



#### APPROVAL SHEET

Title of Dissertation: "TSC (Temperature Sensitive suppressors of the Calcium sensitivity of csg2<sub>4</sub>) Mutants; A Tool to Investigate Sphingolipid Metabolism in the Yeast Saccharomyces cerevisiae"

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#### ABSTRACT

Title of Dissertation: TSC (Temperature Sensitive Suppressors of the Calcium Sensitivity of  $csg2\Delta$ ) Mutants; A Tool To Investigate Sphingolipid Metabolism in the Yeast Saccharomyces cerevisiae

Harry Francis Slife, Jr., Ph.D., 1998

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Sphingolipids are essential components of eucaryotic cell membranes but the basis of this essential nature is not understood. Sphingolipids and their metabolites have been implicated in a wide range of critical cell functions ranging from regulating cellular processes to fulfilling a membrane structural role. Defects in sphingolipid metabolism are a known cause of many diseases.

To gain a more clear understanding of sphingolipid homeostasis, a genetic approach to their study was undertaken in the yeast *Saccharomyces cerevisiae* using the  $csg2\Delta$  mutant. The  $csg2\Delta$  mutant fails to mannosylate sphingolipids and exhibits a Ca<sup>2+</sup> sensitive phenotype not seen in wild type cells. Suppressors to the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  possess mutations in genes prior to of the mannosylation step of sphingolipid biosynthesis resulting in decreased synthesis or altered structure of the toxic, unmannosylated intermediate (IPC-C). Identification of these suppressor genes may help elucidate the reactions occurring in sphingolipid synthesis. A collection of  $csg2\Delta$ suppressor mutants was made which possess a secondary ts conditional lethal phenotype to facilitate cloning by complementation. This collection is referred to as the *tsc* collection or Temperature Sensitive suppressors of the Calcium sensitivity of  $csg2\Delta$ . Fifty-nine ts suppressors were isolated which possess mutations in one of 15 genes (*tsc1-tsc15*).

Of the 15 genes responsible for the tsc mutations, 11 have been identified. TSCI and TSC2 encode subunits of the serine palmitoyltransferase (SPT) and were previously identified as LCB2/SCS1 and LCB1 respectively. TSC3 encodes a previously uncharacterized 80 amino acid protein involved in SPT activity. TSC4 and TSC5 are allelic to FAS2 which encodes the  $\alpha$ -subunit of fatty acid synthase, responsible for the synthesis of palmitoyl CoA in the cell. TSC6 encodes the very long chain fatty acid hydroxylase previously identified as SCS7. TSC7 is allelic to SUR2 which encodes the dihydrosphingosine hydroxylase responsible for the formation of phytosphingosine, the predominant long chain base in yeast sphingolipids. TSC10 encodes 3-ketosphingosine reductase which forms dihydrosphingosine from 3-ketosphingosine. TSC11 encodes an uncharacterized protein with no homology to any known protein or functional domain. TSC14 is TOR2, a phosphatidylinositol kinase which forms PIP from PI and TSC15 has been identified as MSS4, the phosphatidylinositol-4-phosphate 5- kinase that adds a second phosphate to the PI-4-P forming PI-4,5-P2. This discussion is limited to the cloning and characterization of TSC1-7 and TSC11.

# TSC (TEMPERATURE SENSITIVE SUPPRESSORS OF THE CALCIUM SENSITIVITY OF CSG2A) MUTANTS; A TOOL TO INVESTIGATE SPHINGOLIPID METABOLISM IN THE YEAST SACCHAROMYCES CEREVISIAE

by

Harry F. Slife, Jr.

Thesis submitted to the Faculty of the Department of Biochemistry Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1998 In memory of Dr. Troy J. Beeler

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and

To Leslie, Caitlin, Carolyn and Jacob, without your love and support none of this would be possible

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### **ABBREVIATIONS**

3-KS	3-Ketosphinganine
ACP	Acyl-carrier protein
ATCC	American Type Culture Collection
BCS	Bathocuproine sulfate
BME	β-Mercaptoethanol
DHS	Dihydrosphingosine
DNA	Deoxyribonucleic acid
DTT	Dithiothreotol
DAG	Diacylglycerol
EDTA	Ethylenediaminetetracetic acid
EGTA	Ethyleneglycol-bis-tetraacetic acid
ER	Endoplasmic Reticulum
FAS	Fatty Acid Synthase
FOA	5-Floroorotic acid
IPC	Inositolphosphorylceramide
IP <sub>3</sub>	Inositoltrisphosphate
LCB	Long Chain Base
LiOAc	Lithium Acetate
MIPC	Mannosylinsositolphosphorylceramide
M(IP) <sub>2</sub> C	Mannosyldiinositolphosphorylceramide
NADPH	Nicotinamide Adenine Dinucleiotide Phosphate (reduced
	form)
ORF	Open reading frame
PEG	Polyethyeneglycol
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PIP	Phosphatidylinositolphosphate

PIP <sub>2</sub>	Phosphatidylinositoldiphosphate
PMSF	Phenylmethyl Sulfonyl Fluoride
SD	Synthethic Dextrose (media)
SDR	Short-chain Dehydrogenase/Reductase (enzyme family)
SLC	Sphingolipid Compensation (mutants)
SPT	Serine Palmitoyltransferase
TLC	Thin Layer Chromatography
TRIS	Tris(hydroxymethyl)aminomethane buffer
TSC	Temperature Sensitive suppressors of Ca <sup>2+</sup> sensitivity
UAS	Upstream Activating Sequence
VLCFA	Very Long Chain Fatty Acid
YNB	Yeast Nitrogen Base
YPD	Yeast extract, Peptone, Dextrose (media)

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### <u>Chapter 1</u> <u>Introduction</u>

Sphingolipids are essential components of eukaryotic cell membranes though the basis of their essential nature is unknown. Research suggests sphingolipids are involved in altering charge and fluidity of membranes<sup>1</sup>, acting as binding sites for viruses, bacteria and toxins<sup>2, 3, 4</sup>, cell-cell recognition<sup>5</sup>, endocytosis<sup>6</sup>, and participate in cell wall<sup>7</sup> and lipid anchored protein synthesis.<sup>8, 9, 10</sup> Sphingolipid metabolites, including sphingosine, sphingosine-1-phosphate, and ceramide, appear to act as secondary messengers in signal transduction pathways<sup>5, 11, 12</sup>, stress responses<sup>13</sup>, cell proliferation<sup>14</sup>, cell differentiation<sup>15</sup>, initiation of apoptosis<sup>16, 17</sup>, and regulation of membrane proteins.<sup>18</sup> Though the experimental evidence supporting these functions is far from conclusive, it is obvious that sphingolipids are important in normal cellular metabolism.

To further our understanding of sphingolipid metabolism and function we have adopted a genetic approach. A genetic approach involves isolating cells mutated in genes involved in the process being studied and observing the phenotypic consequence of those mutations. The mutated gene is then identified, usually by complementation with a wild type copy of the gene, and homology studies are used to group the gene product into a family of functional proteins. The identity of the protein family provides insight into the reaction the protein is involved in and potentially, identifies reactions in related pathways. We have applied this genetic approach to elucidating the sphingolipid biosynthetic pathway in *Saccharomyces cerevisiae*. Saccharomyces cerevisiae offers some distinct advantages over other systems in the study of sphingolipids. First, powerful genetic techniques are available in this organism which are well characterized and standardized. Second, the *S. cerevisiae* genome is completely sequenced. Finally, the study of sphingolipids in mammalian cells is hampered by the number of sphingolipid species produced. Over 300 different sphingolipids, differing primarily in the complexity of the polar head group, have been identified in mammalian cells.<sup>17</sup> *S. cerevisiae* synthesizes only three mature sphingolipids by way of a common biosynthetic pathway. Since single cell organisms lack the complex structures and specialized tissues most commonly associated with the sphingolipids in mammalian cells only essential sphingolipid functions are probably conserved in this simple eucaryote. Gaining a comprehensive knowledge of sphingolipid homeostasis in a simple eukaryote will provide a better understanding of their role in more complex organisms.

#### Sphingolipid Structure

Sphingolipids are amphipathic molecules composed of a polar head group attached to a hydrophobic ceramide by a phosphodiester linkage (Figure 1). In *S. cerevisiae* the polar head group contains inositol phosphate which may be decorated with mannose or phosphoinositol-mannose producing the three sphingolipid biosynthetic end products, inositolphosphorylceramide (IPC-C), mannosylinositolphosphorylceramide (MIPC), or mannosyldiinositolphosphoryl-ceramide ( $M(IP)_2C$ ) (Figure 2). The ceramide portion of the molecule is composed of a sphingoid base attached, by an amide linkage, to



Figure 1. Sphingolipid structure

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Figure 2. Proposed sphingolipid biosynthetic Pathway in S. cerevisiae.

a fatty acid. The primary sphingoid base in S. cerevisiae is phytosphingosine which is hydroxylated at C<sub>3</sub> and C<sub>4</sub>.<sup>1</sup> The attached fatty acid is 26 carbons in length and is called a very long chain fatty acid (VLCFA) which is hydroxylated on the  $\alpha$  carbon ( $\alpha$ -hydroxyhexaconsanoic acid).<sup>19</sup>

#### Sphingolipid Synthesis

Localization of sphingolipid synthesis in *S. cerevisiae* has been studied using temperature sensitive secretory mutants blocked at various points in the secretory pathway.<sup>20</sup> Accumulation of nascent sphingolipid intermediates in these mutants at their non-permissive condition indicates maturation and transport follows the protein secretory pathway.<sup>21, 22</sup> By correlating the blockage point in vesicle trafficking to the accumulated sphingolipid species it was deduced that synthesis is initiated at the endoplasmic reticulum, continues through the Golgi, and results in mature sphingolipids being highly enriched in the plasma membrane.<sup>23, 21, 22</sup>

Sphingolipid synthesis in yeast requires palmitoyl CoA for the formation of sphingoid base, VLCFA (Figure 2) and phosphatidylinositol (PI) which donates the phosphoinositol head group. Synthesis of palmitoyl CoA is catalyzed by a multi enzyme complex, fatty acid synthase (FAS). FAS catalyzes seven separate reactions to add malonyl CoA subunits to an acetyl CoA molecule.<sup>1</sup> It also codes for the acyl carrier protein (ACP) which acts as a scaffold to assemble FAS subunits. FAS is composed of two multifunctional proteins,  $\alpha$  and  $\beta$ , arranged in an  $\alpha_6\beta_6$  stochiometry.<sup>24, 25, 26, 27</sup> These subunits are multifunctional polypeptides carrying out several different reactions.<sup>28</sup> The

 $\alpha$  subunit is encoded by *FAS2* and is responsible for the  $\beta$ -ketoacyl reductase, and  $\beta$ -ketoacyl synthase activities and also encodes the ACP of FAS.<sup>25</sup> The  $\beta$  subunit is encoded by *FAS1* and contains the five remaining catalytic activities (enoyl reductase, acetyltransacylase, dehydratase, and malonyl and palmitoyl transacylases).<sup>24</sup>

*FAS1* and *FAS2* are genetically distinct, unlinked genes. FAS genes are, however, coordinately regulated to facilitate proper assembly of the multi-protein complex.<sup>28,29</sup> The coordinated expression of *FAS1* and *FAS2* is due to a common upstream activating sequence (UAS) shared by many other yeast genes involved in phospholipid biosynthesis.<sup>30</sup> Mutations in either of these genes adversely affect fatty acid synthesis but heterozygous diploids, produced by mating *fas1* and *fas2* mutants, exhibit a wild type phenotype.<sup>24</sup> This is due to interalleleic complementation where one wild type allele of each FAS gene in the diploid expresses sufficient gene product to compensate for the mutant allele. Similarly, since both Fas1p and Fas2p are multifunctional themselves, intraalleleic complementation has been noted in homozygous diploids if the mutations are in separate functional domains of the mutated FAS gene.<sup>24</sup>

Since palmitoyl CoA plays such a pivotal role in lipid biosynthesis it is possible that availability of this molecule may regulate the biosynthetic pathways for glycerophospholipids and/or sphingolipids. In this way palmitoyl CoA could signal the nutritional status of the cell and thereby control growth by limiting lipid for membrane synthesis or initiation of signals appropriate only when the cell is actively growing.

Palmitoyl CoA condenses with serine forming 3-ketosphinganine in the first committed step of sphingolipid synthesis in yeast. This reaction is catalyzed by serine palmitoyltransferase (SPT) which is a member of the pyridoxylphosphate dependent enzyme family of acyl transferases and is composed of at least 2 subunits encoded by *LCB1* and *LCB2*.<sup>31, 32, 33</sup> Lcb1p and Lcb2p exhibit significant identity (22.4%) over their entire sequence suggesting the proteins are related.<sup>1</sup> A notable difference between the sequences of Lcb1p and Lcb2p is that Lcb2p contains a lysine residue that commonly binds pyridoxal phosphate in this family of enzymes. Lcb1p does not possess this lysine suggesting that Lcb2p contains the catalytic site whereas Lcb1p may have evolved into a regulatory subunit.<sup>32</sup> Null mutations in either of these subunits result in a long chain base auxotrophy and a lack of SPT activity in membranes.<sup>34</sup> The SPT step is thought to be the site of regulation for sphingolipid synthesis in animals.<sup>31</sup> However, additional evidence is needed to support this hypothesis. Currently there is no information available on sphingolipid synthesis regulation.

The product of SPT is 3-ketosphinganine (3-KS). The C<sub>3</sub> keto group of this sphingoid base is reduced by 3-ketosphinganine reductase (Tsc10p) to form the aldehyde, dihydrosphingosine (DHS) in an NADPH dependent manner.<sup>35</sup> In wild type cells, 3-KS does not accumulate to detectable levels indicating that the reductase has very high affinity for its substrate and reduces it immediately upon formation by SPT and that accumulation of the keto form of the LCB may be toxic to the cell.<sup>36, 37</sup> This is supported by experimental results showing that Tsc10p is essential to cell viability. Tsc10p is a member of a short chain dehydrogenase/reductase (SDR) family which includes over 60 enzymes.<sup>71</sup> This family exhibits little sequence identity but possesses a characteristic YXXXK sequence (X=any amino acid) in the catalytic site.<sup>13, 38</sup> Several members of this

family also possess a serine residue 13 amino acids amino terminal to the conserved tyrosine. The placement of these conserved residues is consistent with their participation in proton transfer from the conserved tyrosine to the substrate.<sup>13</sup> SDR enzymes also possess GXXXGXG consensus sequence forming a turn between a  $\beta$  strand and an  $\alpha$  helix bordering the NADPH binding domain known as the Rossman fold.<sup>39, 38</sup>

The product of 3-ketosphinganine reduction, dihydrosphingosine, is hydroxylated at C<sub>4</sub> by Sur2p (dihydrosphingosine hydroxylase) forming phytosphingosine.<sup>19</sup> Sur2p is a member of the oxo-diiron family of lipid hydroxylases/desaturases.<sup>19</sup> SUR2 was originally identified as one of a group of genes which, when mutated, suppressed the pleiotropic phenotypes of rvs/61 mutants.<sup>40</sup> RVS161 was named based on mutants exhibiting reduced viability upon starvation. The rvs161 mutants are characterized by an abnormally decreased cell viability and the presence of an increased budding ratio upon nitrogen or carbon exhaustion compared to a wild-type strain.<sup>40</sup> These cells also exhibit cell lysis when starved and defects in stationary phase cell cycle entry.<sup>40</sup> The cells are sensitive to high salt concentrations and exhibit an inability to grow on non-fermentable carbon sources.<sup>40</sup> Analysis of the sequence of Rvs161p gave no indication as to its function and so suppressor analysis was pursued to isolated other related genes. It is now known that all of the identified suppressors of rvs161 (SUR1, SUR2, SUR4, and SUR5) are involved in sphingolipid biosynthesis.<sup>19</sup> The mechanism for Sur2p hydroxylation of DHS requires reduction of the oxo-diiron center, presumably by the transfer of electrons through a cytochrome b<sub>s</sub>-dependent endoplasmic reticulum electron transport chain, however details remain unclear.1.41

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LCB produced by the yeast sphingolipid biosynthetic pathway condenses with a VLCFA via an amide linkage to form a ceramide. The VLCFA is a product of elongation of palmitoyl CoA by the proteins encoded by ELO2/SUR5 and ELO3/SUR4 and also requires the cytochrome b, endoplasmic reticulum electron transport system.<sup>41,42</sup> ELO2 and ELO3 were identified from the S. cerevisiae genome database as homologs of ELOI, a gene required for elongation of myristate ( $C_{14}$ ) to palmitate ( $C_{16}$ ).<sup>42</sup> Both ELO2 and ELO3 encode integral membrane proteins consistent with the fatty acid elongation activity being membrane associated.<sup>42</sup> Disruption of either gene results in an altered composition of fatty acids and sphingolipids in the cell (our unpublished observations).<sup>42</sup> The  $elo2\Delta$ strain produces reduced levels of sphingolipids with normal length VLCFA whereas  $elo3\Delta$ produces normal levels of sphingolipid but with shorter VLCFA (our unpublished observations). Simultaneous disruption of these genes is lethal.<sup>42</sup> Analysis of single mutants indicates that Elo2p elongates palmitate to a  $C_{22}$  fatty acid in sequential 2 carbon additions. Elo3p acts on a broader range of substrates but the conversion of a C24 to a C26 fatty acid is exclusively catalyzed by this enzyme.<sup>42</sup>

Formation of ceramide is catalyzed by ceramide synthase (sphinganine Nacyltransferase). The gene encoding ceramide synthase, which attaches the VLCFA to the LCB, has not yet been identified. Synthesis of ceramide occurs on the cytosolic side of the ER and requires an VLCFA-CoA as substrate.<sup>1</sup> The activity of ceramide synthase is expected to be very high because precursors are not detected in intact cells.<sup>36, 37</sup> It is not clear if the primary substrate for ceramide synthase is phytosphingosine or dihydrosphingosine; both LCBs are found in cells blocked in ceramide synthesis with inhibitors of the ceramide synthase (see below) and *sur2* mutants do not prevent formation of ceramide.<sup>19</sup>

The action of ceramide synthase is blocked by fuminosins. Fuminosins are toxic compounds found in grain contaminated with the mold *Fusarium moniliforme*. Consumption of contaminated grain containing these compounds has been linked to human esophageal cancer in areas of Africa and the Far East.<sup>43,44</sup> Fuminosins possess a remarkable structural similarity to dihydrosphingosine which led to the hypothesis that this similarity was responsible for their disease causing nature.<sup>44</sup> Fuminosins effect on ceramide synthase was determined by use of radiolabeled sphingolipid maturation studies in the presence and absence of fuminosins as well as direct enzyme activity assays.<sup>44</sup> The use of fuminosins has facilitated the study of the effect of LCB on cellular function by inhibiting the formation of ceramide.<sup>45</sup>

