S. pombe* (C20) and fly (C14 and C16) mirror the substrate utilization of their heterodimers when they are coexpressed with their cognate ssSPTs. These results suggest that the diversity in the sphingolipid backbone chain length is dictated by these subunits. It is not clear whether these small subunits may have coevolved with the fatty acid synthase and elongase machinery. For example, it has been reported that the predominant fatty acid species in *S. pombe* is oleic acid (18:1Δ9) (71) and in *D. melanogaster* C14, C16, C18 free fatty acids are abundant (19). These reports are consistent with the selective preference of PssSPTp (C18) and FssSPTs (C12, C14). Therefore it is possible that the ssSPTs may have evolved to efficiently incorporate the most predominant fatty acid(s) available in the host organism. However, yeast cells heterologously expressing fly and *S. pombe* SPT complexes have no distinct physiological disadvantages despite the fact they show significant accumulations of C14, C16 or C20 containing LCBs. It is true that in all cases, the yeast expressing the heterologous enzymes do accumulate significant amounts of C 18-LCBs. This would argue that, while LCBs of other chain lengths may not hurt, the C18-LCBs are still required. In fact preliminary (Dunn's lab, unpublished data) studies to rescue a yeast

lcb1Δ with C20-LCBs, showed positive complementation, but the LCB profile of the chemically rescued mutants showed presence of C18-LCBs. Therefore it is likely that the positive chemical complementation of yeast SPT knockout mutants by C20-LCBs is actually because of contaminating C18-LCBs.

The substrate preference dictated by human ssSPTa and ssSPTb has been reported to be controlled by a single residue (Met-25 in human ssSPTa, and Val-25 in ssSPTb) (43; 48). A homology alignment of human small subunits with plant and pombe ssSPTs was used to predict the residue responsible for substrate selection in those subunits. Point mutations of those residues showed altered substrate preference of the heterodimers. Surprisingly, the expression of Met19-Val mutants of the AtssSPTb subunit in plant showed the accumulation of significant C20-LCBs, but the plants did not show any phenotype (59). Then again, transgenic plants overexpressing AtssSPTb M19V also have wild type SPTs and lipidomics analysis indicated the presence of C18-LCBs.

Physiological roles of ssSPTs:

Yeast *tsc3Δ* knockout mutants show temperature sensitivity (yeast cells lacking Tsc3p failed to grow at 37ºC) and increased vacuolar fragmentation (Dunn's lab unpublished data), which can be rescued by supplementation of the growth medium with phytosphingosine (PHS), an intermediate in the sphingolipid biosynthetic pathway (32). Rescue of the ts and vacuolar fragmentation phenotypes by the addition of PHS suggests that the only essential function of Tsc3p is to activate the heterodimer. This is consistent with the fact that the *tsc3Δ* suppressor mutants, with mutations in Lcb2p that increase the

basal activity of the mutant heterodimers, suppress both the ts and the vacuolar fragmentation phenotypes (Dunn's lab unpublished data).

Here we have reported for the first time the ssSPT knockout phenotypes of a higher eukaryote, Arabidopsis. Biochemical characterization of AtssSPTa and AtssSPTb indicated that they are functionally redundant with regard to their ability to activate the Arabidopsis LCB1/LCB2 heterodimers and to confer C16-CoA substrate preference. AtssSPTa and AtssSPTb both are ubiquitously expressed, but the expression levels of AtssSPTa are consistently higher compared to AtssSPTb and maximum in pollen (>400 fold), suggesting that AtssSPTa might be playing a very important role in pollen development. Indeed, plants genotyped as heterozygous for AtssSPTa knockout showed 50% pollen lethality, suggesting that such pollen lethality is due to the lack of AtssSPTa transcripts. Consistent with this, homozygous AtssSPTa knockout plant lines could only be recovered if they were expressing an AtssSPTa transgene. Homozygous AtssSPTb knockout plant lines did not show any differences in their growth and pollen development compared to the wild type plants. These results strongly indicate that expression of AtssSPTa, not AtssSPTb, is required for pollen development. However, since expression of an AtssSPTb cDNA under the control of *pAtssSPTa* promoter showed complete rescue of pollen lethality in homozygous AtssSPTa knockout plant lines, confirming that the Arabidopsis ssSPTs are functionally redundant at the level of their biochemical activities. These results are consistent with the previous report showing that homozygous AtLCB1 knockouts are lethal and heterozygous knockout lines are defective in pollen development (12). Overall, these results suggest that the high level of AtssSPTa

transcripts in pollen is required to maintain the high level of *de novo* sphingolipid synthesis that is needed during pollen development (59).