The VLCFA moiety of phytoceramide is hydroxylated at the  $\alpha$  carbon by phytoceramide hydroxylase (Scs7p). This enzyme is another member of the oxo-diiron hydroxylase/desaturase family, similar to Sur2p, and like Sur2p, the reaction is believed to occur on the endoplasmic reticulum, using the cytochrome b<sub>5</sub>-dependent electron transport chain and is not essential for cell viability nor is it required for synthesis of mature sphingolipids. <sup>19,46</sup> The oxo-diiron domain of these enzymes consists of four transmembrane segments. The loops between these segments include histidine motifs specific for this family of hydroxylases/desaturases.<sup>19,46</sup> Interestingly, Scs7p differs from Sur2p in that it possess a cytochrome b<sub>5</sub>-like domain on its N-terminus, and presumably interacts directly with the cytochrome b<sub>5</sub> reductase.<sup>19</sup> The purpose of these

hydroxylations is unknown; they may play a role in localization of ceramides within the cell, or may alter the physical properties of membranes.<sup>19</sup> They may also impact sphingolipid interaction with integral membrane proteins.<sup>19</sup>

An alphabetic IPC nomenclature has been adopted based on the hydroxylation status of LCB and VLCFA and the resulting effect on their hydrophobicity and thus on the migration of IPC species on TLC plates. The more hydrophobic species run further on the TLC plate due to their decreased hydroxylation status. Since *TSC10* is essential all sphingolipid products are hydroxylated on C<sub>3</sub> of the LCB. Mutations in either *SUR2* or *SCS7* result in altered hydroxylation status on C<sub>4</sub> of the LCB and the  $\alpha$ -carbon of the VLCFA.<sup>19</sup> IPC-A results from mutations in both *SUR2* and *SCS7* and is hydroxylated on C<sub>3</sub> of the LCB only, IPC-B is produced by *scs7* mutants and is hydroxylated on C<sub>3</sub> of the LCB, IPC-B' is synthesized by *sur2* mutants and therefore is hydroxylated on C<sub>3</sub> of the LCB and on the  $\alpha$ -carbon of the VLCFA, and in IPC-C is hydroxylated on both the LCB and the VLCFA.<sup>19</sup>

 $\alpha$ -hydroxyphytoceramide is the product of Scs7p modification of IPC. This product is modified by the addition of a phosphoinositol to the head group of the nascent sphingolipid at the ER by inositolphosphorylceramide (IPC) synthase, encoded by the *AUR1* gene.<sup>47</sup> *AUR1* was initially identified as a gene, which when mutated, conferred resistance to the anti-fungal agent aureobasidin.<sup>48,49</sup> The *aur1* mutant link to sphingolipid biosynthesis was determined in a screen of SLC (sphingolipid compensation) mutants which possess a disrupted *lcb1* gene and have acquired the ability to use glycerophospholipids in place of sphingolipids.<sup>47</sup> The screen found a mutant unable to incorporate <sup>3</sup>H-inositol into mature sphingolipids and when provided exogenous phytosphingosine, which bypassed the *lcb1* disruption, it accumulated toxic levels of ceramide.<sup>17,47</sup> The strain also could not tolerate transformation with a plasmid-based *LCB1* gene.<sup>47</sup>

IPC is carried to the Golgi by vesicular transport and mannosylated forming mannosylinositolphosphorylceramide (MIPC).<sup>21, 50</sup> At least two genes are required for mannosylation of IPC, CSG1 and CSG2.<sup>32,50</sup> Mutations in either of these genes leads to an accumulation of IPC-C and confer a Ca<sup>2+</sup> sensitile phenotype. <sup>50</sup> Mutants display normal growth on rich media (YPD) indicating that mannosylation of sphingolipids is not essential to viability.<sup>51, 50</sup> Ca<sup>2+</sup> sensitivity of csg mutants is remediated by increasing the osmolarity of the media indicating that the defect in csg mutants is a weakened cell wall.<sup>1,50</sup> CSG1 and CSG2 were identified in a screen for mutants exhibiting altered Ca<sup>2+</sup> homeostasis.<sup>51</sup> A screen of over 60,000 mutants yielded 18 which displayed increased Ca<sup>2+</sup> sensitivity but tolerance to Sr<sup>2+</sup> indicating this was a Ca<sup>2+</sup> specific defect.<sup>51</sup> Ten of these mutants fell into the CSG1 or CSG2 complementation groups. The two genes were identified by complementation of the Ca<sup>2+</sup> sensitive phenotype by complementation with a plasmid based genomic library. Csg1p is an integral membrane protein which possesses a region of homology to  $\alpha$ -1.6 mannosyltransferase, Ochlp.<sup>50</sup> Ochlp mannosylates proteins in the Golgi, using GDP mannose as a donor.<sup>52</sup> This homology suggests that Csglp may function directly in the mannosylation of IPC. Homology studies using the sequence of Csg2p give no indication as to its function, however, it is an integral membrane protein possessing 9 putative transmembrane domains and an EF-hand

Ca<sup>2+</sup> binding domain.<sup>51</sup> Overexpression of *CSG1* suppresses the Ca<sup>2+</sup> sensitive phenotype of *csg2* mutants and results in increased synthesis of MIPC; however, overexpression of *CSG2* cannot rescue *csg1* mutants.<sup>50</sup> These results suggest that Csg1p may be the mannosylating enzyme in sphingolipid biosynthesis and Csg2p may either be a nonenzymatic subunit in a mannosylation complex or be involved in presenting IPC to Csg1p for mannosylation.<sup>51</sup> To obtain information concerning the function of Csg2p suppressor analysis was conducted yielding first the *scs* (Suppressors of the Calcium Sensitivity of *csg2*Δ) collection. Identification of *SCS1* as a subunit of the enzyme catalyzing the first committed step of sphingolipid biosynthesis indicated that the CSG genes are involved in sphingolipid biosynthesis.<sup>32, 37</sup>

MIPC is further modified in the Golgi with the addition of a second phosphoinositol to the head group of the nascent sphingolipid forming mannosyldiinositolphosphorylceramide ( $M(IP)_2C$ ) which is the end product of sphingolipid synthesis in yeast. This phosphoinositol transfer is catalyzed by inositolphosphotransferase (*IPT1*, M(IP)<sub>2</sub>C synthase). *IPT1* was identified as a homolog of *AUR1* in the *S. cerevisiae* genome.<sup>53</sup> *IPT1* encodes an integral membrane protein containing 7 predicted transmembrane domains.<sup>53</sup> A conserved membrane spanning domain is shared with *AUR1* and is believed to contain the catalytic site.<sup>53</sup> Point mutations in this domain of the *AUR1* gene confer resistance to aureobasidin A.<sup>53</sup>  $M(IP)_2C$  synthase activity is also sensitive to aureobasidin A, but less so than Aur1p (IPC synthase)<sup>53</sup>

#### Sphingolipid Breakdown

Degradation of sphingolipids in yeast is not well understood. The enzymes responsible for cleaving off the polar head group (phospholipases) or hydrolyzing the amide linkage (ceramidases) have not been identified. Free LCB, a metabolite of sphingolipid breakdown and an intermediate in sphingolipid synthesis, is phosphorylated by a kinase which has not yet been identified. Breakdown of phosphorylated LCB is catalyzed by a lyase encoded by DPL1. This lyase cleaves the phosphorylated LCB between C<sub>2</sub> and C<sub>3</sub> resulting in phosphoethanolamine and a C<sub>16</sub> aldehyde.<sup>54</sup> Mutations in dpl1 confer hypersensitivity to exogenous LCB probably due to an accumulation of free LCB which cannot be degraded.<sup>54</sup> Null mutations of *dpl1* rescue the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  (our unpublished observations) probably due to feedback inhibition, by accumulated phosphorylated LCB, on SPT activity, though this has yet to be proven. The phosphorylated LCB can also be recycled back into sphingolipid biosynthesis under the action of a phosphatase, Lcb3p.<sup>55, 56</sup> Overexpression of this gene suppresses the LCB hypersensitivity phenotype of the dpl1 mutants.<sup>56</sup> It is suspected that accumulation of the end products of the kinase and lyase may act in a negative feedback mechanism on SPT activity.

#### Sphingolipid Investigation

Our investigation of sphingolipid biosynthesis stems from the observation that the Ca<sup>2+</sup> sensitive phenotype of *csg* mutants arises from accumulation of IPC-C due to the

block in its conversion to MIPC. Therefore, secondary mutations that decrease the formation of IPC-C or alter its structure suppress the  $Ca^{2+}$  sensitivity phenotype of *csg* mutants. In this way *csg1* and *csg2* mutants provide a powerful means of identifying mutations in sphingolipid synthesis genes.

Using this approach we have isolated a collection of suppressors of the Ca<sup>2+</sup> sensitive phenotype of  $csg2\Delta$  referred to as tsc mutants or Temperature sensitive Suppressors of the Calcium sensitivity of  $csg2\Delta$ . In addition to suppressing the Ca<sup>2+</sup> sensitive of  $csg2\Delta$ , these mutants also possess a conditional lethal temperature sensitivity phenotype. A description of the tsc collection is the topic of this thesis.

### <u>Chapter 2</u> <u>TSC Isolation and Identification</u>

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#### Introduction

To identify genes and proteins involved in sphingolipid metabolism in the yeast Saccharomyces cerevisiae a genetic approach was used with a collection of mutants possessing defects in this pathway. These mutants are suppressors of the Ca<sup>2+</sup> induced death of  $csg2\Delta$  and are temperature sensitive. They are referred to as Temperature Sensitive suppressors of the Calcium sensitivity of  $csg2\Delta$  or *tsc* mutants. Fifty-nine *tsc* mutants have been isolated which are the result of mutations in 15 genes. Here we report on the methods used to isolate and characterize these genes.

#### **Materials and Methods**

*Materials*: Polyethylene glycol was obtained from Fluka, Glucuronidase was obtained from Boehringer Mannheim GmbH, Zymolase 100T was obtained from Seikagaku Corp., Tokyo, *E. coli* competent cells, AG-1 and XL1-blue, were obtained from Stratagene, La Jolla, CA, Restriction enzymes were obtained from New England Biolabs or BRL, all other chemicals were obtained from Sigma.

Yeast Strains used include: CuH3 (Mata, ura3-52 his4-619), DBY947 (Mata ade2-101 ura3-52), TDY2037 (Mata lys2 ura3-52 trp1 $\triangle$  leu2 $\triangle$ ), TDY2038 (Mata lys2 ura3-52 csg2::LEU2<sup>+</sup> trp1 $\triangle$  leu2 $\triangle$ ) TDY2039 (Mata ade2-101 ura3-52 trp1 $\triangle$  leu2 $\triangle$ ) and TDY2040 (Mata ade3 ura3-52 csg2::LEU2<sup>+</sup> trp1 $\triangle$  leu2 $\triangle$ ). All other strains were obtained from these by standard crosses. *Growth Media*. Yeast media components (agar, peptone, yeast extract, YNB) were obtained from DIFCO and media were prepared using standard techniques.<sup>57</sup> Phytosphingosine, purchased from Sigma, was suspended in ethanol at 25 mM and diluted into autoclaved media along with 0.2% tergitol (Non-iodet detergent, NP-40; Sigma).

Requirements for tsc mutant collection. To be included in the tsc mutant collection, strains had to exhibit the following characteristics: (1) Suppression arising from a spontaneously occurring mutation in a  $csg2\Delta$  mutant allowing growth on YPD containing 20 mM Ca<sup>2+</sup>; (2) exhibit a temperature sensitive (ts) phenotype at 37°C; (3) suppression and ts phenotypes are due to the same mutation, and (4) the responsible mutations are recessive.

*Nucleic acid manipulation.* DNA was prepared from yeast by the method of Holm *et al.*, 1986.<sup>58</sup> Plasmid DNA was prepared from *E. coli* using the method of Holmes and Quigley, 1981<sup>59</sup> or using the Jetquick Plasmid Miniprep Spin Kit (Genomed Inc., Research Triangle Park, N.C.). DNA was radioactively labeled using  $[\alpha^{32}P]dATP$  and the Gibco DNA random labeling kit. DNA sequence determination was accomplished by PCR using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI 373A DNA Sequenator, both obtained from Perkin Elmer Corp. *Isolation of the tsc mutants.* Csg2p is an integral membrane protein required for mannosylation of sphingolipids and growth on media containing greater than 10 mM Ca<sup>2+</sup><sup>32</sup>. Second-site suppressors of the Ca<sup>2+</sup> sensitivity of *csg2*Δ were isolated to identify other

genes involved in sphingolipid metabolism. A null allele of csg2, with most of the coding

sequence replaced with a *LEU2* marker gene, was used to isolate suppressors to eliminate true revertants from being identified. Single  $csg2\Delta$  (null) mutant colonies (TDY2038 and TDY2040) were plated on YPD media containing from 20 mM to 100 mM Ca<sup>2+</sup> with (the CJY mutant strains) and without (the LHY mutant strains) 80  $\mu$ M bathocuproine sulfonate at 26° C to obtain independent, spontaneously occurring mutations suppressing the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$ . BCS is a copper chelator. Copper is required by an enzyme which adds a second hydroxylation on the VLCFA moiety of IPC-C forming IPC-D. BCS inhibits the hydroxylation of IPC-C thereby exacerbating the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  mutant cells due to accumulation of IPC-C.<sup>50</sup> A total of 946 independent suppressors of the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  were isolated. These were tested for temperature sensitivity by plating on YPD and incubating at 37°C, a permissive condition for the starting  $csg2\Delta$  strain. The goal was to obtain mutants in which the same mutation causing suppression of Ca<sup>2+</sup> sensitivity simultaneously conferred temperature sensitivity (Figure 3).

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Determining recessiveness. The cloning protocol requires introducing a plasmidbased genomic library into *tsc* mutant haploid cells. The mutations resulting in the *tsc* phenotype must, therefore, be recessive to be complemented by the wild type gene. Dominant mutations are not complemented and their phenotype remains unaltered. To determine if the *tsc* mutation was recessive, haploid suppressors were mated to parental  $csg2\Delta$  strains and the resulting diploids tested for Ca<sup>2+</sup> sensitivity. These diploids were homozygous for  $csg2\Delta$  but heterozygous for the *tsc* mutation. Recessive mutations in a diploid are masked by the wild type gene on the homologous chromosome resulting in a

# **TSC Phenotype**



Figure 3. Common phenotype of *tsc* mutants

 $csg2\Delta$  phenotype. Our screen retained only recessive mutants.

Linkage analysis; ts vs suppression. Using conditional lethal, ts suppressors of  $csg2\Delta$ , allows for use of a positive selection scheme to identify a *tsc* complementing clone. This is true only if the mutation suppressing the Ca<sup>2+</sup> sensitivity and conferring the ts are in the same gene and therefore do not independently segregate in meiosis.

Two events of major genetic consequence occur during meiosis; genetic recombination and chromosome segregation.<sup>60</sup> The frequency of a recombination event occurring between two genes, resulting in their segregation, is directly related to the physical distance between those genes on a chromosome.<sup>60</sup> To determine if the mutations resulting in suppression and ts were linked in the *tsc* collection, mutants were mated to parental  $csg2\Delta$  strains and the diploids sporulated. The resulting 4 spores, or tetrad, are haploid and are the product of a single meiotic event. The spores are encapsulated in a glycoprotein complex, the ascus, which can be digested with glucuronidase and the spores removed by micro-manipulation and individually phenotypically evaluated in tetrad analysis. Tetrad analysis of the *tsc* mutants indicated that all but one, *tsc6*, exhibited co-segregation of ts and suppression phenotypes indicating the mutations are linked.

Of the original 946 suppressors isolated 59 met the criteria of possessing a linked, conditional lethal ts phenotype which was recessive.

Grouping of tsc mutants into complementation groups. To determine how many genes were responsible for the 59 *tsc* mutants they were mated to one another and their ability to complement the mutant phenotype in the diploid was determined. Being that all the *tsc* 

Complementation	Strain	Identity
Group		
TSC1	LHYa38, a64, a67, a137; LHYa21, a45,	LCB2/SCS
	a47, a121, a127, a155, a163; CJYa5,	
	a19; CJYa1, a2, a24	
TSC2	LHYa22, a24, a26, a81; CJYa3, a4, a7,	LCB1
	al2, al4, a31	
TSC3	LHYa60; CJYa16, a18; CJYa8, a11	YBR058 a
TSC4	СЈҮаб	FAS2
TSC5	СЈҰа30	FAS2
TSC6	CJYa32	SCS7
TSC7	LHYa11, a28, a105; LHYa34	SUR2
TSC8	LHYa33, a37; LHYa33, a111, a116,	
	al 57, al 66	SIN3
TSC9	LHYa39; LHYa38, a43, a61, a101,	
	al 37, al 41	RPD3
TSC10	LHYa56, a108, a109	YBR265W
TSC11	LHYa53	YBR093C
TSC12	LHYa4	RPD3
TSC13	LHYa15	YDL015C
TSC14	LHYa82	TOR2
TSC15	LHYa86	MSS4

Table 1. The TSC Mutant Collection Complementation Groups

mutations, by definition, are recessive only those diploids homozygous for the mutant gene exhibit a *tsc* phenotype; this indicates that the parental haploids both have defects in the same gene. Those haploids failing to complement one another were grouped into complementation groups (Table 1). To date 15 complementation groups are known. Eight of the groups contain only one member suggesting that the collection is far from saturated.

Screening of the tsc mutants with scs mutants. Two collections of  $csg2\Delta$  suppressors have been isolated in our laboratory. The first, Suppressors of Calcium Sensitivity or scs mutants, were isolated based on their suppression phenotype. The second is the *tsc* collection, which exhibits the secondary phenotype of temperature sensitivity. Seven complementation groups exist in the *scs* collection; two have been identified (*SCS1*, *SCS7*). Members of each *tsc* complementation group were mated to members of each *scs* complementation group. The resulting diploids were tested for suppression and temperature sensitivity to determine if any of the *scs* mutants were defective in the same genes as the *tsc* mutants. Wild type *SCS* genes already identified were transformed into *tsc* mutants to determine complementation directly. This screen identified *TSC1* as *SCS1* and *TSC6* as *SCS7*.

*Identification of the TSC genes.* Mutants were transformed with a plasmid-based genomic libraries carrying nutritional markers (YCp50 *URA3* or pRS200 *TRP1*) using the LiOAc method of Geitz *et al.* (1995).<sup>61</sup> Four independently produced banks of the YCp50 library were used. Transformants were selected first as nutritional prototrophs, to indicate presence of the plasmid, and then replica plated to YPD at 37°C. Colonies

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growing at 37° were tested for Ca<sup>2+</sup> sensitivity on YPD and SD media containing 20 mM Ca<sup>2+</sup>. Transformants were then grown on YPD at 26° C to allow the transformed yeast strain to lose the plasmid. Selection of *ura3* segregants from the YCp50 library was done on plates containing 5-flouroorotic acid (FOA)<sup>1</sup>. Segregants from the *TRP1* (pRS200) library were selected from YPD + Ca<sup>2+</sup> plates at 26° C. The resulting segregants, which have lost the plasmid, were tested again at 37° and on 20 mM Ca<sup>2+</sup> media to determine if their complementing phenotype was plasmid linked. If the segregants reverted to a ts, Ca<sup>2+</sup> resistant phenotype it was concluded that the complementing phenotype was plasmid linked. Complementing plasmids were harvested from yeast genomic preps and amplified in *E. coli*. Insert sequence was determined by hybridization of a [ $\alpha$  <sup>12</sup>P]dATP labeled *Sau3*AI digest of the complementing clone to a blot of the contiguous clone collection of the yeast genome (ATCC) or by sequencing.

Contig hybridization. Approximately 100 ng of complementing plasmid DNA was digested with Sau3AI. The digest was randomly labeled with  $[\alpha^{32}P]ATP$  using the Gibco random labeling kit recommended protocol with the following changes: unlabeled dCTP substituted for dATP and  $[\alpha^{32}P]dATP$  for labeled dCTP. Hybridization membranes containing DNA from physically mapped overlapping clones covering the

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<sup>&</sup>lt;sup>1</sup>5-Flouroorotic acid (FOA) is an analog of Orotic acid, an intermediate in uracil and thymine biosynthesis. FOA is readily taken up by yeast cells and enters the uracil/thymine biosynthetic pathway and is converted to 5-flourouracil. This metabolite blocks the methylation of uracil that forms thymine which is essential. A cell which is a uracil auxotroph cannot transform FOA to 5-flourouracil and so survives on plates containing FOA. If the transformed cells have lost the URA3 containing plasmid they will grow on FOA plates (Boeke, *et al.* 1984).<sup>62</sup>

entire genome of Saccharomyces cerevisiae were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The membranes were incubated at 68°C in a roller bottle containing prehybridization fluid (0.9 M NaCl, 0.09 M sodium citrate, 100 µg/ml denatured salmon sperm DNA, and 1mg/ml each of polyvinylpyrrolidone, ficoll, and bovine serum albumin, pH 7.0 (Denhardt's reagent)) for 2 hours. The Sau3AI labeled digest obtained above was added with 1 M EDTA to make a final concentration of 0.01 M EDTA. The hybridization reaction was carried out at 68°C overnight. The membranes were washed by successive incubations in 0.3 M NaCl, 0.03 M sodium citrate and 0.5% SDS, pH 7.0; 0.3 M NaCl, 0.03 M sodium citrate and 0.1% SDS at room temperature for 5 min then in 15 mM NaCl, 1.5 M sodium citrate and 0.5% SDS in the roller bottle at 68°C for 2 hours. The dried membranes were then exposed to X-ray film (XAR5 Kodak) and the resulting autoradiogram identified the region of the S. cerevisiae genome that hybridized to the labeled probe. Each spot on the ATCC contiguous array is assigned an alphanumeric designator which assigns a clone number to the spot. This clone number is cross referenced in the Saccharomyces cerevisiae database available on the internet at http://genome-www.stanford.edu /Saccharomyces/, and the region of the genome harboring the clone can be easily identified. Restriction enzyme digests of the complementing DNA were made to identify the size of the plasmid insert and align it with the region of the genome.

Subcloning. Once the complementing fragment was isolated subclones of the complementing insert were constructed using specific restriction enzyme digests to isolate ORFs which were then ligated into shuttle vectors and transformed into the
evaluated tsc mutant to determine their ability to complement the tsc phenotype.

Sequencing. Direct sequencing of the plasmid insert is an alternative to using radio-labeled plasmid digests in identifying the insert sequence on complementing constructs. Primers were constructed flanking the vector insert site (BamHI) of YCp50, and the bluescript T3 and T7 primers (Stratagene, LaJolla, CA) were used to amplify the DNA inserted into the polylinker of pRS200. PCR sequencing techniques were used to obtain sequence of several hundred nucleotides into the genomic DNA insert using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Corp.). In a PCR eppendorf tube 500 ng/µL of template complementing plasmid DNA, 3.2 pM of primer, and 8 uL of Terminator Ready Reaction Mix were combined and the total volume brought to 20 µL with dH<sub>2</sub>O. The tubes were then placed in a thermalcycler using a protocol of 25 cycles consisting of 96°C for 30 sec., 50°C for 15 sec. and 60°C for 4 min. Upon completion the mix was held at 4°C until removed. The extension products were then purified using STE SELECT-D, G-50 Spin columns for purification of DNA (5 Prime > 3 Prime Inc., Boulder CO.). The columns are inverted several times to resuspend the gel then allowed to drain. The gel was washed with 1 mL of water and allowed to drain and then dried by spinning at 1000 g for 2 min. The column was placed in a collection tube and the 20 µL of the PCR product was layered on top of the gel bed. The columns were then spun at 1000 x g for 2 min. The eluate was then loaded onto an ABI Sequenator (Perkin Elmer Corp.). This sequence was then used in a BLASTN search of the yeast genome and the homologous region identified. Subclones are then made by restriction enzyme digesting or by PCR-amplifying defined regions to identify the

complementing gene within the insert.

Direct sequencing of the complementing clone holds significant advantages over using the hybridization technique. First, it eliminates the use of isotope. Second, the resulting sequence identifies the exact beginning and end points of the insert eliminating the need to map the complementing plasmid. Third, the sequence identifies the orientation of the insert, with respect to the vector, which aids in construction of subclones.

Linkage analysis. To test whether the complementing ORF is the wild type allele of the *tsc* mutant gene, the locus corresponding to the complementing gene was marked with an auxotrophic marker. This was accomplished by subcloning the complementing gene in an integrating plasmid (eg., pRS306) and linearizing the plasmid at a unique restriction site in the midst of the coding sequence for the gene. The linearized construct was transformed into a parental  $csg2\Delta$  haploid (TDY2038 or TDY2040). Linearized DNA integrates by homologous recombination in the yeast genome at the locus of the cut gene.<sup>63</sup> The integration results in tandem repeats of the genomic sequence on the plasmid flanking the integrated plasmid, including the *URA3* marker, in the case of pRS306. The marked strain was then mated to the *tsc* mutant being investigated and the resulting diploids were sporulated and dissected. The marked locus corresponds to the *tsc* locus if the products of meiosis exhibit uniform segregation of the *tsc* phenotype away from the auxotrophic marker.

Null mutant construction. Null tsc mutants were constructed by replacing regions of the coding sequence of the complementing ORF with auxotrophic marker sequence (URA3, TRP1, LEU2). The disrupted ORF was then excised from the plasmid by restriction digestion and the linearized fragment was transformed into wild type and  $csg2\Delta$  haploid and diploid strains. Transformants were selected on SD auxotrophic media. The DNA fragment integrates into the yeast genome to disrupt the ORF locus by a homologous recombination double crossover event.<sup>63,72</sup> Integrations are performed in both haploid and diploid strains because disruption of essential genes results in a lethal phenotype in haploid cells. Obtaining transformants in only diploid cells is a strong indication that the disrupted gene is essential for vegetative growth. Diploid transformants were sporulated and dissected and tetrad analysis was used to determine if products of meiosis inheriting the disrupted allele were viable, and if so, whether they exhibit a *tsc* phenotype. If none of the viable products of meiosis exhibit the marker prototrophy and spore viability is consistent with a 2:2 lethal segregation, it is concluded that the disrupted gene is essential for spore germination.

## tsc6 Investigation

CJY $\alpha$ 32 (*tsc6-1*, *csg2* $\Delta$ ) was found to have a mutation in *SCS7*, a cytochrome b-5 dependent  $\alpha$ -hydroxylase which acts on the very long chain fatty acid moiety of phytoceramide.<sup>19</sup> The mutant was dropped from the *tsc* collection when linkage analysis revealed that the ts and suppression phenotypes were not linked. However, the identification of TSC6 demonstrates another means at our disposal to evaluate members of the tsc mutant collection; sphingolipid analysis by thin layer chromatography.

The scs7 mutant was originally isolated as a suppressor of the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  and cloned by complementation of this phenotype with a plasmid-based genomic



Figure 4. TLC sphingolipid pattern of scs7, tsc6 and  $csg2\Delta$  mutants (IPC-B inositolphosphorylceramide unhydroxylated on the LCB; IPC-C inositolphosphorylceramide hydroxylated on the LCB; MIPC-B mannosylated form of IPC-B; MIPC-C mannosylated form of IPC-C, M(IP)<sub>2</sub>C Mannosyldiinositolphosphorylceramide.

library. Scs7p belongs to a family of desaturases/hydroxylases that contain an oxo-diiron domain.<sup>19, 46</sup> This domain contains four transmembrane segments. The members of this family exhibit a characteristic pattern of histidine residues spaced between the transmembrane segments. The loop between the second and third transmembrane segment contains an  $HX_{3,4}HX_{8-31}HX_{2,3}HH$  motif. After the fourth segment a  $HX_{2,3}HH$  or  $HX_{2,3}HX_{13-39}HX_{2,3}HH$  motif is found.<sup>46, 19</sup> Sphingolipid analysis of CJY $\alpha$ 32 (*tsc6-1*, *csg2* $\Delta$ ) showed a similar TLC sphingolipid pattern to *scs7*, *csg2* $\Delta$  mutants in that both accumulated a more hydrophobic IPC species designated IPC-B (Figure 4).<sup>19</sup> This pattern is due to the loss of the hydroxylation on the VLCFA moiety of the ceramide. Consistent with the other suppressors of *csg2* $\Delta$ , mutations in *SCS7* suppress by reducing the accumulation of IPC-C in a *csg* mutant, which is unable to mannosylate sphingolipids. The seemingly subtle difference of the loss of a hydroxylation on the VLCFA of the ceramide alters the structure sufficiently to suppress the toxic response to Ca<sup>2+</sup>.

The similarity of the TLC patterns of tsc6 and scs7 prompted transforming CJY $\alpha$ 32 (tsc6-1,  $csg2\Delta$ ) with a plasmid bearing a wild type SCS7 gene and testing for its ability to complement the suppression and ts phenotype of tsc6. The tsc6-1,  $csg2\Delta$ mutant was transformed with both a high copy and single copy plasmid borne SCS7. Both complemented the Ca<sup>2+</sup> resistance of tsc6-1,  $csg2\Delta$  but did not alter the ts phenotype, suggesting the ts mutation was in another gene. The TLC pattern of the transformed tsc6-1,  $csg2\Delta$  mutant with pSCS7 also suggested complementation of the IPC-B hydroxylation defect by exhibiting a  $csg2\Delta$  pattern of accumulated IPC-C (Figure Complementation in the diploid was tested by mating an scs7 mutant to a tsc6 mutant and determining complementation of the suppressing phenotype. The diploid remained Ca<sup>2+</sup> resistant providing additional evidence that *TSC6* was allelic to *SCS7*.

Definitive evidence of TSC6/SCS7 allelism came from linkage analysis obtained by mating a *tsc6*,  $csg2\Delta$  mutant to a null mutant of scs7,  $csg2\Delta$  in which the coding sequence of SCS7 was disrupted by insertion of a *URA3* gene. The resulting diploid was sporulated and dissected and tetrad analysis revealed that all products of meiosis were  $Ca^{2+}$  resistant with a uracil protrophy segregating 2:2 in each tetrad. Any crossing over at the *SCS7* locus did not relieve the suppressing phenotype indicating the *tsc6* suppressing mutation is co-located with the *scs7* suppressing mutation.

#### Discussion

The  $csg2\Delta$  mutation results in a failure to mannosylate sphingolipids leading to an accumulation of inositolphosphorylceramide (IPC-C).<sup>32</sup> The accumulation of this intermediate is toxic in the presence of high calcium, though the molecular basis for this sensitivity is not known. Isolating spontaneously occurring suppressors of the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  provides a means of collecting mutated strains whose defects are in genes involved in the sphingolipid biosynthetic pathway.

Two collections of  $csg2\Delta$  suppressors have been isolated. The first is referred to as the *scs* or Suppressors of Calcium Sensitivity of  $csg2\Delta$  collection.<sup>32</sup> These mutants were isolated solely on there ability to suppress the  $csg2\Delta$  Ca<sup>2+</sup>sensitive phenotype. The second group is the *tsc* collection which are temperature sensitive (ts) suppressors of the

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 $Ca^{2+}$  sensitivity of  $csg2\Delta$ . This discussion is limited to the *tsc* collection.

Suppressing mutants that carry a conditional lethal ts phenotype are experimentally valuable for several reasons. They provide a positive selection scheme for cloning the mutated gene because complementing clones can be evaluated by their ability to complement the ts lethal phenotype rather than invoking a  $Ca^{2+}$  sensitive lethal phenotype (negative selection). Conditional lethal mutants have a non-permissive condition (37°) which is used as a switch to invoke the mutation at a critical point in analysis possibly uncovering defects which are not apparent at the permissive condition. Suppressors of the temperature sensitivity of these mutants will identify a new collection of mutants possessing defects in genes related to sphingolipid metabolism. Finally, the *tsc* mutant collection is expected to identify genes involved in sphingolipid synthesis which are essential because the mutations are lethal at 37°C.

Temperature sensitive suppressors of the Ca<sup>2+</sup> sensitive phenotype of  $csg2\Delta$ mutants were isolated on media containing Ca<sup>2+</sup> and complementing clones were isolated from a genomic library. Complementing genomic regions were identified by sequencing or hybridization to an array of the yeast genome. Specific complementing open reading frames were identified by subcloning and linkage analysis. Using this technique 946 suppressors of the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  were isolated of which 59 were found to have a linked recessive ts phenotype. Fifteen genes were found responsible for these 59 mutants by complementation analysis. The mutated genes which suppress  $csg2\Delta$ decrease the rate of synthesis or alter the structure of IPC-C suggesting that the levels of this intermediate in the cell in the presence of Ca<sup>2+</sup> is the cause of the  $csg2\Delta$  phenotype. A description of the specific techniques used and the results obtained in the identification and characterization of 7 of the *tsc* complementation groups follows.

# Chapter 3 TSCI/TSC2

# Introduction

Many of the *tsc* mutants of *Saccharomyces cerevisiae* possess defects in genes related to sphingolipid biosynthesis in yeast. As part of our investigation of sphingolipid homeostasis, *TSC1* and *TSC2* were cloned and characterized. *TSC1* and *TSC2* encode subunits of serine palmitoyltransferase which catalyzes the first committed step of sphingolipid biosynthesis in yeast. This chapter describes the identification of *TSC1* and *TSC2*.

## Methods

The ts phenotype of tsc1 and tsc2 mutants is rescued by phytosphingosine. Mutants in the TSC1 and TSC2 complementation groups were tested on YPD media containing 2, 10, and 20  $\mu$ M phytosphingosine and incubated at 26°C and 37°C to determine if the ts phenotype was complemented exogenous LCB.

#### TSC1 is allelic to SCS1/LCB2.

Complementation in the diploid, scs1/tsc1. A CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) mutant was mated to a 28R (scs1) mutant. Diploids were selected on SD auxotrophic media (no adenine or lysine) and tested for their ts and Ca<sup>2+</sup> sensitive phenotype on YPD at 37°C and YPD + 20 mM Ca<sup>2+</sup> at 26°C. Since both scs1 and tsc1-1 mutations are recessive, the suppressing phenotype will only be manifest if they are mutated in the same gene and cannot complement one another in the diploid.

Transformation of tsc1 with pSCS1<sup>+</sup> (pCZ1). The CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) mutant was transformed with a pRS316 (URA3) plasmid containing the coding sequence for SCS1, (pCZ1) constructed in our laboratory previously.<sup>32</sup> Transformation was accomptished using the LiOAc method of Geitz *et al.*(1995), and transformants selected on SD media lacking uracil. Individual transformants were then tested for their Ca<sup>2+</sup> sensitive and ts phenotypes on YPD media with and without 20 mM Ca<sup>2+</sup> at 26°C and 37°C.

Linkage analysis tsc1/scs1. SCS1 in a pRS306 vector<sup>1</sup> was linearized using a unique AvrII site, 200 bp into the insert sequence but outside the SCS1 coding region.<sup>37</sup> Homologous recombination of the linearized fragment results in an auxotrophic marker, URA3, from the vector, being integrated at the SCS1 locus in a wild type strain (CuH3). This marked strain was then mated to CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) and the resulting diploids were sporulated and dissected. The products of meiosis were evaluated to determine the segregation pattern of the uracil prototrophy and temperature sensitivity.

# Cloning of TSC2: TSC2 is allelic to LCB1

Mating tsc2 to the scs collection; failure to complement in the diploid. The  $csg2\Delta$  suppressors CJYa3 (tsc2-1), CJYa4 (tsc2-2), CJYa7 (tsc2-3), CJYa12 (tsc2-4), CJYa14 (tsc2-5), and CJYa31(tsc2-6), all members of the TSC2 complementation group, were mated to 28R (scs1, csg2 $\Delta$ ) and 5L (scs2, csg2 $\Delta$ ) mutant strains and the resulting diploids selected on SD media lacking adenine and lysine. The diploid growth on YPD media

<sup>&</sup>lt;sup>1</sup> The shuttle vector containing SCS1 was previously constructed in our laboratory by Anna Zhao (see reference 37) and referred to as pCZ1; 8'-2#3 SpeI/pRS306.

with and without 100 mM Ca<sup>2+</sup> at 26°C and 37°C was used to determine if the mutations are allelic to *tsc2*.

*Transformation with genomic library.* The CJYa12 (*tsc2-4, csg2*∆) mutant was transformed with a YCp50 (*URA3*) based yeast genomic library using the LiOAc/SS-DNA/PEG method of Gietz *et.al.* (1995). Transformants were selected on synthetic media lacking uracil (SD-URA). Transformation efficiencies were estimated at less than 1000 transformants per genomic library bank. At two days these transformants were replica plated to YPD media and incubated at 37°C to select those transformants that had acquired the ability to grow at 37°C (TS<sup>+</sup>). Three candidate colonies grew at 37°C within 24 hours (B2,2; B2,3; B2,4).