The failure to produce pollen complicates analysis of the role of the AtssSPTs in vegetative growth. To address this, AtssSPTa was placed under control of a promoter that is expressed in pollen but not in vegetative tissues. Expression of AtssSPTa under the control of a pollen- and ovule (meiotic cell)-specific-*AtDMC1* promoter, showed that AtssSPTa is also required for vegetative growth. No transgenic plants genotyped as homozygous AtssSPTa knockout were recovered. There were however a few, where wild type AtssSPTa transcripts could be detected in leaves, presumably because of leaky expression of *AtDMC1* promoter. These plants were small just like the heterozygous AtLCB1 knockout plants. It is not clear why expression of hypomorphic SPT resulted in fully developed but dwarf plants (59). The other subunit of SPT, AtLCB2 has also been implicated in the pollen development of plant (99).

Surprisingly, over-expression of AtssSPTa resulted in increased SPT activity. This is the first report which indicates that, under normal physiological conditions, AtssSPTa is limiting and raises the possibility that this may be the case in other organisms as well. Consistent with this finding, AtssSPTa overexpression showed increased fumonisin B1 sensitivity and conversely AtssSPTa knockdown lines showed resistance. This suggests that tight regulation of the AtssSPTs is required to maintain proper sphingolipid homeostasis in plants (59). It is not known whether the same is true for other higher eukaryotes, but experiments are underway to knock out these genes in mice.

Not much is known about the roles of ssSPTs in fly growth and development. It has been reported that the sphingolipid profile changes throughout developmental (38). Characterization of the FssSPTs in yeast revealed not only that all three are bona fide ssSPTs, but also that the different small subunits activate the fly LCB1/LCB2 heterodimer to different extents and confer distinct acyl-CoA preferences, which is consistent with the expression profiles of these small subunits during fly development (Table 4.1). A report showed that, a read through mutation in the FssSPTc gene is responsible for male cytokinesis defects or the 'ghiberti' phenotype, reminiscent of the pollen defect in plants. This is the first direct evidence of the roles of ssSPTs in any physiological defects in fly (38). Other genes that cause the same phenotype are involved in sphingolipid trafficking (such as TRAPII, Rab11), suggesting that this is due to a lack of sphingolipids. This is consistent with our findings which showed that FssSPTghi is a loss of function mutation of the FssSTc wild type gene. A hypomorphic fly LCB2 gene (Lace) rescued the growth and developmental defects of a hyperactive MAPK kinase fly mutant (2). This raises the interesting possibility that proper sphingolipid homeostasis is required for normal growth and development, mediated, at least in part, by MAPK kinase signaling. It has recently been demonstrated that yeast SPT activity is responsive to the mTOR signaling pathway, mediated by the Orm family of proteins (Orm1p and Orm2p in yeast) as discussed in the introduction. It is interesting to speculate that the hyperactivated MAPK kinase pathway may inappropriately derepress the Orms and lead to excessive SPT activity; this would explain why the hypomorphic mutations in fly LCB2 rescue the hyperactive MAPK kinase fly mutant.

Unlike the situation in *S. cerevisiae*, knocking out the *S. pombe* ssSPT (PssSPT) has been reported to be lethal (58). This is consistent with the low or undetectable *in vitro* enzymatic activity of the pombe Lcb1p/Lcb2p heterodimer in yeast, but it is also possible that the sphingolipid requirements to maintain viability in *S. pombe* are higher than in *S. cerevisiae*. It is still unclear if ssSPTs have any other functions apart from SPT activation, but there is currently no evidence to suggest that, because ssSPT null phenotypes can be chemically complemented by PHS in yeast. Analogous to that, the AtssSPT null mutant phenotype strongly resembles the phenotypes associated with reduced expression of AtLCB1 or AtLCB2.

Identification of the residue that confers the chain length specificity of the heterodimer in human has been successfully used to predict the residues that have the same function in AtssSPTs and PssSPTp. However such phylogenetic approach failed to identify such residues in FssSPTs and in CssSPT. This is because FssSPTa and FssSPTc both have a preference for shorter (C12-, C14-) CoAs and they have Met in the predicted location. HssSPTa and AtssSPTs both have Met, which confers a C16-CoA preference for their cognate heterodimer. HssSPTb has a Val, which has been reported to confer a C18 preference with the human LCB1/LCB2aheterodimer, whereas PssSPTp, which shows a C18 preference of its cognate heterodimer, has Leu. These observations suggest that dictating the substrate preference is a combinatorial function of both the ssSPTs and their cognate heterodimers. Possibly both the small subunit and the heterodimer form the interface that actually determine the substrate preference.

PHYLOGENETIC ANALYSIS OF SPT:

LCB1 and LCB2 subunits:

Eukaryotic SPT is a complex enzyme, with multiple subunits, which can exists in multiple isoforms. Eukaryotic LCB1 and LCB2 subunits share considerable homology with the bacterial SPT subunit, which raises an interesting aspect about the origin of eukaryotic subunits. It is possible that both the LCB1 and LCB2 subunit might share a common ancestor with the bacterial SPT. Therefore a phylogenetic approach was used to study the diversity of these two proteins (Fig 6.1). Topology of this phylogenetic tree indicates that the LCB1 and LCB2 subunits belong to two distinct clades. Within each clade, the mammalian, metazoan, nematode, fungal and plant subunits show tight clustering. Probably such topological similarity between the LCB1 and LCB2 cladogram indicates that both subunits have coevolved with the species.