Testing of candidates. The TS<sup>+</sup> TSC2 candidates were grown on YPD media incubated at 26°C and placed on FOA to select for those that had lost the plasmid. They were also plated on SD (-URA) to insure plasmid maintenance. Cells with and without the plasmid were tested for temperature sensitivity and Ca<sup>2+</sup> sensitivity. The complementing plasmid was harvested by extracting genomic DNA from the transformed *tsc2* strain using the method described by Holm *et al.*, 1986, and amplified in *E. coli*. The plasmids were purified from *E. coli* using the Jet Quick Plasmid Miniprep Kit (Genomed Inc., Research Triangle Park, N.C.). A purified plasmid from each candidate was transformed back into the CJYa12 (*tsc2-4*, *csg2*\Delta) mutant to test for complementation. A *Sau3A* digest was performed on plasmids prepared from each of the complementing candidates and run on an ethidium bromide 1% agarose gel. The band pattern for all plasmid digests were indistinguishable indicating that the plasmids all contained the same genomic insert. One representative (B2,4) was selected for further analysis.

Hybridization of the TSC2 candidate to the ATCC contiguous blot. A Sau3A digest of the CJYa12 (tsc2-4,  $csg2\Delta$ ) complementing candidate plasmid B2,4 was randomly labeled with [ $\alpha^{32}$ P]ATP. The labeled probe was then hybridized to the ATCC contiguous genome array using the modified Southern blot procedure as described in methods (chapter 2). The resulting autoradiograph identified two overlapping clones, ATCC numbers 70282 and 71151, corresponding to a region of chromosome XIII containing *LCB1*. Since previous experience with *SCS1* indicated that mutations in SPT can suppress  $csg2\Delta$ , *LCB1* was investigated first.

Investigation of LCB1 as TSC2.

Transformation with pLCB1 Two members of the TSC2 complementation group (CJYa4 and CJYa7) were transformed with a plasmid harboring  $LCB1^2$ , using the LiOAc method of Geitz *et al.*(1995). Transformants were obtained on SD media lacking uracil. Eight individual colonies were selected from the transformation media and plated on YPD media with and without 100 mM Ca<sup>2+</sup> at 26°C and 37°C.

Linkage Analysis. An 1123 bp ClaI/EcoRI amino terminal fragment of LCB1, was bandisolated from the YEpLCB1-4 plasmid. The fragment was ligated into pRS306 and transformed into XL-1 Blue (Stratagene) E.coli competent cells. Transformants were

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The YepLCB1 plasmid was graciously donated by Dr. Robert Lester, University of Kentucky. The plasmid harboring *LCB1* is referred to as YepLCB1-4, derived from the 2 µplasmid Yep429.

screened using blue/white selection on LB+Ampicillin ( $50\mu g/mL$ ) agar media containing XGAL/IPTG. Candidate plasmids were purified from *E. coli*, and plasmids containing the 1123 bp insert were identified by restriction enzyme mapping. The restriction enzyme map of *LCB1* was obtained from the *Saccharomyces cerevisiae* genome database. The predicted fragment size and number were calculated and it was determined that *MscI* should linearize the construct and produce a band of 5504 bp when run on an agarose gel. Sixteen of the plasmid preps were digested with *MscI* and 11 produced the predicted band pattern. Two of the confirmed plasmids were linearized within the coding sequence of *LCB1* with *MscI* and transformed into a WT haploid (CuH<sub>3</sub>) using the LiOAc method of Gietz *et.al* (1995). Transformants were selected on SD media lacking uracil, indicating that the linearized fragment, containing the *URA3* marker gene, had integrated into the genome of CuH<sub>3</sub>. The marked strain was mated to two *tsc2* mutant strains (CJYa4 and CJYa7) and the resulting diploids were sporulated and dissected.

# Results

Isolation of tsc1 and tsc2 mutants. Complementation analysis identified 16 independently isolated alleles of tsc1 and 10 alleles of tsc2. The tsc1 and tsc2 mutants represent 44% of the tsc mutants isolated, indicating that mutations in subunits of SPT occur frequently in suppression of the Ca<sup>2+</sup> sensitivity of csg2 $\Delta$ .

*Phytosphingosine rescue of tsc1 & tsc2*. The ts phenotype arising from mutations in serine palmitoyltransferase, is rescued by exogenously added LCB (eg., phytosphingosine). To test for LCB rescue of *tsc1* and *tsc2* mutants, strains were plated



Figure 5. The effect of phytosphingosine on the ts phenotype of *tsc1* and *tsc2* mutants (phytosphingosine media contains 1% tergitol buffer).

on YPD media containing 2, 10, and 20  $\mu$ M phytosphingosine and incubated at 26°C and 37°C. All alleles of *tsc1* and *tsc2* tested were rescued at 37°C by the addition of phytosphingosine to the growth media, consistent with the mutations being in the sphingolipid biosynthetic pathway prior to the formation of LCB (Figure 5). The ts phenotype of *tsc1* and *tsc2* was rescued by concentrations of phytosphingosine as low as 2  $\mu$ M in the media. Concentrations greater than 12  $\mu$ M were toxic in the absence of 1% tergitol (NP-40) to act as a buffer.

#### Identification of TSC1

The scs1/tsc1 diploid phenotype. A 28R (scs1, csg2 $\Delta$ ) mutant and a 5L (scs2, csg2 $\Delta$ ) mutant suppressor of csg2 $\Delta$  were mated to CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) and the ts and suppressing phenotypes of the resulting diploids used to determine if the tsc1 mutation was allelic to either of these two scs mutants. Both tsc and scs mutations are recessive, therefore the mutant phenotype is only manifest if the diploid is homozygous for the mutation. A 28R/CJY $\alpha$ 1 (scs1/tsc1-1) diploid failed to grow at 37° C and grew well on media containing 100 mM Ca<sup>2+.</sup> This failure to complement one another suggests that mutations in tsc1 and scs1 are in the same gene.

Transformation of tsc1 with pSCS1. CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) was transformed with a plasmid-based, wild type copy of SCS1 (pCZ1) to determine if it would complement the ts phenotype. The transformed CJY $\alpha$ 1 (tsc1-1, csg2 $\Delta$ ) strain acquired the ability to grow at 37°C, therefore, the SCS1 sequence complemented the ts phenotype of the tsc1-1 mutant. This indicates that either the mutation in tsc1 is in SCS1, and the wild type copy of the gene is actually complementing the mutation, or that an extra copy of SCS1

overcomes the defect in *tsc1* mutants.

Linkage analysis tsc1/scs1. To distinguish whether tsc1 has a mutation in SCS1 or is suppressed by an extra copy of SCS1, linkage analysis was performed. The SCS1 locus was marked with a URA3 gene in a wild type strain as described in the methods section. The haploid marked strain was then mated to a CJY $\alpha$ 1 (tsc1-1) haploid and the diploids were sporulated, dissected, and analyzed. A parental diatype was observed in all 12 tetrads evaluated, indicating linkage between the URA marked SCS1 locus and the tsc1 mutant locus. This evidence coupled with the complementation data lead to the conclusion that the tsc1 mutation resides in SCS1.

#### Identification of TSC2.

scs1 and scs2 mutants complement tsc2 mutants in a diploid. Allelic recessive mutations fail to complement one another in a diploid. To determine if tsc2 was allelic to scs1 or scs2 members of the TSC2 complementation group were mated to scs1 mutant 28R and scs2 mutant 5L. The resulting diploids were tested for Ca<sup>2+</sup> sensitivity and temperature sensitivity. All of the diploids obtained from these crosses exhibited Ca<sup>2+</sup> sensitivity and were not temperature sensitive. The suppressing phenotype of both parental strains and the ts phenotype of the tsc2 mutants was complemented. These results indicate that the defect in tsc2 mutants is not allelic to scs1 or scs2.

Cloning of TSC2. To identify the mutated gene in the TSC2 complementation group, CJYa12 (tsc2-4,  $csg2\Delta$ ) was transformed with a plasmid-based genomic library as described in methods (chapter 2). The complementing plasmid obtained from the genomic library contained a fragment of yeast genomic sequence from chromosome XIII containing *LCB1*. Since *LCB1* encodes a subunit of serine palmitoyltransferase and mutations in the other subunit of SPT, (*SCS1/LCB2*), suppress the Ca<sup>2+</sup> sensitive phenotype of  $csg2\Delta$ , *LCB1* was considered first. A Yep24 based plasmid, containing *LCB1*, was transformed into CJYa12 (tsc2-4,  $csg2\Delta$ ) and complemented both the ts and suppressing phenotypes. Curing the tsc2-4 mutant of the plasmid reverted the phenotype to temperature sensitive and Ca<sup>2+</sup> resistant indicating the complementation was plasmid linked.

Linkage analysis. To distinguish whether tsc2 has a mutation in LCB1 or is suppressed by an extra copy of LCB1, linkage analysis was performed. The LCB1 locus was marked by integrating a vector containing the URA3 gene and the amino terminus of LCB1. The marked strain was crossed to CJYa12 (tsc2-4,  $csg2\Delta$ ) and the diploids were sporulated, dissected, and analyzed. Segregation of the ts phenotype away from the URA+ prototrophy was seen in the 12 tetrads analyzed. These results indicate there was no crossover between the mutated tsc2 locus and the marked LCB1 locus in meiosis. Coupled with the complementation data this indicates TSC2 is allelic to LCB1.

## Discussion

The *tsc1* and *tsc2* mutants possess defects in subunits of serine palmitoyl transferase (SPT). SPT is a pyridoxal phosphate dependent enzyme which catalyzes the formation of 3-ketosphinganine by the condensation of serine with palmitoyl CoA and is composed of at least two subunits (*LCB1* and *LCB2/SCS1*) (Figure 2).<sup>64</sup> This reaction constitutes the first committed step of sphingolipid biosynthesis in yeast. *TSC1* was

identified as SCS1 by mating a tsc1 mutant to a scs1 mutant. The resulting diploids failed to grow at 37°C and continued to suppress the Ca<sup>2+</sup> sensitive phenotype of their common  $csg2\Delta$  background. The conclusion was confirmed by complementation of tsc1 with a plasmid carrying SCS1\* and by linkage analysis. SCS1 was identified previously in our laboratory as a suppressor of the Ca<sup>2+</sup> sensitive phenotype of  $csg2\Delta$  and is also known as LCB2 which was a gene identified in a screen for mutants requiring exogenous long chain base for viability.<sup>32,65</sup> TSC2 was identified by subcloning sequence from a complementing plasmid isolated from a plasmid-based genomic library and is allelic to LCB1. LCB1 was also identified in a screen for LCB auxotrophs.<sup>31</sup> The two subunits of SPT, Lcb1p and Lcb2p, exhibit 23% identity and 47% similarity in primary sequence indicating these proteins are related.<sup>31</sup> Both proteins are required for SPT activity suggesting that they may form a complex. A significant difference in their sequence is the lack of a characteristic lysine at the predicted pyridoxal phosphate binding site in Lcb1p suggesting that Lcb2p may be the catalytic subunit and Lcb1p has evolved into a regulatory subunit.<sup>32</sup> Phenotypes arising from mutations in either subunit can be reversed with the exogenous addition of a long chain base such as phytosphingosine.

The *tsc1* and *tsc2* mutants are the first conditional lethal mutants reported in SPT subunits. This makes these mutants especially valuable in that other genes in the sphingolipid biosynthetic pathway may be identified by isolating suppressors to the ts phenotype of *tsc1* and 2. Another potential use for these mutants is for the isolation of synthetic lethal mutations which alone convey no phenotype but when combined with a mutation in SPT activity, are lethal. This group of mutants may identify genes involved

in the regulation of sphingolipid biosynthesis such as those which result in an upregulation of an enzyme downstream in the sphingolipid biosynthetic pathway.

The SPT ts mutants are also being used to investigate the Ca<sup>2+</sup> sensitivity imposed by mutations in *csg1* and *csg2*. The data, outlined below, suggests that Ca<sup>2+</sup> may upregulate sphingolipid synthesis and that this results in toxic IPC-C levels in *csg* mutants. Rescue of *csg2* mutants is accomplished by decreasing sphingolipid synthesis through mutations in genes in the biosynthetic pathway (*tsc/scs* collection) or by adding exogenous myristate to the media which may act as a negative feedback regulator of fatty acid synthesis, reducing available substrate to SPT. SPT is an essential enzyme in yeast that exhibits a high degree of substrate specificity for palmitoyl CoA.<sup>36</sup> The SPT mutants, *tsc1* and *tsc2*, are sensitive to exogenous myristate (C<sub>14</sub>) in the media presumably due to the combined effect of decreased SPT substrate and decreased SPT activity resulting in insufficient sphingolipid synthesis for viability. If this is the case, and Ca<sup>2+</sup> upregulates sphingolipid biosynthesis, Ca<sup>2+</sup> might be expected to rescue the *tsc1* and *tsc2* mutants grown in the presence of myristate. This was found to occur and strongly suggests that Ca<sup>2+</sup> is a positive regulator of sphingolipid synthesis (Figure 6).



Figure 6. Effect of myristate ( $C_{14}$ ) on the growth of *tsc1*, *tsc2* and *csg2* $\Delta$  mutants at 26°C.

# Chapter 4 TSC 3

#### Introduction

Many of the *tsc* mutants possess defects in genes related to sphingolipid biosynthesis in yeast. As part of our investigation of sphingolipid homeostasis, the *TSC3* gene was cloned and characterized. *TSC3* is a small, previously uncharacterized open reading frame located on the long arm of chromosome II. This open reading frame has yet to be given a positional indicator but is located between YBR058C and YBR059C. This chapter describes the identification of *TSC3*.

# Methods

## Cloning of TSC3

Phytosphingosine rescue.  $csg2\Delta$ ,  $tsc3 csg2\Delta$ , and wild type strains were grown in liquid YPD overnight at 26°C to an OD of approximately 1.0. The cultures were diluted into 20% glucose to an OD of 0.1 and serially diluted 1:2 across the wells of a 96 well microtiter plate. The cells were spotted onto YPD, YPD+20 mM Ca<sup>2+</sup>, YPD+20  $\mu$ M 3ketosphingosine, YPD+20  $\mu$ M dihydrosphingosine, and YPD+20  $\mu$ M phytosphingosine and incubated at 26°C and 37°C.

Transforming tsc3-2 with genomic library. The CJYa16 (tsc3-2, csg2 $\Delta$ , ura3, trp1) mutant was transformed with 4 URA3 marked plasmid-based genomic libraries and one TRP1 genomic library using the LiOAc method of Gietz et.al (1995). 300  $\mu$ l of the transformation prep was plated on each of three SD plates lacking uracil or tryptophan to

select those cells which took up a plasmid. One of the 300 µl plates was immediately placed at 37°C and the other two were incubated at 26°C. After two days one of the transformation plates incubated at 26°C was shifted to 37°C and the other was replica plated to an identical SD plate and to a YPD plate and both incubated at 37°C. Transformation efficiency was estimated at approximately 30,000 transformants per 300µl plate. TS<sup>+</sup> colonies were selected 3 days post transformation and streaked out on selective media consisting of YPD and SD lacking uracil or tryptophan at 26°C and 37°C for 2 days. The candidates were allowed to lose the complementing plasmid while growing on YPD at 26°C. Loss of the plasmid was verified on FOA as previously described in methods (chapter 2) for the URA<sup>+</sup> library candidates. TRP candidates were grown on YPD + 20 mM  $Ca^{2+}$  to select for those that had lost the transformed plasmid. Growth from FOA and YPD+Ca<sup>2+</sup> was plated on auxotrophic SD media to confirm loss of the plasmid and on YPD at 26°C and 37°C with and without 20 mM Ca<sup>2+</sup> to determine plasmid linkage to the complementing phenotype. Several candidate transformants from both the URA<sup>+</sup> libraries and the TRP<sup>+</sup> library demonstrated the desired complementing phenotype. Eight URA<sup>+</sup> and four TRP<sup>+</sup> plasmids were characterized further. Genomic DNA was harvested from those exhibiting a complementing phenotype using the method of Holm et al. (1986). Genomic DNA was then transformed into competent E. coli to amplify the complementing plasmid. The plasmid was purified from E. coli using the Jet Quick Plasmid Miniprep Spin Kit (Genomed Inc., Research Triangle Park, N.C.). Plasmids were cut with EcoRV and SalI run on a 1% agarose ethidium bromide gel to determine how many plasmid species were obtained from the genomic banks. Two

plasmid species were identified in the URA<sup>+</sup> and in the TRP<sup>+</sup> candidates. Representative plasmids from each species were transformed back into CJYa16 (tsc3-2,  $csg2\Delta$ ). In each case only one of the plasmid species complemented the ts phenotype of tsc3-2.

Sequencing of insert in TRP<sup>+</sup> complementing plasmid. The ends of the complementing fragment of the pRS200 TRP<sup>+</sup> plasmid (p2-1) were sequenced using the T3 and T7 bluescript primers (STRATAGENE La Jolla, CA) and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, Great Britain) as described in the methods section (chapter 2). The pRS200 based complementing plasmid contained a 7400 bp insert containing the sequence between coordinates 353,836 and 361,219 of chromosome II. This region of the genome includes one complete previously identified open reading frame (YBR059C) and two partial ORFs (YBR058C, YBR060C).

Hybridization of the URA<sup>+</sup> complementing plasmid to the ATCC genomic contiguous array. A complementing URA<sup>+</sup> plasmid (p6-1) was hybridized to the ATCC contiguous clone array as described in methods (chapter 2) to determine its chromosomal position. The autoradiograph identified ATCC clones 70048 and 70247, which are overlapping regions of chromosome II, that also include the YBR059C and neighboring open reading frames.

Subcloning of TSC3

*Kpn1/Pst1 YBR059C subclone*. A 4684 bp *PstI/Kpn*I fragment ranging from the amino terminus of YBR058C to the carboxy-terminus of YBR060C, including the entire coding sequence of YBR059C and the intergenic regions between the open reading

frames, was band isolated from the TRP<sup>+</sup> complementing plasmid (p2-1) (Figure 7). The fragment was ligated into the *PstI/KpnI* sites of pRS314 and amplified in *E. coli*. The plasmid construct was confirmed by restriction enzyme digests and transformed into CJYa16 (tsc3-2,  $csg2\Delta$ ).

Bg/II/BamHI subclones. The Bg/II/BamHI subclones were used to evaluate both YBR058C and YBR059C (Figure 7). A Bg/II/BamHI digest of the pRS200 TRP<sup>+</sup> complementing plasmid (p2-1), cut once at the carboxy-terminus of YBR059C and once at the insertion site of the yeast genomic DNA to the vector sequence (Figure 7). This digest liberated a 4400bp band containing YBR059C and a 7785bp band containing the vector, the amino terminus of YBR058C and the inter-genic region between YBR058C and YBR059C. The 4400bp fragment was band isolated and ligated into a BamHI digest of pRS314. The 7785bp vector + YBR058C band was also isolated and religated (Bg/II/BamHI leave compatible single stranded base-pair ends). Both constructs were amplified in *E. coli*, verified by restriction digests and transformed into CJYa16 (tsc3-2,  $csg2\Delta$ ).