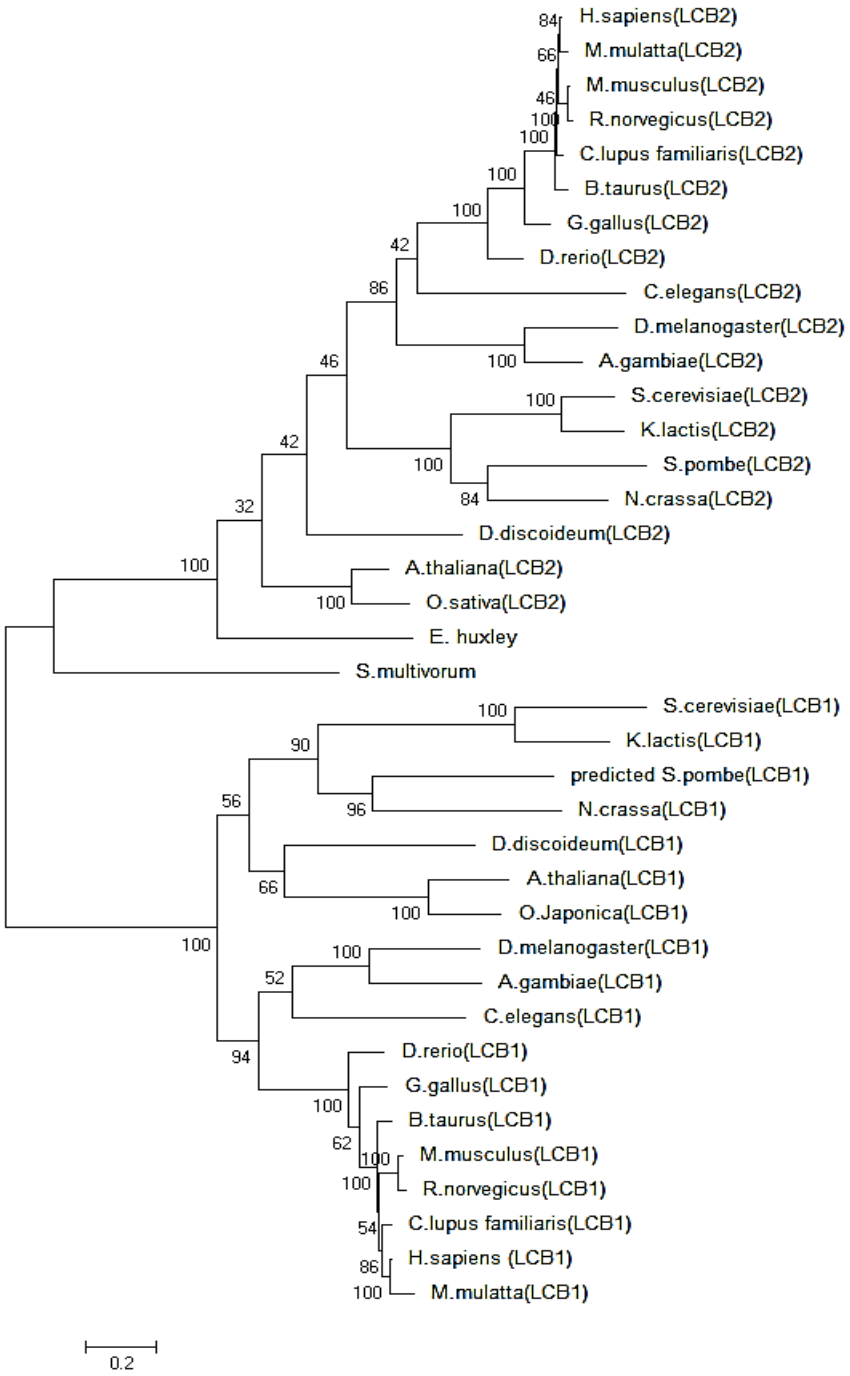

Figure 6.1. Molecular Phylogenetic analysis by the Maximum Likelihood method of the LCB1 and LCB2 subunits.

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (57). The tree with the highest log likelihood (-28784.5200) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and

BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 38 amino acid sequences. There were a total of 1599 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (97).

ssSPTs:

Relative conservation:

It is not yet clear whether or how the different ssSPT subunits influence the heterodimer. They may help the heterodimers fold properly to form a more efficient catalytic interface, they may directly participate in catalysis, or they may affect both structure and function. Alignment of the ssSPTs (Fig 6.2.) shows a high degree of divergence, but the central region or the area surrounding the predicted transmembrane domain contains the following conserved motifs:

- $Y_{xx}Y$ (7 of 9) (considering the gapped alignment of AtssSPTs)
- WER (8 of 9)
- FNS (6 of 9)

Deletion mutants of human ssSPTs indicated that the region surrounding the transmembrane is sufficient for catalytic activation (48). However, further deletion destabilizes the protein making it experimentally difficult to address whether binding and activation are dictated by two separate domains within this region. Deletion mutants lacking the first 10 amino acids from the N-termini of the human ssSPTs were stably expressed and activated the heterodimer, whereas deletion mutants lacking 19 amino

acid from the N-terminus, which includes the conserved $Y_{xx}Y$ motif, were stably expressed but failed to activate (48). These results indicate that this motif ($Y_{xx}Y$) might be important for the function of ssSPTs.

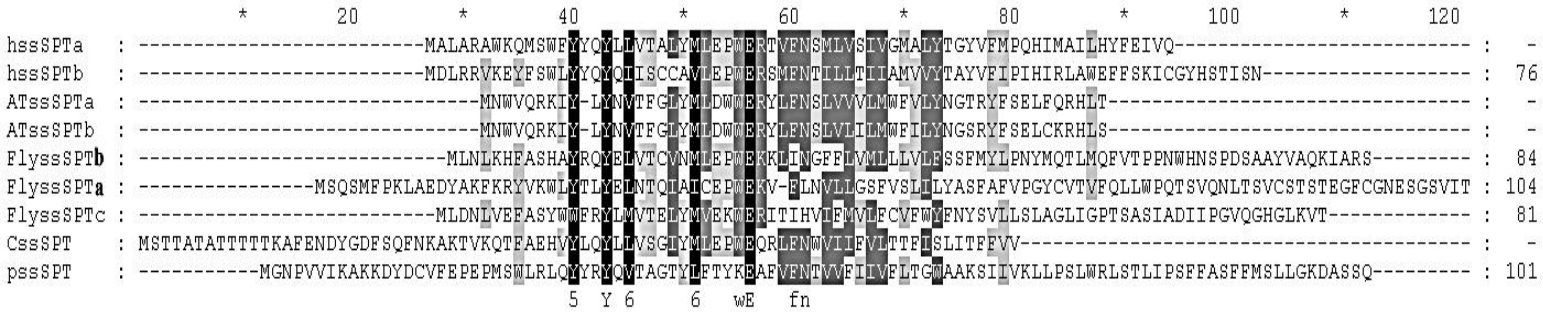

Figure 6.2. Amino acid alignment of ssSPTs

In contrast to the LCB1 and LCB2 subunits, no ssSPT homologs were identified in prokaryotes, which points to the possibility that the origin and evolution of these proteins is confined only within the eukaryotes. Moreover, the limited homology of ssSPTs within the eukaryotes restricted the identification of ssSPTs homologs in different species. Interestingly in mammals, the two different isoforms of the ssSPTs (ssSPTa and ssSPTb) belong to individual clades (Fig 6.3). This is consistent with the topology of the LCB1, LCB2 cladogram. However, representatives of other domains in eukaryotes, such as plant *(A. thaliana*), fungi (*S. cerevisiae* and *S. pombe*) and metazoan ssSPTs (*D. melanogaster*) do not show species specific clustering. Inferences from the phylogenetic topology of ssSPT subunits is highly challenging because of their small sizes and because the number of ssSPT isoforms within a species varies. It is also not clear whether the ssSPTs share a common prokaryotic ancestor. One possible explanation of such phylogenetic divergence of the ssSPTs is lateral gene transfer early during evolution and possible gene duplications.