*YBR058C BstEII Frameshift.* The coding sequence of YBR058C was disrupted at the unique *Bst*EII site at codon 530 of the 803 codon coding sequence. The *Bst*EII restriction enzyme leaves a 5 bp overhang which was filled in and blunt end ligated using 1  $\mu$ I of PCR nucleotide mix (10 mM of each dNTP, Boehringer Mannheim Corp., Indianapolis, IN), 1  $\mu$ I Klenow fragment and 1  $\mu$ I T4 DNA ligase (New England Biolabs, Beverly, MA). The reaction produces a frameshift mutation in the sequence of YBR058C (Figure 7). Verification of the construct was determined by the loss of the



Figure 7. Subclones and complementation effects of the *tsc3* complementing TRP<sup>\*</sup> plasmid (p2-1).

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BstEII site.

YBR059C Nrul Frameshift. To disrupt the sequence of YBR059C a 10 bp Xhol linker was ligated into a blunt end Nrul site in the TRP<sup>+</sup> complementing plasmid (p2-1) (Figure 7).

This results in a 10 base pair frameshift at codon 180 of 1108 in YBR059C. The plasmid was amplified in *E. coli* and harvested using the Jet Quick column method. Verification of the construct was made by a *Xho*I digest which linearizes p2-1 but cuts twice in a successful disruption giving two bands of 6385 bp and 5800 bp. The plasmid carrying the frameshift in YBR059C was transformed into CJYa16 (*tsc3-2, csg2* $\Delta$ ).

*YBR058C/YBR059C intergenic region*. The intergenic region between YBR058C and YBR059C was screened for potential small (<100 amino acids) open reading frames that would not be annotated in the *S. cerevisiae* database. Two putative ORFs are present in the intergenic region potentially encoding proteins of 80 (*Orf a*) and 84 (*Orf b*) amino acids respectively. A 900 bp *Bg/II/PstI* fragment that contains both small ORFs was band isolated from the *KpnI/PstI* subclone of p2-1 and ligated into a *Bam*HI/*PstI* digest of pRS314 (Figure 7). The verified construct was transformed into CJYa16 (*tsc3-2, csg2*\Delta).

Orf a and Orf b subclones. Each ORF was independently subcloned by generating PCR fragments and ligating them into pRS314 (Figure 7). Primers for  $Orf a^{\prime}$ 

<sup>1</sup> 

Orf a forward primer 11383 FPA, 5'-GGCCCTCGAGGCTCGCAATTTGACAGAA-3' reverse primer 11384 RPA, 5'-GGCCGGATCCTTGCCTCCAGCTTATACTA-3'

corresponded to regions 115 bp upstream and 35 bp downstream of the coding sequence and incorporated a XhoI and BamHI site in the sequence respectively and amplified a fragment of 381 bp. The Orf  $b^2$  primers were 84 bp upstream and 20 bp downstream and incorporated PstI and BamHI sites, and amplified a fragment of 458 bp. The PWO DNA Polymerase protocol was followed (Boehringer Mannheim, Indianapolis, IN). Briefly, two master mix tubes were prepared for each reaction. Master mix I contained 500 ng of yeast genomic DNA (wild type TSC3), 10  $\mu$ l of 3  $\mu$ M reconstituted up and downstream primers, 2 µl of 10 mM PCR nucleotide mix (Boehringer Mannheim), in a final volume of 50 µl. Master mix II contained 10 µl of PCR buffer + MgSO<sub>4</sub>, 0.5 µl of PWO DNA polymerase (both from the Boehringer Mannheim PWO kit) and the total volume also was brought to 50 µl with dH<sub>2</sub>O. Master mix I and Master mix II were combined and put into the thermocycler. The thermocycler protocol was set as a step down series of annealing temperatures starting at 54°C and stepping down to 48°C in single degree increments. Each step was run for 4 cycles except the last which was run for 10 cycles. Each cycle includes a 1.5 min 94°C melting, 1.0 min annealing at the stepped temperature, and a 72°C, 3.0 min elongation step. Ten percent (10 µl) of the PCR products were run on a 1% agarose gel to determine the yield of the product. The PCR products were cut with the restriction enzymes whose sites were designed in the primer sequence and ligated into pRS314. The plasmids were amplified in E. coli and verified

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*Orf b* forward primer 11385 FPB, 5'-GGCCCTGCAGTAGTATTTAGTATGCCTTC-3' reverse primer 11390 RPB2, 5'-GGCCGGATCCGTAGTGCATCCAGTAGTGG-3'

by restriction enzyme digests. Both constructs were transformed into CJYa16 (*tsc3-2*,  $csg2\Delta$ ).

YBR059C disruption construct. To disrupt the coding sequence of YBR059C, a TRP1 marker was inserted in the midst of the ORF. A 4684 bp PstI/KpnI fragment was band isolated from the TRP<sup>+</sup> complementing plasmid (p2-1). The fragment was ligated into pUC19 and the plasmid was amplified in E. coli. The plasmid construct was confirmed by restriction enzyme digests and then digested with Bg/II and XbaI to eliminate 3275 bp of YBR059C coding sequence. 20 µl of the Bg/II/XbaI digest was blunt ended by adding 1 µl PCR nucleotide mix, 1 µl of DNA polymerase I, 1 µl Klenow fragment, and 2.5 µl of Klenow buffer and incubating at room temperature for 15 minutes. Two µl of Xhol linker, 1 µl of T4 ligase, and 3 µl of ligase buffer were added to the reaction and incubated at 12°C for 4 hours. The ligase was inactivated by incubating the reaction mixture at 65°C for 10 minutes. The ligation mix was digested with Sall, which cuts within the 3275 bp BgIII/XbaI fragment removed to construct the knockout. Any re-assembly of the YBR059C coding sequence would be cut by the SalI digest and would not be amplified in E. coli. The digest was transformed into E. coli and six transformants were selected and plasmids purified using the Jet Quick Rapid Plasmid Prep Kit (Genomed, Research Triangle, N.C.). The verified plasmids were cut with XhoI and ligated to a Xhol-ended TRP1 PCR fragment which replaced much of the YBR059C coding sequence with TRP1. The plasmid was amplified in E. coli and the desired construct was verified by restriction enzyme digests. The disrupting allele was liberated from the pUC19 vector by cutting the plasmid with KpnI and PstI and was used to

replace the YBR059C wild type allele by a single gene disruption.<sup>72</sup> The disrupting construct was transformed in both wild type and  $csg2\Delta$  haploids and diploids using the LiOAc method of Gietz *et al.* (1995). Both haploids and diploids were transformed to determine if the elimination of YBR059C conferred a lethal phenotype. Disruption of one allele of the diploid would not be lethal. However, if the gene is essential haploid disruptants would not be recovered. Haploid transformants were recovered and integration of the disruption cassette at the YBR059C locus was confirmed by mating the haploid  $csg2\Delta$ , YBR059C::*TRP1* with a CJYa16 (tsc3-2,  $csg2\Delta$ ) mutant. The resulting diploids were sporulated and dissected. Tetrad analysis showed segregation of the TRP prototrophy from the ts phenotype of tsc3 indicating no crossover occurs between the tsc3locus and the site of integration, indicating that the integration occurred at the YBR059C locus.

Carboxy-terminal truncation of Orf a. The Orf a PCR product was cut with XhoI and BstZ17I (blunt unique cutter in carboxy terminus of Orf a) (Figure 8) and ligated into a XhoI/SmaI digest of pRS316. This construct eliminates the last 20 bp of the coding sequence of Orf a. The plasmid was amplified and purified in E. coli, verified by restriction enzyme digests, and transformed into CJYa16 (tsc3-2,  $csg2\Delta$ ).

Orf a disruption. The Orf a PCR product was digested with BamHI and XhoI and ligated into Bluescript  $KS^+$  (Stratagene, La Jolla, CA). The plasmid construct was amplified and verified, then linearized in the midst of the coding sequence of Orf a with HindIII at codon 36 (Figure 8). The HindIII site was filled in with Klenow fragment and dNTPs and a SalI linker was ligated in at the blunt ended HindIII site. The plasmid was





Figure 8. Disruption constructs of *TSC3*. (X-XhoI, Bs-BsrGI, H-HindIII, S-SaII, Bz-BstZ1107I, B-BamHI)

amplified in E. coli and the desired construct was confirmed by the lack of a HindIII site and the presence of a Sall site. The verified construct was linearized with Sall and an 1174bp TRP1 fragment with XhoI ends was ligated into the site. The ligation was cut with Sall to eliminate constructs that had not acquired the TRP1 insert from being amplified in E. coli. The purified plasmid was harvested from E. coli, verified by restriction enzyme digests, and cut with BamHI/XhoI to liberate the 1555 bp Orf a disruption construct from the vector. The fragment was transformed into wild type and  $csg2\Delta$  haploids and diploids selecting for replacement of the Orf a wild type allele by the TRP1 marked disrupting allele. Verification of integration at the Orf a locus was accomplished by obtaining genomic DNA from transformants and using it as the template for PCR amplification of the Orf a locus. The presence of the TRP1 insert increased the expected size of the PCR product from 390bp to 1564bp. Both haploid and diploid transformants were recovered that possess the TRP1<sup>+</sup> insert. Haploid ORFa::TRP1,  $csg2\Delta$  strains were then tested on selective media at 26°C and 37°C to determine their phenotype.

Amino-terminal Orf a Frameshift in Orfa::TRP1. The Orf a::TRP1 construct leaves the amino terminus of Orf a intact. To disrupt both the amino and carboxy termini of Orf a, a frame shift mutation was introduced at codon 9 of the Orf a::TRP1 allele by cutting the plasmid with BsrGI (Figure 8). The 4 bp, 5' overhang was filled in with dNTPs and Klenow fragment as previously described, and the blunt ended product religated shifting the ORF out of frame early in the coding sequence. The construct was amplified in E. coli and verified based on the loss of the BsrGI site. The verified construct was cut from the vector with *Bam*HI and *Kpn*I and transformed into wild type and  $csg2\Delta$  haploids and diploids as described above. Genomic DNA was harvested from diploid and haploid integrated transformants and used as template for PCR amplification of *Orf a*. The PCR product was digested with *Bsr*GI, *Hin*dIII, and *Xba*I, alone and in combination to identify integrations that had both acquired the *TRP1* disrupted *Orf a* allele and the frameshift at the *Bsr*GI site.

Linkage Analysis. Tetrad analysis revealed genetic linkage between the TSC3 and LYS2 genes demonstrating that TSC3 resides on chromosome II. TSC3 was more precisely mapped by measuring the crossover frequency between the tsc3-2 allele and other marker genes near LYS2 on chromosome II (Table 2).

Cloning the tsc3 mutant alleles. Wild type (TDY2037) and tsc3, csg2 $\Delta$  mutant alleles (LHYa60, (tsc3-1), CJYa16,(tsc3-2), CJYa18,(tsc3-3), CJYa8, (tsc3-4), CJYa11, (tsc3-5)) were grown up in 20 ml of liquid YPD at 26°C overnight to an OD of 1.5. Genomic DNA was harvested from the strains<sup>3</sup> and used to PCR amplify the Orf a alleles from each of the tsc3 mutants using the same primers and protocol described for subcloning Orf a (TSC3). The PCR products were cut with BamHI/XhoI and ligated into pRS316. The plasmids were amplified in *E. coli*, verified by restriction digests, and transformed into CJYa16 (tsc3-2, csg2 $\Delta$ ). Only the TSC3 PCR product obtained from the wild type (TDY2037) genomic DNA complemented the tsc3-2, csg2 $\Delta$  mutant.

Sequencing of tsc3 Orf a alleles. Each of the tsc3 mutant alleles was sequenced using

<sup>&</sup>lt;sup>3</sup> Current Protocols in Molecular Biology, section 13.11, Vol. 2

	csg2::LEU2+	LYS2 <sup>+</sup>	sec18	tsc3
			(ts@ 30°C)	(ts@ 37°C)
	PD/TT/NPD	PD/TT/NPD	PD/TT/NPD	PD/TT/NPD
csg2::LEU2	100/0/0	17.5/65/17.5	32 / 64 / 4	45 / 54 / 0
LYS2+	17.5/65/17.5	100/0/0	66 / 34 / 0	35.5/62 / 0
sec18	32 / 64 / 2	66 / 34 / 0	100/0/0	64 / 36 / 0
tsc3	45 / 54 / 0	32.5/62 / 2	64 / 36 / 0	100/0/0

**Table 2.** Segregation patterns of crosses used to map TSC3 genomic location. (PD =parental diatype; TT = tetratype; NPD = non-parental diatype)

the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, Great Britain). The reactions were done using three combinations of primer and template. In reaction 1, the PCR product obtained for each allele (see above) was used as the template and the PCR primers were used as sequencing primers. The primers in this reaction were too close to the start and stop codons of Orf ato provide reliable sequence of the entire Orf a. Therefore, in reaction 2 the pRS316 plasmids, into which the Orf a fragments were subcloned, was used as the template and primers in the vector sequence (T3 and T7 primers from Stratagene) were used to sequence the insert. This strategy provided good sequence of the ends of the open reading frame. The final reaction was a combination of the two previous reactions. The vector based PCR product was used as template as in reaction 2 but the Orf a PCR primers were used as in reaction 1. Using these reactions the entire Orf a nucleotide sequence was obtained on both strands for each mutant allele.

SPT activity assay. The tsc3 mutants are rescued by exogenous long chain base suggesting their defect may be in serine palmitoyltransferase activity (Figure 9). To determine if the mutation in tsc3 is effecting SPT activity membranes were isolated from tsc3 mutants and SPT activity determined by measuring the incorporation of <sup>3</sup>H-labeled serine into long chain base.

Membrane Preparation. 750 ml of exponentially growing wild type,  $csg2\Delta$ , and tsc3 mutant cells (26°C) (OD<sub>600</sub> of .8-1.2) were harvested and washed in dH<sub>2</sub>O. The cells were resuspended by vortexing in 2 ml/gm wet weight of 50 mM Tris (pH=7.5), 1 mM EGTA, 1 mM PMSF, 1 mM BME. Glass beads were added to  $\frac{1}{2}$  inch below the

meniscus and the cell walls were disrupted by vortexing 8x for 30 sec with cooling on ice between vortexing. The solution was transferred to a centrifuge tube and the glass beads washed several times, vortexed and spun until the supernatent was clear. The supernatents were pooled and centrifuged at 4000 g for 10min. The supernatant was transferred to a high speed centrifuge tube for pelleting at 40000 g for 40 min. The pellet was resuspended using a dounce homogenizer and respun at 40000 g for 40 min. This final pellet was resuspended at 1 ml/gram (cellular wet wt.) in 50 mM Tris (pH=7.5), 1 mM EGTA, 1 mM PMSF, 1 mM BME, 33% Glycerol and stored at -80°.

Assay of serine palmitoyltransferase. A reaction cocktail containing final concentrations of 0.1 M Hepes (pH=8.3), 5 mM DTT, 2.5 mM EDTA, 1 mM Serine ( $10\mu$ Ci/ml <sup>3</sup>H Serine), 50  $\mu$ M pydridoxal-5'-phosphate, membrane protein (to 1mg/ml), and 0.2 mM palmitoyl-CoA was reacted at 37° for 10 minutes. The reaction was started by the addition of protein and palmitoyl-CoA and after 10 min. it was quenched by addition of NH<sub>4</sub>OH to 0.25 M. The LCB was extracted by successive additions of 1.5 ml of CHCl<sub>3</sub>:Methanol (1:2), 30  $\mu$ g of dihydrosphingosine carrier, and 1 ml CHCl<sub>3</sub> with vortexing after each addition. 2 ml of 0.5 M NH<sub>4</sub>OH was added, vortexed and centrifuged briefly. The upper aqueous layer was aspirated off and the lower layer was washed two times with 2 ml of 30 mM KCL. 10% of the sample was dried and counted and the remainder was dried and analyzed by TLC using CHCl<sub>3</sub>:Methanol:2 M NH<sub>4</sub>OH (40:10:1) as the developing solvent. Each lane was scraped off in 20 equal portions and counted in a scintillation cocktail.

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### Results

Isolation of tsc3 mutants. The tsc3 mutants were isolated as outlined in methods (chapter
2). Five tsc3 mutants were isolated, as they represent 8.5% of the tsc collection, suppressing ts mutations in TSC3 occur at a relatively high frequency.

*Phytosphingosine rescue.* The ts phenotype of *tsc* mutants (eg. *tsc1* and *tsc2* mutants) that are deficient in SPT activity are reversed by exogenous LCB. Therefore, representative mutants from each complementation group were tested for LCB reversal of temperature sensitivity as a screen for mutations in LCB synthesis. Serial dilutions of the *tsc* mutants were spotted on YPD media containing 3-ketosphingosine, dihydrosphingosine or phytosphingosine to determine if exogenous long chain base would rescue their ts phenotype. All the *tsc3* mutants were rescued at 37°C on YPD media containing exogenous long chain base suggesting that Tsc3p is involved in sphingolipid biosynthesis early in the pathway prior to the formation of LCB (Figure 9).

These experiments revealed 2 phenotypic classes among the tsc3,  $csg2\Delta$  mutants. Group 1 consists of LHYa60 (tsc3-1), CJY $\alpha$ 8 (tsc3-4), and CJY $\alpha$ 11 (tsc3-5) which grow on all concentrations of LCB tested at both 26°C and 37°C. Group 2 includes CJYa16 (tsc3-2) and CJYa18 (tsc3-3) which are sensitive to exogenous LCB at 26°C but are rescued at 37°C. Sequence data from these mutants does not explain the findings (see below). Further investigation is required to understand why tsc3-2 and 3-3 exhibit these unique phenotypic characteristics.

Cloning the TSC3 gene. The TSC3 gene was cloned by isolating a pRS200 TRP<sup>+</sup>

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Figure 9. Phytosphingosine rescue of tsc3-2 at 37°C.

plasmid that complemented the ts phenotype from a yeast genomic library and identifying the yeast insert by sequencing, using primers in the vector sequence flanking the insert site. The sequence of the complementing clone was compared to the SGD (*Saccharomyces* Genome Database) and found to reside on chromosome II. YCp50 based URA<sup>+</sup> complementing plasmids were hybridized to the ATCC contiguous yeast genome collection and were found to contain sequence from this region as well. Since the *tsc3* mutations were genetically mapped to this region of chromosome II (see below), it could be concluded that the complementing plasmids carry the wild type *TSC3* gene rather than a dose-dependent suppressor.