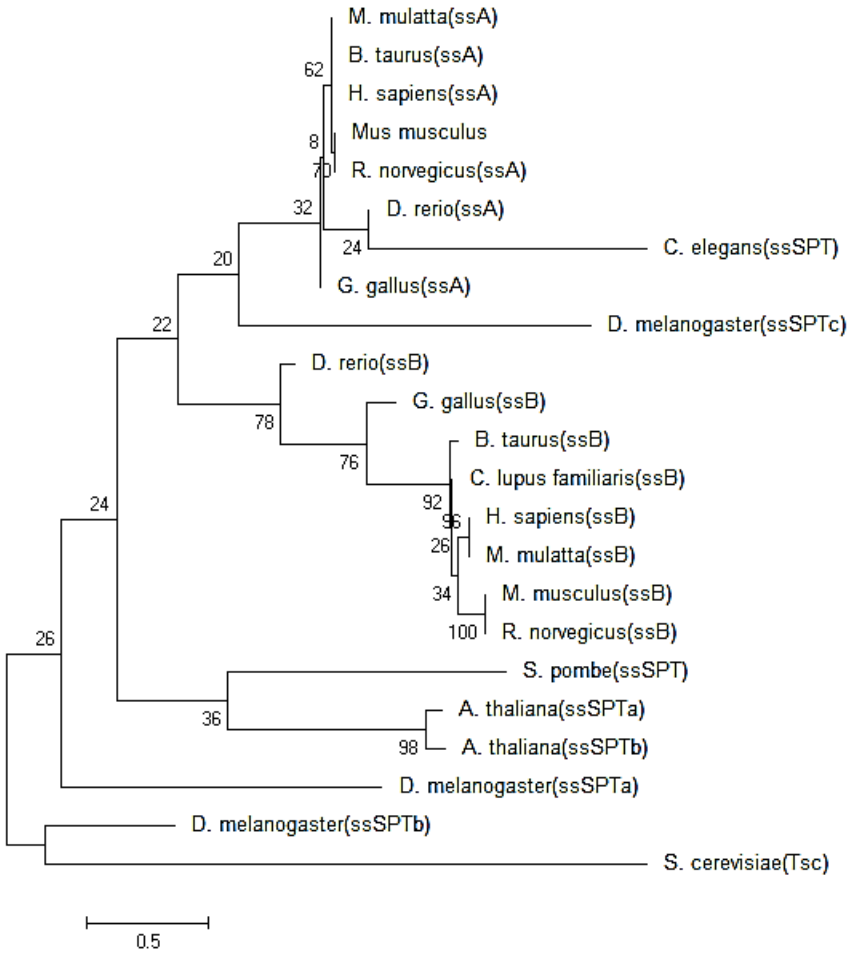

Figure 6.3.Evolutionary relationships of ssSPTs

The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 6.03584771 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 23 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 29 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

An amino acid sequence alignment of characterized ssSPT subunits shows that the central region encompassing the transmembrane domain has the maximum conservation (Fig 6.2). Taken together, the observation suggests that the smallest

ssSPTs(AtssSPTs) characterized to date show weak activation of other eukaryotic heterodimers (Dunns' lab unpublished data), and the studies with human deletion mutants, it appears that the conserved central domain of the ssSPTs is sufficient to activate the SPT heterodimer (48), and that each ssSPT does so by a similar mechanism.

These studies indicate that the small subunits, present throughout eukaryotes, activate the SPT heterodimer(s) and confer distinct substrate preferences. Although they show a high degree of divergence, they share a conserved domain and likely activate their cognate SPT heterodimer by a common mechanism. In the case of plants, functionally redundant ssSPTs are differentially expressed to meet differing requirements for *de novo* synthesis of the same sphingolipid species during development. In the case of flies and mammals, ssSPT isoforms that drive the synthesis of LCBs of different chain lengths are present, presumably naturally selected to allow for modulation of both the levels and species of sphingolipids to fulfill unique requirements during growth, development and execution of tissue specific functions. This is consistent with the observation that in higher eukaryotes, they play important roles in maintaining sphingolipid homeostasis (59), and show developmental specific expression (Table 4.1).

It is still not clear how these subunits have evolved, or if the members of such a diverse family of protein came from a common ancestor, but this study indicates that the ssSPT family of proteins likely originated to allow regulation of the activity of the eukaryotic SPT heterodimer. Since ssSPT can be loosely correlated with the phylogenetic topology of the highly conserved LCB1 subunit it is tempting to speculate that diversity of ssSPT family of proteins is because of coevolution.

As mentioned earlier, some human diseases have been associated with defects in the different SPT subunits. 'Hereditary Sensory Neuropathy Type 1' is caused by the accumulation of a neurotoxic intermediate as a result of a mutation in the human LCB1 and LCB2 subunits (83). It has also been reported that decreased expression of the human LCB2 subunit is associated with Alzheimer's disease (37) and increased SPT activity has been associated with cancer (106). This suggests that the identification and characterization of the ssSPTs will advance the understanding of different diseases. Moreover, the identification of such novel subunits from different organisms will allow us to use to those organisms to model human diseases.

CHAPTER 7: Materials and Methods

YEAST STRAINS GROWTH: AND EXPRESSION PLASMIDS:

Yeast strain TDY9113 (*Mat α tsc3*Δ::NAT *lcb1*Δ::KAN 1 *ura3 leu2 lys2 trp1⊿)* lacking endogenous SPT was used for expression and characterization of different ssSPT subunits from different species. The mutant was cultured in YPD containing 15 μM phytosphingosine (PHS) and 0.2% tergitol. The ssSPT (cDNAs) open reading frames were inserted after the 3x HA tag in pADH1 (61).