Subcloning TSC3. The complementing chromosome II insert of the smaller pRS200 candidate plasmid contained one complete ORF, YBR059C. A subclone containing this open reading frame was constructed in a yeast centromeric shuttle vector and was transformed into CJYa16 (tsc3-2,  $csg2\Delta$ ) (Figure 7). However, the subclone failed to complement the ts phenotype indicating that YBR059C is not the TSC3 gene.

A series of additional subclones were constructed either by subcloning defined restriction fragments or by generating PCR fragments. Figure 7 summarizes the subcloning results. Only those subclones containing a 381 bp intergenic region between YBR058C and YBR059C of chromosome II complemented the ts phenotype of the tsc3mutants. This region contains a small potential open reading frame of 240 bp (unannotated in the SGD due to its small size), which we refer to as Orf a, and which we demonstrate does encode Tsc3p (Figure 10A). The predicted protein product is 80 amino acids long and possesses a hydrophilic amino terminal 37 amino acids and a hydrophobic carboxy terminal 43 amino acids capable of spanning a lipid bilayer twice (Figure 10B). Sequence of tsc3 mutant alleles.

The 80aa ORF complements tsc3 mutants. The role of Orf a was investigated by obtaining the Orf a coding sequences by PCR from wild type and the tsc3 mutants and determining if, when transformed into a tsc3 mutant, they altered the ts phenotype. Each of the subcloned tsc3 alleles were transformed into CJYa16 (tsc3-2, csg2 $\Delta$ ). The tsc3 mutant-derived Orf a sequences failed to complement CJYa16 (tsc3-2*ib*0s12.0v1P, csg2 $\Delta$ ), but the construct containing the Orf a derived from the wild type DNA, complemented the ts phenotype of the CJYa16 (tsc3-2, csg2 $\Delta$ ). These results indicate that the 240 bp Orf a contains the mutation that results in the temperature sensitive and the suppressing phenotypes of the tsc3 mutants.

Sequencing the tsc3 alleles. To determine the nature of the mutations that lead to the ts phenotype of the tsc3 mutants, each of the amplified tsc3 alleles were sequenced. The sequencing data indicates that all the tsc3 alleles possess mutations in the hydrophobic domain of Tsc3p (Figure 10B). The region of sequence mutated in 4 of the 5 mutant alleles lies in a stretch of T's in the sequence encoding two phenylalanine and a valine residue in the hydrophobic domain (Figure 10C). While wild type TSC3 has 8 T's, in the tsc3-1 and tsc3-4 there are 9 T's. This frameshift mutation leads to substitution of amino acids 67-80 as well as an additional 39 amino acids added to the carboxy-terminus of the protein product. The tsc3-3 allele has only 7 T's in the poly-T region which causes a frameshift mutation at codon 63, altered sequence for 5 amino acids and then a premature stop. The tsc3-5 allele contains an additional CT immediately following the poly-T region of the carboxy-terminus. This also results in a frameshift mutation which alters sequence carboxy-terminal to the mutation and introduces a premature stop 5 codons past the insertion. In the *tsc3-2* allele, a point mutation results in a premature stop at codon 39. This mutation differs from the others because the altered sequence is not in the poly-T region of the gene sequence. The location of the mutations in the *TSC3* primary sequence is shown in Figure 10B and C.

*Characterization of TSC3.* To gain a more clear understanding of the possible role Tsc3p may be playing in the cell, sequence disruptions of *TSC3* were constructed (Figure 8). Three constructs were made with the disruptions introduced at different locations in the coding sequence. The disrupted alleles of *TSC3* were tested for cell viability to address the question of Tsc3p being essential at both 26°C and 37°C or, as suggested by the *tsc3* mutant alleles, is only required at 37°C.

Carboxy-terminal truncation of Orf a. All of the mutations in the tsc3 alleles were found in the hydrophobic carboxy-terminal domain of the predicted protein and lead to major alterations in the encoded protein, raising the question of whether or not TSC3 is essential for viability at 26°C. Using a unique restriction enzyme site in this region, it was possible to construct a truncated Tsc3p similar to the predicted protein length of tsc3-3 and tsc3-5. The carboxy terminal 20 bp of a wild type Orf a coding sequence was eliminated and the remainder of the sequence ligated into a vector and transformed into CJYa16 (tsc3-2, csg2 $\Delta$ ) allele. The construct failed to complement the ts phenotype of tsc3-2 suggesting that minimally, the carboxy terminal domain is required for functioning at 37°C.

## TSC3 Mutant and Wild Type Sequence and Proposed Structure

A. TSC3 nucleotide and amino acid sequence (Wild Type).

**B.** Proposed Tsc3p structure in a membrane and amino acid sequence of *tsc3* mutants.



C.

Poly-T nucleotide sequence in tsc3 mutants

**VFFLTIF Amino** Acids WT ATGTTTTTTTTTCTCACAATTTT tsc3-1 ATGTTTTTTTTCTCACAATTTT - Additional T tsc3-3 ATGTTTTTTTTCTCACAATTTT - Deletion of a T tsc3-4 ATGTTTTTTTTTCTCACAATTTT - Additional T tsc3-5 ATGTTTTTTTTCTCACAATTTT - Additional CT

Figure 10. Sequence information of tsc3 mutants.

Disruption of Orf a. To determine if Tsc3p is essential to cell viability at 26°C the coding sequence was disrupted by the insertion of a *TRP1* gene. The linearized *tsc3::TRP1* disrupting allele was used to replace the wild type *TSC3* gene in both haploid and diploid wild type and  $csg2\Delta$  cells. Verification of the knockout was made by PCR. *tsc3::TRP1* disruptions in haploid  $csg2\Delta$  strains grew on SD media lacking tryptophan, suggesting that *TSC3* is not essential at 26°C. The haploid *tsc3::TRP1* disruptants were, however, ts and suppressed the Ca<sup>2+</sup> sensitivity of the  $csg2\Delta$  mutant (Figure 11).

Amino-terminal disrupted knockout of tsc3. Since the disrupting allele of tsc3 was constructed by inserting the TRP1 marker at codon 36, which when integrated into the TSC3 coding sequence, leaves an intact amino-terminal hydrophilic domain, we tested whether the amino terminus of Tsc3p is essential for viability at 26°C. A disruption construct that introduced a frameshift mutation into the amino terminal sequence of Tsc3p was produced. This disrupting allele was constructed by filling in a BsrGI site in codon 9 of TSC3 in the tsc3::TRP1 allele. The construct was integrated into wild type and csg2 $\Delta$  cells and verified by PCR and restriction enzyme digests as described in the methods section. Growth of haploid integrated transformants, expressing no Tsc3p, confirms that Orf a is not essential at 26°C. The tsc3::TRP1-NH<sub>3</sub> frameshift codon 9 construct exhibits a similar phenotype to tsc3 mutant alleles (Figure 11). The disrupted strains, however, were not tested on media containing exogenous LCB to determine which group of tsc3 alleles the knockout resembles.



Figure 11. Growth phenotype of tsc3::TRP1 in haploid and diploid cells with and without the amino terminal disruption (NH<sub>3</sub>X).

Linkage analysis. The frequency of crossover between two loci is directly related to the genomic distance between them.<sup>60</sup> The genetic map distance between TSC3 and other marker genes in the S. cerevisiae genome was determined by mating the strains indicated in Table 2, sporulating the diploids and conducting tetrad analysis on the dissected spores. The markers used included the ts phenotype of *tsc3* at 37°C, the *lys2* auxotrophy,  $csg2::LEU2^+$  prototrophy, and the sec18 ts phenotype at 30°C. A total of 117 tetrads were analyzed. The distance in centimorgans was calculated using 1cM=3.3 kb for chromosome II. Using this ratio, TSC3 is predicted to be in a 30 kb region of chromosome II, surrounding YBR050C. The cloned complementing TSC3 gene was eventually found to be 18 kb away in the vicinity of YBR058C. Together, the subcloning and linkage data is conclusive genetic proof that the cloned gene is allelic to TSC3. Serine palmitoyltransferase assay of tsc3 membranes. Phytosphingosine rescue of tsc3 mutants indicates that TSC3 plays a role in sphingolipid biosynthesis early in the biosynthetic pathway, prior to the formation of LCB. To determine if Tsc3p plays a role in serine palmitoyltransferase function, membranes were isolated from tsc3 mutants and SPT activity was measured in the microsomal cell fraction. The assay was run in the presence and absence of NADPH. The presence of NADPH allows reduction of the product of SPT activity, 3-ketosphingosine, to be reduced to dihydrosphingosine. In the absence of NADPH all counts should be in 3-ketosphinganine. Results show that when cells are grown at the permissive temperature (26°C) and the enzyme activity assay is performed at the non-permissive temperature (37°C) there is no measurable SPT activity in *tsc3* mutant microsomal cell fractions in the presence or absence of NADPH,

indicating that Tsc3p does play a role in SPT function (Figure 12). Based on the primary structure of the small protein, it is possible that it may act as a scaffold to facilitate assembly of the other two known SPT subunits (Tsc1p and Tsc2p) at the endoplasmic reticulum membrane. Another possible function of Tsc3p may be that the hydrophobic carboxy-terminus of Tsc3p may interact with the hydrophobic tail of palmitoyl CoA and present the fatty acyl-CoA to the enzyme for catalysis. Co-immunoprecipitation and two hybrid system experiments are currently underway to investigate these possibilities.

### Discussion

The *tsc3* mutants were found to possess mutations in an unannotated and previously uncharacterized open reading frame located on the long arm of chromosome II between YBR058C and YBR059C. Tsc3p is required for optimal activity of serine palmitoyltransferase, since *tsc3* mutant membranes lack SPT activity and their ts phenotype is rescued by addition of exogenous phytosphingosine, a downstream product of SPT. However, the precise role of Tsc3p in SPT activity remains to be determined.

Tsc3p is composed of 80 amino acids possessing a hydrophilic amino terminus and a hydrophobic carboxy-terminal domain. The hydrophobic domain is of sufficient length to span a lipid bilayer twice and may anchor Tsc3p in the endoplasmic reticulum. It is interesting to consider whether the hydrophilic domain might interact with the Tsc1p/Lcb2p and Tsc2p/Lcb1p subunits to assemble an intact SPT molecule on the ER membrane.



Figure 12. SPT activity expressed in counts per minute of <sup>3</sup>H-serine incorporated into long chain base in membranes harvested from  $csg2\Delta$  and tsc3 mutant cells.

Sequence analysis of the *tsc3* mutant alleles indicates that the most common mutations in *tsc3* result from frameshift mutations in a string of T nucleotides that encode two phenylalanine and one valine residue present in the hydrophobic carboxy-terminus. These mutations result in either a premature stop codon, a truncated protein, or a readthrough mutation resulting in an extended tail. The string of T nucleotides appears to be a hotspot for mutations perhaps due to slippage during DNA replication. The phenotype of these mutant alleles can be divided into two categories based on their rescue by exogenously added LCB. The null mutant is viable indicating that the gene product is not essential for cell viability.

Experiments to address the role of Tsc3p in SPT activity and its interaction with other proteins are currently underway and include making use of the yeast 2 hybrid system to determine if Tsc3p interacts with Lcb1p and Lcb2p.

### <u>Chapter 5</u> <u>TSC4/TSC5</u>

### Introduction

Many members of the *tsc* mutant collection possess defects in sphingolipid biosynthesis. The *tsc4* and *tsc5* mutants were characterized as part of our investigation of sphingolipid homeostasis in yeast. Both the *tsc4* and *tsc5* mutants are defective in the *FAS2* gene which encodes the  $\alpha$  subunit of the multi-enzyme complex of fatty acid synthase.<sup>26</sup> This complex synthesizes palmitoyl CoA which is required for the formation of long chain base (LCB), very long chain fatty acids (VLCFA), and PI, all of which are required for sphingolipid biosynthesis.<sup>25, 36, 42</sup> Here we describe the cloning and characterization of *TSC4/TSC5*.

### Methods

Identification of TSC4. There was a single mutant representative of the TSC4 complementation group, CJYa6 (tsc4-1,  $csg2\Delta$ ).

Transformation with the genomic libraries. The TSC4 gene was cloned from a  $YCp50 (URA3^{+})$  based genomic library. The CJYa6 (tsc4-1, csg2 $\Delta$ ) strain was transformed with 4 independently isolated banks of this library following the protocol outlined in methods (chapter 2). Transformants were selected as uracil prototrophs on synthetic media lacking uracil (SD-Ura). The transformation efficiency exceeded 10,000 transformants for each of the 4 banks. After two days the transformants were replica plated from the SD-Ura plates onto YPD and incubated at 37°C.

*Testing candidates.* Seven fast growing colonies were selected from the YPD plates at  $37^{\circ}$ C plates and tested for Ca<sup>2+</sup> sensitivity on SD-Ura+100mM Ca<sup>2+</sup>. Two candidates, B2,5 and B4,3, exhibited Ca<sup>2+</sup> sensitivity and when forced to lose the *URA3* plasmid on media containing FOA, they reverted back to a ts phenotype. Genomic DNA was harvested from these candidates using the method of Holm *et al.* (1986) and the plasmids were amplified and purified by passage through *E. coli* as described previously.<sup>59</sup>

Hybridizing the complementing clone to the ATCC contiguous genome array. A Sau3A digest of the candidate B2,5 plasmid was randomly labeled with  $[\alpha^{32}P]dATP$  and hybridized to the ATCC contiguous yeast genome array as described in methods (chapter 2). The autoradiogram of the hybridized blot indicated that B2,5 contained sequence from 3 overlapping clones, corresponding to ATCC numbers 70067, 71156, and 70258, on chromosome XVI. Restriction enzyme mapping of the complementing plasmid insert indicated that the B2,5 plasmid contains about 14.7 kb of yeast genomic sequence harboring 6 complete open reading frames from YPL229W to YPL234C. This region of chromosome XVI contains the 5682 bp gene *FAS2* (YPL231W), which encodes the  $\alpha$ -subunit of fatty acid synthase.

Subcloning of B,5. To identify the responsible open reading frame on the complementing plasmid, a series of subclones of the B2,5 genomic insert were constructed.

BstEII subclone. A 9000 bp BstEII fragment was purified from the B2,5 complementing plasmid and ligated into a pRS316 plasmid into which a BstEII site had



Figure 13. Subclones of *tsc3* complementing plasmid, B2,5

been introduced on an unrelated restriction fragment. This construct eliminated 800 bp from the carboxy terminal coding region of *FAS2* as well as the complete coding sequences of YPL230W and YPL229W (Figure 13).

*Eco*RI *subclone*. An *Eco*RI fragment was deleted from the B2,5 plasmid thereby eliminating YPL230W and YPL229W and leaving the amino terminal 1100 bp of *FAS2* as well as the ORFs YPL234C-YPL232W (Figure 13).

Linkage analysis. A 3500 bp EcoRI fragment was isolated from B2,5 containing the carboxy terminus of FAS2. This fragment was ligated into the EcoRI site of pRS306 and linearized with SphI. The linearized fragment was integrated into TDY2038 ( $csg2\Delta$ ) and the resulting haploid transformant was mated to CJYa6 ( $csg2\Delta$ , tsc4-1). The diploid was sporulated and dissected. Tetrad analysis was performed to determine the segregation pattern of uracil prototrophy, temperature sensitivity, and Ca<sup>2+</sup> sensitivity (Figure 14A). Identification of TSC5. There was a single mutant representative of the TSC5 complementation group, CJYa30 (tsc5-1,  $csg2\Delta$ ).

Complementation of the ts of tsc4 and tsc5 with exogenous myristate and palmitate. The tsc mutants were tested for their ability to grow on YPD media containing exogenously added fatty acids myristate ( $C_{14}$ ), palmitate ( $C_{16}$ ), stearate ( $C_{18}$ ), and lignocerate ( $C_{24}$ ) at 37°C. Each of the fatty acids was added to YPD media to a final concentration of 0.5mM in the presence of 1% tergitol (NP-40).

Complementation of tsc5 with the TSC4 B2,5 plasmid. Fatty acid synthase is composed of two subunits. To determine if the tsc5 mutation was in the  $\alpha$  or  $\beta$  subunit of fatty acid synthase, the CJY $\alpha$ 30 (tsc5-1, csg2 $\Delta$ ) strain was transformed with the tsc4-1, csg2∆ complementing B2,5 plasmid using the method of Geitz et al. (1986).

FAS2 linkage to tsc5. The SphI linearized FAS2 construct in pRS306 described above was integrated into a  $csg2\Delta$  strain (TDY2040) and mated to CJY $\alpha$ 30 ( $csg2\Delta$ , tsc5-1). The resulting diploids were sporulated and dissected and the segregation of temperature sensitivity, uracil prototrophy, and Ca<sup>2+</sup> sensitivity assessed (Figure 14A).

Confirmation of URA3 insertion in the FAS2 locus. The FAS2 marked  $csg2\Delta$  strain was mated to a  $fas2\Delta$  strain (DTY10A fas2::LEU2)<sup>1</sup> and the resulting diploids sporulated and dissected. Due to the auxotrophic requirement of exogenous fatty acid in the media by the  $fas2\Delta$  the dissection was performed on media containing 0.5 mM C<sub>14</sub> in 1% tergitol. The tetrads were evaluated for segregation of the uracil prototrophy and the C<sub>14</sub> auxotrophy.

Tetrad analysis from tsc4/tsc5 diploids. A diploid was made by mating CJYa6 (tsc4-1,  $csg2\Delta$ ) to CJYa30 (tsc5-1,  $csg2\Delta$ ). The diploid was sporulated and dissected and the products of meiosis evaluated as to temperature sensitivity and Ca<sup>2+</sup> sensitivity. If the defects in both mutants are in the same gene all products of meiosis must demonstrate the mutant phenotype. There can be no co-segregation of mutant loci.

#### Results

Isolation of tsc4 and tsc5 mutants. The isolation of the tsc4 and tsc5 mutants were as

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The DTY10A *fas2* strain was graciously donated by Dr. Charles Martin, Bureau of Biological Research, The State University of New Jersey, Rutgers, Piscataway, N.J.

described in methods (chapter 2).