CLONING AND EXPRESSION OF SPT SUBUNITS IN YEAST:

LCB1 and LCB2 genes were PCR amplified, with suitable restriction sites (Table 7.1), and cloned into pAL2-TRP vector. *C. elegans* LCB1 and LCB2 was a generous gift from Dr. Kevin O'connell's laboratory at NIH. *S. pombe* LCB1, LCB2 and *D. melanogaster* (fly) LCB1 and LCB2 were a generous gift of Dr. Napier's laboratory (Rothamsted Research, UK). Plant (*A. thaliana*) LCB1, LCB2a and LCB2b subunits were the gifts from Dr. Cahoon's laboratory (University of Nebraska, USA). All the LCB1s are C-terminally Flag tagged. *S. pombe* LCB2 is C-terminally Myc tagged. *D. melanogaster* and *A. thaliana* LCB2 subunits are are N-terminally Myc tagged, and *C. elegans* LCB2, which is untagged. pAL2-TRP was constructed for divergent constitutive expression of LCB1-FLAG and Myc-LCB2a or Myc-LCB2b by replacing the Gal1 and Gal 10 promoters of pESC-TRP (Stratagene) with the yeast *LCB2* and *ADH* promoters respectively (59).

AtssSPTa and AtssSPTb were PCR amplified using specific primers from the At-CDNA library (Table 7.1). PssSPT and Porm were amplified from the genomic DNA

prepared from wild type 972 *S. pombe* strains (a generous gift from Dr. Chattopadhyay at NIH), since it does not have any intron. FssSPTa and FssSPTb clones were a generous gift from Dr. Jonathan Napier's laboratory. A FssSPTc clone obtained from The Drosophila Genomics Resource Center (DGRC) was used as a template to PCR FssSPTc. CssSPT was synthesized using 1 forward and 3 reverse primers. Each primer has 10 nucleotide overlap with the next primer sequence. PCR was performed as per standard protocol. The PCR product was then amplified with CssSPTF and CssSPTR primers and cloned into a yeast expression plasmid (pADH) with N-terminally 3X HA tag.
The AtssSPTb M19V mutation was introduced by QuikChange mutagenesis (Stratagene). Similarly, PssSPT L41M mutation was introduced by Quickchange mutagenesis.

COMPLEMENTATION ASSAY:

Activation of different species specific SPT heterodimers by ssSPTs were tested by complementation assay as described in previous studies (43; 59). Yeast cells heterologously expressing different species specific SPT isoforms were grown on SD+PHS and exponentially growing cells were harvested at 0.2 OD_{600nm} and serially diluted at 1:5 into a microtitter plate. Cells were phrogged on different media and grown for 4-5 days at indicated temperatures.

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LONG CHAIN BASE (LCB) ANALYSIS:

5 ODs_{600nm} of yeast cells were harvested from exponentially growing cultures heterologously expressing different SPT subunits. Cells were washed with H_20 and dried

under N_2 in glass screw cap tubes. 1ml of 1N Methanolic HCL containing 0.5 μM C17-SPH was added. Samples were boiled in the screw cap tube for 30 minutes in water bath. After brief cooling on ice, 1ml of 1% NaCL was added and followed by vortexing. 2ml of Hexane: Ether (1:1 v/v) was added, vortexed, and spun in a clinical centrifuge. The upper organic layer was aspirated off followed by the addition of 0.25ml of 10N NaOH, and vortexed again for proper mixing. 1.75ml of Hexane was added, vortexed and centrifuged. 1.5ml of top organic layer was transferred using a glass pipette directly into the HPLC vials and dried under N_2. Samples were derived for HPLC analysis by adding 80μl of Methanol: 190mM TEA (20:3) and 20μl of $A_{CC}Q$ TagFluor Reagent (Waters) incubated for 30-60 minutes (48; 102). Injection volumes were adjusted to inject 3 ODs worth of extracted LCBs. The HPLC parameters are described in Tsegaye et al 2007 (102)

25 mg of adult flies were used for the extraction of total LCBs. Culture tubes containing larval flies were treated with 15% glucose, and the fly larvae were extracted from the media dried and 25 mg of dry weight was used for the extraction of total LCBs by the above method.

PREPARATION OF MICROSOMES:

Yeast microsomes were prepared from exponentially growing cells that were pelleted, washed in TEGM (50mM Tris-HCl, pH 7.5, 1mM EGTA, 1mM _mercaptoethanol) and resuspended in TEGM containing 1 mM PMSF, 2 mg/ml pepstatin A, 1 mg/ml leupeptin, and 1mg/ml aprotinin. Glass beads were added to the meniscus, and cells were disrupted by repeated (four times, 1 min each) cycles of vortexing with cooling on ice between. Unbroken cells, beads, and debris were removed

by Centrifugation (10,000 $_X$ *g*, 10 min), and the low speed supernatant was centrifuged at 100,000 $_X$ *g* for 40 min. The crude microsomal pellet was homogenized in TEGM and spun at 100,000 $_X$ *g* for 40 min to obtain the microsomal pellet. The pellet was homogenized at 5–8 mg/ml in TEGM containing 33% glycerol and stored at 80 °C.

Fly microsomes were prepared from 50mg of dry weight, followed by homogenization in TEGM containing 1 mM PMSF, 2 mg/ml pepstatin A, 1 mg/ml leupeptin, and 1mg/ml aprotinin. The microsomes were prepared as above.

SPT ASSAY:

SPT was assayed as described (49), using 50μM different chain length of CoAs (Decanoyl CoA, Lauroyl CoA, Myristoyl CoA, Palmitoyl CoA, Stearoyl-CoA or Arachidonyl CoA) except as mentioned. A final concentration of 20μM BSA was supplemented. Assays were done using 200 μg of microsomal protein, unless stated otherwise, at 37 ºC.

PROTEIN-PROTEIN INTERACTIONS:

Immunoprecipitations were conducted as described (9) with minor modifications. Microsomal membrane proteins were prepared from yeast cells expressing Flag-tagged Lcb1, Myc-tagged Lcb2 with HA-tagged ssSPTs. Microsomal membrane proteins were resuspended at 1 mg/ml in IP buffer (50 mM Hepes-KOH, pH 6.8, 150 mM KOAc, 2 mM MgOAc, 1 mM $CaCl_2$, 15% glycerol) supplemented with 1 mM PMSF, 2 mg/ml Pepstatin A, 1mg/ml leupeptin and 1 mg/ml protinin and solubilized using 1% digitonin at 4°C for 2.5 hours. 1 ml of solubilized microsomes were incubated with 25 μl of anti-FLAG beads (Sigma) at 4°C for 4 h and the beads were washed four times with IP buffer containing 0.1% digitonin. The bound proteins were eluted in IP buffer

containing 0.25% digitonin and 200 μg/ml FLAG peptide, resolved on a 4-12 % Bis-Tris NuPAGE gel (Invitrogen) and detected by immunoblotting.

MEMBRANE ASSOCIATION ASSAYS:

The assays were conducted as described (Harmon et al, 2013) with minor modifications. Microsomes prepared from yeast cells expressing HA-tagged ssSPTs (different homologs), LCB1, and LCB2 were incubated on ice in buffer containing 1 M NaCl, 0.2 M Na2CO3, 5 M urea, 0.4% Nonidet P-40 or 2% Triton X-100 for 60 min. The samples were subjected to centrifugation at 100,000 x g for 30 min, and equal proportions of the supernatants and pellets were resolved by SDS-PAGE.

GLYCOSYLATION CASSETTE MOBILITY SHIFT ASSAYS

Microsomal protein (20 μg, with or without EndoH treatment) was resolved by SDS-PAGE and HA-AtssSPTb was detected by immunoblotting as described in Kimberlin et al (59).

PHYLOGENETIC ANALYSIS:

Homologs of LCB1 and LCB2 subunits from different species were identified 'HomoloGene' (http://www.ncbi.nlm.nih.gov/homologene/) from NCBI. The amino acid sequences of the respective genes were downloaded in FASTA format. Phylogenetic analysis was done using MEGA 5.2.2. First, sequences of those homologs were aligned by 'MUSCLE' with Gaps. The Phylogenetic tree was constructed using 'Maximum likelihood' analysis, using the bootstrap value of 100.

Candidate homologs of ssSPTs were identified by 'tblastn' from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Different species were selected and the filter for masking low complexity regions was turned off. The 'Hit' list of candidate genes was then manually verified. Phylogenetic analysis of ssSPTs was performed using MEGA 5.2.2 using the same parameters.

Table 4.1. Expression profiles of different fly small subunits during development.

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	Moderate-high expression (hrs)	
	Expression goes up at	**Declines at**
FssSPTa	Embryo 08-10hr	Larva L3 12hr old
FssSPTb	• Larva L3 puffstage 1-2 • Adult female 05day	• White prepupae new • Adult female 30day
FssSPTc	Embryo 00-02hr	Adult 30day

Table 7.1. List of primers used in this study.