Phytosphingosine fails to rescue ts of tsc4 and tsc5. If only LCB synthesis were limited by the reduced palmitoyl-CoA pool in the tsc4-1,  $csg2\Delta$  and tsc5-1,  $csg2\Delta$  mutants, then exogenous phytosphingosine would be expected to rescue the ts lethal phenotype (Figure 1). However, the *tsc4* and *tsc5* mutants are not rescued by the addition of exogenous phytosphingosine. This may be due to several factors. Palmitoyl CoA is the primary fatty acid building block in the cell, required in many other pathways other than sphingolipid biosynthesis. Exogenously added LCB may satisfy the sphingolipid requirement of the cell but not the other palmitoyl CoA requirements, which may be of a higher priority. Enzymes in critical pathways may have lower Km's and therefore a higher affinity for the limited palmitoyl CoA. When the cell is in an enriched environment and palmitoyl CoA levels are high, enzymes with a high Km, and lower affinity, would have substrate to act on. When concentrations of palmitoyl CoA are low, in a nutritionally-deprived environment, only high affinity enzymes are able to act on the limited substrate, supporting vital requirements. In this way the level of palmitoyl CoA may act as an intracellular indicator of nutritional status. Another possible explanation for the failure of LCB to rescue tsc4 and tsc5 is that the sphingolipid biosynthetic pathway requires palmitoyl CoA at two points; in the formation of LCB and in the formation of the VLCFA attached to form the ceramide. Exogenous LCB only satisfies half of this requirement. It may be the formation of VLCFA which is limiting the synthesis of sphingolipid.

Identification of TSC4 as FAS2. To identify the mutated gene in the CJYa6 (tsc4-1,

 $csg2\Delta$ ) mutant, a plasmid-based genomic library was transformed in as described in methods (chapter 2). Complementing plasmids were harvested from two clones. Three representative plasmids from each candidate were transformed back into the CJYa6 (tsc4-*I*,  $csg2\Delta$ ) mutant and all complemented both ts and suppression of Ca<sup>2+</sup> sensitivity. Identification of FAS2 as the complementing ORF. The complementing plasmid was hybridized to the ATCC contiguous yeast genome array and the blot identified a region of chromosome XVI containing a 5700 bp open reading frame identified as FAS2. Subcloning of the complementing plasmid indicated that the FAS2 sequence is responsible for the complementation of the tsc4-1,  $csg2\Delta$  phenotype (Figure 13). Linkage analysis. To determine if the gene that is mutated in tsc4-1,  $csg2\Delta$  is allelic to FAS2, linkage analysis was performed. The FAS2 locus was marked with a URA3 gene in a  $csg2\Delta$  strain, as described in methods and mated to the CJYa6 ( $csg2\Delta$ , tsc4-1) mutant. The resulting diploid was sporulated and dissected and tetrad analysis indicated complete linkage of the uracil prototrophy, temperature resistance, and Ca<sup>2+</sup> sensitivity in 9 of 9 tetrads analyzed. The failure to recover any URA<sup>+</sup> ts products of meiosis indicates that the FAS2 gene is allelic to the tsc4 mutant locus (Figure 14A).

Exogenous fatty acid rescue. Since the tsc4 mutant harbors a ts allele of fas2, rescue of the ts phenotype of the tsc4-1, csg2 $\Delta$  mutant by exogenously added fatty acid was tested. In addition, all the unidentified tsc mutants were tested for rescue by exogenous fatty acid, since it was considered possible that other genes required for fatty acid synthesis (eg. FAS1) might also be represented in the tsc mutant collection. Exogenous C<sub>14</sub> and C<sub>16</sub> rescued CJYa6 (tsc4-1, csg2 $\Delta$ ) and CJYa30 (tsc5-1, csg2 $\Delta$ ) at 37°C, indicating that both mutants possessed defects in the formation of fatty acid (Figure 15). Exogenously added  $C_{18}$  and  $C_{24}$  did not alter the ts phenotype of CJYa6 (*tsc4-1*, *csg2*\Delta) or CJYa30 (*tsc5-1*, *csg2*\Delta) or any of the other *tsc* mutants.

#### Identification of TSC5

Transformation of tsc5 with FAS2. CJY $\alpha$ 30 (tsc5-1, csg2 $\Delta$ ) was transformed with the FAS2 containing B2,5 plasmid. The B2,5 plasmid complemented both the suppression and the temperature sensitive phenotypes of CJY $\alpha$ 30 (tsc5-1, csg2 $\Delta$ ) suggesting that either FAS2 was a dose-dependent suppressor or that tsc4 and tsc5 were both defective in FAS2 and were displaying intraallelic complementation.

To determine whether the mutation in CJY $\alpha$ 30 was in the *FAS2* gene, linkage analysis was performed. A CJY $\alpha$ 30 (*tsc5-1, csg2* $\Delta$ ) haploid was mated to a wild type strain in which the *FAS2* locus was marked with *URA3* as described above. The diploid was sporulated, dissected, and analyzed for segregation of the temperature sensitive, Ca<sup>2+</sup> sensitive, and uracil phenotypes. In 10 out of 10 tetrads analyzed, temperature sensitivity segregated away from Ca<sup>2+</sup> sensitivity and uracil prototrophy. The results indicate linkage between the marked *FAS2* locus and the *tsc5-1* mutation. This result demonstrates that both CJY $\alpha$ 30 (*tsc5-1, csg2* $\Delta$ ) and CJYa6 (*tsc4-1, csg2* $\Delta$ ) harbor mutations in the *FAS2* gene.

Intraallelic complementation. Data indicating tsc4 and tsc5 are in separate complementation groups appears to contradict the linkage data suggesting both strains are fas2 mutants (Figure 14B). Fas2p contains multiple functional domains. Each of these catalytic domains is subject to mutation. In a diploid, recessive mutations in distinct

**A**.

### tsc 4 and 5 Linkage to Fas2



B.

the tsc4xtsc5 diploid.

## tsc4 x tsc 5 Phenotype Complementation in Diploid



**Figure 14.** A. Representative tetrad dissection of tsc4 and  $tsc5 \times csg2\Delta$  diploids where the FAS2 locus has been marked with a URA3<sup>+</sup> marker gene. B. Phenotype of  $csg2\Delta$ , tsc4, and tsc5 haploids and the complementation of the suppression and ts phenotype in

# Myristate (C14) Rescue of tsc4 / tsc5





Figure 15. The rescue of the ts phenotype by exogenous  $C_{14}$  (myristate 0.5mM+1% tergitol)

domains of the same gene will complement one another and appear to be in separate genes. Such is the case with tsc4/tsc5 heterozygous diploids. Both possess mutations in FAS2, yielding a defective  $\alpha$ -subunit of fatty acid synthese, but these mutations are apparently in distinct domains of the protein. When they are mated, the diploid possesses alleles which code for both mutant fas2 forms; one from tsc4 and the other from tsc5. If the alleles are both equally transcribed and translated, the catalytic activity of the mutant tsc4 Fas2p domain would be complemented by the TSC4 domain of the tsc5 Fas2p allele and vice versa. This phenomenon is referred to as intraallelic complementation. Tetrad analysis of tsc4 x tsc5 diploids. Mutations can only co-segregate in the products of meiosis if the cross-over occurs between the mutations. If the mutations in *tsc4* and *tsc5* are in the same gene, though the gene is large, the frequency of crossover is very small. The cM to kbp ratio for chromosome XVI is 0.37 indicating that a crossover event between extreme ends of a gene the size of FAS2 would occur in approximately 2% of crosses. To test the allelism of *tsc4/tsc5* mutations, a heterozygous diploid was sporulated, dissected and analyzed to determine if any products of meiosis had lost the ts/suppressing mutation. In 12 of 12 tetrads analyzed all the products of meiosis exhibited a *tsc* phenotype of temperature sensitivity and Ca<sup>2+</sup> resistence indicating that the tsc4 and tsc5 mutations did not crossover and co-segregate. This result demonstrates that tsc4 and tsc5 are allelic; segregation of mutations did not occur because of the linkage between the mutated domains of the FAS2 gene.

### Discussion

The *tsc4* and *tsc5* mutants were found to harbor mutations in the *FAS2* gene which encodes the  $\alpha$ -subunit of a multienzyme complex, fatty acid synthase (FAS). Both mutations occur in the same gene; their failure to fall into the same complementation group is due to intraallelic complementation where mutations occur in distinct domains of a multidomain protein.

Fatty acid synthase catalyzes the formation of palmitoyl CoA ( $C_{16}$ ). Palmitoyl CoA sits at a critical point in lipid biosynthesis; it is required for synthesis of glycerophospholipids as well as sphingolipids. Our investigation of *tsc4* and *tsc5* indicates that limiting the availability of palmitoyl CoA to the cell suppresses the Ca<sup>2+</sup> sensitivity of *csg2* $\Delta$ , presumably because of reduced flux through the sphingolipid biosynthetic pathway. This fact suggests that the cell prioritizes the use of limited palmitoyl CoA possibly by the Km of downstream enzymes. One hypothesis is that levels of palmitoyl CoA may signal the cell as to nutritional status so that when levels are limiting the higher affinity enzymes, possibly catalyzing synthesis of glycerol based phospholipids have priority, leaving little substrate for sphingolipid biosynthesis. The mutations we identified in *FAS2* artificially impose this decreased palmitoyl CoA synthesis and therefore suppress *csg2* $\Delta$  by decreasing the synthesis of IPC-C, while still providing sufficient synthesis of other palmitoyl CoA-dependent products to maintain viability at 26°C.

The *tsc4/tsc5*, *csg2* $\Delta$  mutant ts phenotype is rescued by the addition of exogenous

myristate (C<sub>14</sub>). This result suggests that myristate can substitute for essential functions provided by palmitate, lacking in the *tsc4* and 5 mutant at the nonpermissive condition of 37°C. Myristate also suppresses the Ca<sup>2+</sup> sensitivity of *csg2* $\Delta$ . One possible explanation for this finding is that myristate acts as a negative regulator of fatty acid synthase reducing palmitoyl CoA synthesis. Reduced levels of palmitoyl CoA (C<sub>16</sub>) decrease long chain base synthesis. SPT demonstrates significant substrate specificity for palmitoyl CoA and so exogenous myristate (C<sub>14</sub>) is not incorporated into sphingolipids.<sup>36</sup> Palmitoyl CoA also is required for VLCFA synthesis and so reduced C<sub>16</sub> synthesis results in decreased ceramide formation.<sup>42</sup> Reduced sphingolipid synthesis decreases IPC-C formation and rescues *csg2* $\Delta$  on Ca<sup>2+</sup> containing media.

The mutations in *tsc4* and *tsc5* impact many other pathways other than sphingolipid biosynthesis. This fact, coupled with their conditional lethal ts phenotype, may make them useful tools for future studies of lipid homeostasis in yeast.

### Chapter 6 TSC7

### Introduction

Many of the *tsc* mutants of *Saccharomyces cerevisiae* possess defects in sphingolipid biosynthesis. As part of the study of this mutant collection, *TSC7* was cloned and characterized. Mutants in the TSC7 complementation group possess mutations in the *SUR2* gene which encodes the long chain base hydroxylase that forms phytosphingosine from dihydrosphingosine (Figure 2).<sup>19</sup> This chapter describes the cloning and characterization of *TSC7*.

### Methods

The ts phenotype of the tsc7 mutants is rescued by phytosphingosine. Mutants in the tsc7 complementation group were tested on YPD plates containing 10  $\mu$ M phytosphingosine at 26°C and 37°C to determine if the ts phenotype was rescued by exogenous LCB. tsc7 mutant transformed with wild type sphingolipid genes. LHYa11 (tsc7-1, csg2 $\Delta$ ) was transformed with plasmids containing wild type copies of CSG2, LCB1, and SCS1 following the LiOAc method of Gietz et al., 1995. Transformants were selected and tested on YPD and SD media with and without 20 mM Ca<sup>2+</sup> at 26°C and 37°C. Cloning tsc7. The LHYa11 (tsc7-1, csg2 $\Delta$ ) strain was transformed with a YCp50 (URA3) plasmid-based genomic library using the LiOAc method of Gietz et al., (1995). Approximately 8,000 transformants were screened, and 12 candidates were obtained after replica plating the transformants to YPD at 37°C for 2 days. These TS<sup>+</sup> candidates were

plated on YPD and SD media lacking uracil with and without 20 mM Ca<sup>+2</sup> at 26°C and 37°C to select those whose suppression phenotype had also been complemented. Two candidates (B2,4 and B4,13) exhibited a  $csg2\Delta$  phenotype (temperature resistant and Ca<sup>2+</sup> sensitive) and reverted to a *tsc* phenotype when cured of the complementing plasmid on FOA. Genomic DNA was harvested from the B2,4 and B4,13 transformants using the method of Holm *et. al* (1995) and transformed into competent *E. coli* cells to amplify the complementing plasmids. Repeated attempts to amplify B4,13 were unsuccessful for unknown reasons. B2,4 plasmids were harvested from *E. coli* using the Jet Quick rapid plasmid prep kit (Genomed, Research Triangle, N.C.), and transformed into LHYa11 (*tsc7-1, csg2*\Delta).

Hybridizing the B2,4 complementing plasmid to the genome contiguous collection. The B2,4 tsc7 complementing plasmid was digested with Sau3A, randomly labeled with [ $\alpha$  <sup>32</sup>P]dATP, and then hybridized to the ATCC contiguous yeast genome array as described in methods (chapter 2). The autoradiogram identified the ATCC clone 70360 which corresponds to a region of chromosome IV. Restriction mapping indicated the plasmid insert contained 13,700 bp of chromosome IV sequence with coordinates from 1,050,482 to 1,064,182 containing 7 complete open reading frames including YDR294C to YDR300C and the amino terminus of YDR301W.

Subcloning of TSC7

Isolation of YDR294C (Sphingosine-1-phosphate lyase). YDR294C, present on the B2,4 complementing plasmid, was known to play a role in sphingolipid breakdown and so was investigated first.<sup>54</sup> A construct containing YDR294C was available from work done previously in our lab.<sup>1</sup> A 4600bp *Eco*RI fragment containing YDR294C was band isolated from this construct and was ligated into an *Eco*RI digest of pRS316 and amplified in *E. coli*. The construct was verified by restriction enzyme digest, and was transformed into LHYa11 (*tsc7-1*, *csg2* $\Delta$ ).

BamHI subclones of B2,4. Two plasmids were constructed from the B2,4 complementing clone. A BamHI restriction enzyme digest liberated a 5400 bp fragment containing YDR298C (ATP5) through YDR300C (PRO1) and an 8300 bp fragment containing the vector and YDR294C (DPL1) through YDR297W (SUR2). Both fragments were band isolated. The 5400 bp excised fragment was ligated into a BamHI digest of pRS316. The 8300 bp vector band was religated and both were amplified in *E.* coli and verified by restriction enzyme digests. Both constructs were transformed into LHYa11 (tsc7-1,  $csg2\Delta$ ) (Figure 16).

Isolation of YDR297W (SUR2). The coding sequence of SUR2 was obtained by PCR using primers designed with a Sal1 site 387 bp upstream, and an Xba1 site 353 bp downstream of the coding sequence that amplify a fragment of 1770  $bp^2$ . The PCR product was obtained using the standard protocol outlined in methods (chapter 2), cut

ł

2

YDR294C was cited in the literature as coding for a lyase of phosphorylated long chain base.

SUR2 PCR primers: upstream - #9131 SUR2 5'-GGCCGTCGACCGACCTCCTGTTT CCTATTGTCTT-3'; downstream -#9132 SUR21 5'GGCCTCTAGAATGTTCGTGTATCCAG GCAAACTT 3' Primer designed and

<sup>5&#</sup>x27;GGCCTCTAGAATGTTCGTGTATCCAG GCAAACTT-3'. Primers designed and maintained by Ken Gable.



Figure 16. Subcloning of the *tsc7* complementing plasmid B2,4

with the designed restriction enzymes, and ligated into the Sall/Xbal sites of the polylinker of pRS316. The construct was amplified in *E. coli* and plasmids verified by restriction enzyme digests. The verified construct was transformed into all of the alleles of the *tsc7* mutant.

Isolating tsc7 alleles of SUR2. PCR fragments carrying the coding sequence of SUR2 were obtained using the previously described primers and genomic DNA isolated from tsc7 mutants as template. The Pwo (Pyrococcus woesei) DNA Polymerase protocol was followed (Boehringer Mannheim, Indianapolis, IN). Briefly, two master mix tubes were prepared for each reaction. Master mix I contained 500 ng of yeast genomic DNA obtained from either wild type TSC7 or one of the tsc7 mutants, 10  $\mu$ L of 3  $\mu$ M reconstituted up and downstream primers, 2 µL of 10 mM PCR nucleotide mix (Boehringer Mannheim), and volume brought to  $50\mu$ L with dH<sub>2</sub>O. Master mix II contained 10 µL of PCR buffer + MgSO<sub>4</sub>, 0.5 µL of Pwo DNA polymerase (both in Boehringer Mannheim PWO kit) and the total volume also brought to 50 µL with dH<sub>2</sub>O. Master mix I and Master mix II were combined and put into the thermocycler. The thermocycler protocol was set as a step-down series of annealing temperatures starting at 54°C and stepping down to 48°C in single degree increments. Each step was run for 4 cycles except the last which was run for 10 cycles. Each cycle includes a 1.5 min 94°C melting, 1.0 min annealing at the stepped temperature, and a 3.0 min, 72°C elongation step. Ten percent (10  $\mu$ L) of the PCR products were run on a 1% agarose gel to determine the yield of the product. The PCR products were cut with the restriction enzymes whose sites were designed in the primer sequence (Sall, Xbal) and ligated into

pRS316. The constructs were transformed into *E. coli* and the purified plasmids were verified by restriction enzyme digests. The plasmids were transformed into  $csg2\Delta$  and  $csg2\Delta$ ,  $sur2\Delta$  mutant cells.<sup>3</sup>

Tryptophan availability influences ts of tsc7 mutants. The tsc7 mutants, all trp1 $\Delta$ tryptophan auxotrophs, were tested for temperature sensitivity in the presence and absence of exogenous tryptophan by adding exogenous tryptophan to YPD media in final concentrations of 90, 130, and 220  $\mu$ M and by transforming each of the tsc7 mutants with the pRS314 plasmid which contains the TRP1 gene. Transformants and controls were streaked out on YPD media at 26°C and 37°C.

Demonstrating linkage between trp auxotrophy and ts. An LHYa11 (tsc7-1,  $csg2\Delta$ ) strain transformed with a pRS314 plasmid was mated to an LHY $\alpha$ 34 (tsc7-4,  $csg2\Delta$ ) strain to produce a homozygous tsc7 mutant diploid that would randomly segregate the extrachromosomal pRS314 plasmid harboring the *TRP1* gene in meiosis. Diploids were selected on SD media lacking adenine and lysine to eliminate haploids. Diploids were grown on YPD plates for 2 days then a small innoculum was placed in sporulation media and shaken at 30°C for 4 days. The tetrads were then dissected and evaluated for segregation of ts and TRP<sup>+</sup>.