Primer Name	Gene	Restriction site	Sequence
ATL1F (Forward)	ATLCB1	Not1	5'-CCCGGG GCGGCCGCATGGCTTCGAATCTC-3'
ATLCB1 (Reverse)	ATLCB1	Spe1	5'-CCCGGGACTAGTGACTTGAGTAGAA-3'
ATLCB2a (Forward)	ATLCB2a	Sal1	5'-CCCGGGGTCGACATGATTACGATCCCA-3'
ATLCB2a(Reverse)	ATLCB2a	Kpn1	5'-GGGCCCGGTACCTTAATCCAATTTGAT-3'
ATLCB2b (Forward)	ATLCB2b	Sal1	5'-CCCGGGGTCGACATGATAACGATTCCT-3'
ATLCB2b (Reverse)	ATLCB2b	Kpn1	5'-GGGCCCGGTACCTCAATC CAGCTTGAT-3'
PL1F(Forward)	S.Pom LCB1	EcoR1	5'-GGGCCC GAATTC ATGAGTTACTCATATCCCTTTTTT
PL1R(Reverse)	S.Pom LCB1	Avr2	5'-GGGCCC CCTAGG ACTTGATTAATAACCTTATG
PL2F(Forward)	S.Pom LCB2	BamH1	5'-GGGCCC GGATCC ATGGCTCAAGCGGACTTTGTTTCC
PL2R (Reverse)	S.Pom LCB2	Sal1	5'-GGGCCC GTCGAC TGTGTAAGTGCATCATGGAC
FLCB1 (Forward)	Fly LCB1	Not1	5'-CCCGGG GCGGCCGCATGGTGGCCATCCAA-3'
FLCB1 (Reverse)	Fly LCB1	Spe1	5'-CCCGGGACTAGTAGGACGGAGCTGGA-3'
FLCB2 (Forward)	Fly LCB2	Sal1	5'-CCCGGGGTCGACGGCAATTTCGACGGC-3'
FLCB2 (Reverse)	Fly LCB2	Kpn1	5'-GGGCCCGGTACCCTAGTAAATGACGGG-3'
Cl1F (Forward primer)	C.e LCB1 (SPTL1)	NotI	5'-ATAAGAAT GCGGCCGC ATG GGA TTT CTA CCA GAT TCG
Cl1R (Reverse primer)	C.e LCB1 (SPTL1)	SpeI	5'-GG ACTAGT AA GAA TTT ATG AGC AAC AAC TCG
Cl2F(Forward primer)	C.e LCB2 (SPTL2)	BglII	5'-CCCGGG AGATCT ATG TCT CGA CGC ACC GAT TCT
Cl2R(Reverse primer)	C.e LCB2 (SPTL2)	XhoI	5'-CCCGGG CTCGAG CCA CTC GAT TTT CTG ATT TTT
At3F (Forward primer)	AtssSPTa	XhoI	5'- CCCGGGCTCGAGCAACTGGGTTCAACGC
At3R (Reverse primer)	AtssSPTa	XhoI	5'- CCCGGGCTCGAGTCATGTCAAATGCCT
ScF1 (Forward	AtssSPT	XhoI	5'-CCCGGG CTCGAG C AACTGGGTTCAA

primer)	b		
ScR (Reverse primer)	AtssSPTb	XhoI	5'-CCCGGG CTCGAG TCATGAAAGATGCCT
PF1 (Forwad primer)	PssSPT	XhoI/Nhe1	5'-CCCGGGCTCGAGGGCTAGCATGGGCAATCCAGTTGTTATT
PR1 (Reverse peimer)	PssSPT	XhoI/Xba1	5'-CCCGGGCTCGAGTCTAGATTACTGAGAAGATGCATCCT
FsCF (Forward primer)	FssSPTc	XhoI	5'-CCCGGG CTCGAG A ATG CTT GAC AAC CTC GTG GAA
FsCR (Reverse peimer)	FssSPTc	XhoI	5'-CCCGGG CTCGAG TTA GGT GAC CTT GAG CCC ATG
Primer1 (Forward)	CssSPT	XhoI	5'-CCATGGCTCGAGAATGTCTACGACGGCTACAGCAACCAC CACCACAACAAAAGCATTCGAAAATGACTATGGGGATTTCA GCCAGTTCAACAA
Primer2 (reverse)	CssSPT	NA	5'-GAGACTAGTAGATATTGAAGATAGACGTGCTCGGCGAACGT TTGCTTTACAGTTTTTGCCTTGTTGAACTGGCTGAAATC
Primer3 (reverse)	CssSPT	NA	5'-AGAGACTAGTAGATATTGAAGGGAATCTACATGCTTGAGCC TTGGGAGCAGCGTCTCTTCAACTGGGTTATCATCTTTGT
Primer4 (reverse)	CssSPT	XhoI	5'-CCATGGCTCGAGTTAGACGACAAAGAAGGTGATTAGGGAAAT GAATGTTGTGAGCACAAAGATGATAACCCAGTT
CssSPTF (Forward)	CssSPT	XhoI	5'- CCCGGG CTCGAG A ATG TCT ACG ACG GCT ACA GCA
CssSPTR (reverse)	CssSPT	XhoI	5'- CCCGGG CTCGAG TTA GAC GAC AAA GAA GGT GAT
PormF (Forward primer)	Porm	XhoI/Nhe1	5'-GGGCCCCTCGAGGGCTAGC ATGGGCAGCAGTAGT
PormR (reverse)	Porm	XhoI/Xba1	5'-CCCGGGCTCGAGTCTAGACTAACGAGGAATACT

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