### **Results**

*tsc7 mutant isolation.* The *tsc7* mutants were isolated as described in the methods section (chapter 2). Four independent suppressor mutants make up the *TSC7* complementation

<sup>&</sup>lt;sup>3</sup>The sur  $2\Delta$  is disrupted with the *TRP1* gene, constructed previously.

group accounting for 7% of the tsc mutant collection.

*Phytosphingosine rescue of tsc7.* As described above, rescue of the *tsc* mutants by exogenous phytosphingosine was tested in an initial screen to test whether the mutants appear to be deficient in LCB synthesis. The ts phenotype of the *tsc7* mutants is rescued by exogenous phytosphingosine in the media at a concentration of 10  $\mu$ M. The phytosphingosine rescue of *tsc7* suggests the defect in these mutants is early in the sphingolipid biosynthetic pathway.

Transforming tsc7 with previously identified sphingolipid genes. LHYa11 (tsc7-1, csg2 $\Delta$ ) was transformed with wild type copies of several genes known to play a role in sphingolipid biosynthesis (CSG2, LCB1, SCS1) to determine is any of these complemented the mutant phenotype. The results showed that tsc7-1, csg2 $\Delta$  is no longer ts in a CSG2 background. The other genes had no effect on the tsc7 mutant phenotype. That is, the ts phenotype of mutants in the tsc7 group was dependent on the presence of the csg2 $\Delta$  mutant allele.

Cloning TSC7. The TSC7 gene was cloned from a plasmid based genomic library by complementing the ts phenotype. The complementing sequence resides on chromosome IV, as determined by hybridizing a labeled digest to the ATCC contiguous clone array of the yeast genome. Subclones of the complementing fragment indicated that the SUR2 gene, included on the complementing plasmid, was required for complementation (Figure 16). SUR2 codes for the long chain base C<sub>4</sub> hydroxylase responsible for converting dihydrosphingosine to phytosphingosine. These results are consistent with the rescue of the ts phenotype of *tsc7* by exogenous long chain base. However, previous

characterization of the SUR2 gene (Haak *et.al* 1997) indicated that  $sur2\Delta$  mutants are viable and are not ts. Therefore, it was unexpected that sur2 mutants would be recovered in the *tsc* mutant collection.

Characterizing tsc7. Several possibilities could explain why tsc7 mutants exhibit a ts phenotype while a sur2 $\Delta$  does not. One possibility is that a mutant gene, linked to SUR2, may be responsible for the ts phenotype. Another possibility is that an unlinked gene, present in all the parental strains, and therefore all products of meiosis, may manifest a ts phenotype in the environment of a csg2 $\Delta$ , sur2 double mutant. Finally, a specific sur2 allele, that is recessive but confers a ts phenotype, may be responsible. To investigate these possibilities and determine which was correct a series of experiments were conducted.

The SUR2 coding sequence was obtained from each of the tsc7 mutants by PCR. The alleles were ligated into a shuttle vector and transformed into a  $csg2\Delta$ , sur2::TRP1(double knockout). This double knockout does not exhibit a ts phenotype but does suppress the Ca<sup>2+</sup> sensitivity in a  $csg2\Delta$  background. The tsc7 mutant sur2 alleles were transformed into the double knockout mutant to determine if, (1) the suppression phenotype was complemented, indicating that the mutation in tsc7 was not in SUR2, and (2) the mutant allele would confer a ts phenotype on the double knockout indicating that it was the specific mutant allele of sur2, in the tsc7 group that was responsible for the ts and suppression phenotypes. The results showed that the sur2 alleles obtained from tsc7mutants neither altered the suppression phenotype nor did they confer a ts phenotype. This suggests that the tsc7 mutants possess a suppressing mutation in SUR2 but that it is not the mutation responsible for the ts phenotype.

Control transformations of tsc7 alleles (TDY2038/2040 csg2 $\Delta$  based, trp1 $\Delta$ ) with plasmids containing a TRP1 sequence, rescued the ts phenotype. Recent evidence suggests that phytosphingosine influences the uptake of tryptophan from the media probably by down-regulating transcription of the high affinity tryptophan transporter, TAT2.66 To determine if the trp1 auxotrophy might also be responsible for the ts phenotype of tsc7, members of the TSC7 complementation group were tested on YPD media at 37°C with and without exogenous tryptophan. All the tsc7 mutants were rescued by tryptophan in concentrations as low as 90  $\mu$ M (Figure 17). To determine if providing the cell the ability to synthesize tryptophan endogenously would also rescue the ts phenotype, LHYa11 (tsc7-1,  $csg2\Delta$ ) was transformed with a pRS314 (TRP1) plasmid and streaked out with the untransformed LHYa11 (tsc7-1,  $csg2\Delta$ ) strain on YPD at 37°C. The ability to synthesize tryptophan also complemented the ts phenotype suggesting the effect of the mono-hydroxylated long chain base on growth phenotype was due to transport of tryptophan across the cell membrane. The transformed LHYa11 (tsc7-1,  $csg2\Delta$ ) strain was then mated to LHY $\alpha$ 34 (tsc7-4,  $csg2\Delta$ ) forming a homozygous diploid. The diploid was sporulated and dissected to determine the segregation pattern of TRP<sup>+</sup> and temperature sensitive spores. All spores that were TRP<sup>+</sup> grew at 37°C. All tryptophan auxotrophic spores were temperature sensitive. These data indicate that the sur2 mutation in tsc7 is synthetically lethal at 37°C with  $csg2\Delta$  when in a trp1 auxotrophic strain and that a mutation in sur2 alone is sufficient to suppress the Ca2+ sensitive phenotype of the  $csg2\Delta$  mutant.



Figure 17. The effect of exogenous tryptophan and transformation with a *TRP1* containing plasmid on the ts phenotype of tsc7,  $csg2\Delta$  mutants.

#### Discussion

The tsc7 mutants possess a defect in SUR2 which codes for the long chain base C4 hydroxylase that forms phytosphingosine from dihydrosphingosine.<sup>19</sup> SUR2 is located on chromosome IV (YDR297W) and was originally isolated in a screen for suppressors of rvs161/rvs167 mutants.<sup>40</sup> The rvs mutants exhibit impaired survivability under starvation conditions.<sup>40</sup> When cloned, the RVS gene sequences gave no indication as to their function and so a group of suppressors to both mutants were isolated and characterized in an attempt to explain the rvs pleiotropic phenotype. Four genes were identified (SUR1-SUR4) that, when mutated, suppress the pleiotropic defects of rvs161 and rvs167 single mutants and the double mutant. Subsequently, a fifth gene (SUR5), was identified. SUR genes are involved in sphingolipid biosynthesis.<sup>19</sup> SUR1 is homologous with CSG1 required for mannosylation of IPC.<sup>50</sup> SUR2 encodes dihydrosphingosine hydroxylase forming phytosphingosine.<sup>19</sup> SUR3 has not been identifed. SUR4 and SUR5 are homologous to ELO3 and ELO2 respectively, which elongate fatty acids to the C<sub>26</sub> VLCFA which is found in an amide linkage with LCB in sphingolipids.42

Sur2p is a cytochrome-b5 dependent member of the oxo-diiron family of hydroxylases/desaturases which catalyzes the hydroxylation at C4 of dihydrosphingosine forming phytosphingosine.<sup>19</sup> The hydroxylation reaction occurs on the endoplasmic reticulum. The *sur2* $\Delta$  phenotype (synthesis of sphingolipids containing

dihydrosphingosine rather than phytosphingosine as the long chain base) can circumvented by the addition of exogenous phytosphingosine.<sup>21</sup>

Transformation of a tsc7 strain with a CSG2-containing plasmid complements the ts phenotype. Disruption of SUR2 with a TRP1 insert suppresses the  $Ca^{2+}$  sensitivity of  $csg2\Delta$  but does not confer a ts phenotype. The ts phenotype of tsc7 is only evident in a tryptophan auxotrophic (trp1) strain suggesting that the sur2, csg2 mutants are unable to transport tryptophan normally. It has been shown that trp auxotrophic strains exhibit increased sensitivity to phytosphingosine at 26°C and that this increased sensitivity is due to tryptophan starvation of the cell.<sup>66</sup> This same study indicated that overexpression of the tryptophan high affinity transporter, Tat2p, could overcome the phytosphingosine sensitivity indicating that the transporter activity is compromised when phytosphingosine is present.<sup>66</sup> Data presented here suggests that the C<sub>4</sub> hydroxylation of LCB, and/or the mannosylation of sphingolipids, is also required for proper functioning or the plasma membrane localization of Tat2p so that an mono-hydroxylated LCB ceramide results in tryptophan starvation in a sur2, trp1, csg2 mutant at 37°C. Consistent with this hypothesis, the ts phenotype of *tsc7* is eliminated when tryptophan is added to the media indicating that either a low affinity transporter can supply needed tryptophan if the media concentration is high enough or Tat2p is compensated by added tryptophan. The ts phenotype can also be complemented by providing the *TRP1* gene on an extrachromosomal plasmid; when the cells synthesize tryptophan endogenously they do not require Tat2p.
## Chapter 7 TSC 11

### Introduction

Many mutants in the *tsc* collection of suppressors of the Ca<sup>2+</sup> induced death of  $csg2\Delta$  possess defects in the biosynthesis of sphingolipids. As part of a study to analyze members of the *tsc* collection, the *TSC11* gene was cloned and characterized. *TSC11* is a previously uncharacterized essential gene located on chromosome V at YER093C. This chapter describes the cloning and characterization of *TSC11*.

### Methods

*Cell viability assay.* Wild type,  $csg2\Delta$ , and LHYa53 (tsc11-1,  $csg2\Delta$ ) cells were grown overnight at the permissive condition of 26°C in liquid YPD. The cells were diluted to a starting OD 600 of 0.1 and split into two tubes and growth rates at 37°C and 26°C were determined. OD 600 readings were taken at hourly time points and aliquots were plated on YPD media at 2 hour intervals for colony counts from both 26° and 37° cultures. *Identification of tsc11*. The LHYa53 (tsc11-1,  $csg2\Delta$ ) mutant was transformed with a YCp50 based genomic library as described in the methods section (chapter 2). Twelve transformants, able to grow at 37°C, were selected as candidates for having acquired a plasmid containing the *TSC11* gene. They were plated on SD media lacking uracil to insure they possessed a YCp50 plasmid and on YPD + 50mM Ca<sup>2+</sup> to assess their suppression phenotype. Seven candidates exhibited a  $csg2\Delta$ -like phenotype of temperature resistance and Ca<sup>2+</sup> sensitivity and grew on SD(-URA). These candidates

were plated on FOA and SD(-URA) to select those which had lost the plasmid and those that retained a plasmid respectively.<sup>62</sup> Cells which had lost the plasmid were tested for reversion back to the tsc11 phenotype (temperature sensitive and able to suppress the Ca2+ sensitivity of the  $csg2\Delta$  mutation) to determine if their complementing phenotype was plasmid linked. All the candidates reverted to the *tsc11* phenotype (temperature sensitive and able to suppress the Ca<sup>2+</sup> sensitivity of the  $csg2\Delta$  mutation) when grown on FOA while growth from the SD(-URA) plates retained the  $csg2\Delta$  phenotype (temperature resistant and Ca<sup>2+</sup> sensitive). Genomic DNA was harvested from the transformed candidates using the method of Holm, et al. (1986). Genomic DNA was transformed into E. coli competent cells and the complementing plasmids were harvested using the Jetquick Plasmid Mini-prep Spin kit (Genomed Inc., Research Triangle, N.C.). The complementing plasmids from seven candidates were digested with Sau3A and the pattern of restriction fragments indicated that there were two distinct plasmids among the seven candidates. One of each of the plasmids was transformed into LHYa53 (tsc11-1,  $csg2\Delta$ ) and both complemented the *tsc11* phenotype.

Hybridizing of complementing plasmid to ATCC contig. An  $[\alpha^{32}P]$ dATP labeled Sau3A digest of a complementing plasmid, p3-4, was hybridized to the ATCC contiguous yeast genome array as described in the methods section (chapter 2). The radiolabeled probe hybridized to regions corresponding to ATCC overlapping clone numbers 70660, 70838, 70118, 70835 identifying a region of chromosome V. Restriction enzyme mapping of the complementing insert showed that it contained approximately 11,200 bp of chromosome V and included the sequence of 5 complete open reading frames (YER091C-YER094C)

(Figure 18).

Subcloning of p3-4. Various subclones of the p3-4 plasmid were constructed to determine which ORF complements the *tsc11* mutant (Figure 18).

A PvuII digest of p3-4 liberated a 5900 bp fragment from the middle of the insert. The linearized plasmid was religated eliminating the coding sequence of YER091C (MET6), YER092W, and most of YER093C (Figure 18). The religated plasmid was amplified in *E. coli*, as decribed previously, and the confirmed construct transformed into the *tsc11-1*,  $csg2\Delta$  mutant.

A BamHI digest of p3-4 liberated a 4000 bp band containing all of YER092W and most of YER091C. This fragment was band isolated and ligated into a centromeric plasmid, pRS316. The construct was amplified in *E. coli* and the confirmed plasmid was transformed into the tsc11-1,  $csg2\Delta$  mutant.

Notl/SpeI isolation of YER093C. A Notl/SpeI double digest of the complementing plasmid liberated a 5600bp fragment containing all of YER092W and YER093C. This fragment was band isolated and ligated into pRS316, amplified in *E. coli*, and the confirmed plasmid was transformed into the LHYa53 (tsc11-1,  $csg2\Delta$ ) mutant. *Construction of a knockout of YER093C/Linkage analysis of YER093C to tsc11*. A 3500 bp *Xhol/Xba*I YER093C internal fragment was ligated into Bluescript (KS+). An internal 1100 bp *Eco*RI/*Mun*I fragment was replaced with an 1176 bp *Eco*RI fragment containing the *TRP1* gene. The disrupting YER093C allele, was cut from the vector with *KpnI/Not*I sites in the polylinker region of the vector, and transformed into a  $tsc11-1/csg2\Delta$ heterozygous diploid, a tsc11 homozygous diploid, a wild type haploid, and a  $csg2\Delta$ 



Figure 18. Subclones of the *tsc11* complementing plasmid p3-4. (B-BamHI, P-PvuII, S-SpeI, Xa-XbaI, RI-EcoRI, M-MunI, Xo-XhoI, N-NotI)

haploid. TRP1<sup>+</sup>transformants were selected.

YER093C required for vegatative growth. A csg2::LEU2 homozygous diploid was cotransformed with the linearized YER093C::TRP1 construct and the pRS316 NotI/SpeI tsc11-1, csg2D complementing construct and selected on SD media lacking both uracil and tryptophan. The transformants were sporulated, dissected and analyzed to assess the segregation of uracil and tryptophan auxotrophs and the prototrophic requirement for viability. TRP1/URA3 products of meiosis were isolated and grown on YPD at 26°C for 2 days to allow plasmid segregation. Colonies from the YPD plate were plated on FOA at 26°C to select for those YER093C::TRP1 integrants that had lost the Tsc11-1 covering plasmid.

Caffeine sensitivity. To determine if the tsc11-1,  $csg2\Delta$  mutant strain exhibited the caffeine sensitive phenotype, it was plated on YPD media containing 1, 6 and 18 mM caffeine to test for sensitivity at 26°C.

Long chain base/ceramide analysis. Wild type,  $csg2\Delta$ , LHYa53 (tsc11-1,  $csg2\Delta$ ), and LHYa53 (tsc11-1,  $csg2\Delta$ ) containing a plasmid based wild type copy of YER093C were grown in 500 mL of liquid SD overnight. The plasmid-containing LHYa53 (tsc11-1,  $csg2\Delta$ ) strain was grown in SD lacking uracil to maintain the plasmid. 100mL of each strain, equivalent to 30 OD 600 units of cells, were recovered at 0, 1, 3, or 5 hours and long chain bases and ceramides analyzed.

Long chain base analysis. The cells were spun and washed 2 times with  $dH_2O$  in a preweighed glass conical tube. The tubes were then reweighed to obtain the wet weight

of cells used at each time point (used for normalizing the loading of TLC plates). The pellet was resuspended in 200 µL of 0.5 M NH<sub>4</sub>OH by vortexing. Chloroform:Methanol (1:2) (2 mL) and glass beads to half the total volume were added to the conical tube and vortexed for 1 minute. The tubes were sonicated in a water bath for 15 minutes and the supernatent was decanted into a clean glass tube and spun in a clinical centrifuge to pellet glass beads and cellular debris. The supernatent was extracted with sequentially added 1 mL of chloroform, 2 ml of 0.5 M NH<sub>4</sub>OH, and 0.2 ml 3 M KCL solution. Aqueous layer was washed with 2 ml of 0.3 M KCL. After each wash the top layer was aspirated off and discarded. The remaining solution was dried under N<sub>2</sub> and resuspended in Chloroform:Methanol:NH4OH (40:10:1) to the same concentration based on the wet weight of the cells. The extracted LCBs were spotted onto a silica gel TLC plate and the plate was developed in CHCl<sub>3</sub>:CH<sub>3</sub>OH:NH<sub>4</sub>OH (40:10:1). The plate was allowed to dry at room temperature and was then sprayed with a 0.2% ninhydrin solution in ethanol and allowed to air dry. Sphingosine, dihydrosphingosine, and phytosphingosine were run as standards to identify the sample spots on the plate.

*Ceramide analysis.* At each time point (0, 3, 5 hours) a 50 ml sample of cells were spun and washed and the wet weight of the cells determined for use in normalizing loading of the TLC plate. The cells were resuspended in 5 ml of hexane and glass beads were added to one-half the total volume of liquid in the tubes. The cells were disrupted by vortexing for 7 minutes, cooling the tubes on ice at 1 minute intervals, and the lysate was decanted into a clean tube. The remaining glass beads and cellular debris were washed with 2 ml of hexane and the wash was pooled with the lysate. The combined

material was spun in a clinical centrifuge and the supernatent was transferred to a clean tube and dried under N<sub>2</sub>. The dried material was suspended in 200  $\mu$ l of ethanol : dH<sub>2</sub>O : ether : pyridine (15:15:5:1) and 20 µl of 1 M KOH in methanol and incubated at room temperature overnight. Acetic acid (1 M) in methanol was added to a ratio of 1:10 and the mixture was dried under  $N_2$ . The dried mixture was then desalted with 900  $\mu$ L of water-saturated butanol. 600 µl of dH<sub>2</sub>O was added and the mixture vortexed. The upper butanol phase was transferred to a clean glass tube and re-extracted with 300 µl of water. The butanol phases were dried under N<sub>2</sub> and resuspended in hexane:ethanol (95:5) for loading on the TLC plate. The sample concentrations were normalized based on wet cell weight to assure the same amount of material was loaded per lane on the TLC plate. The TLC plate was developed with chloroform:methanol:acetic acid (95:4.5:0.5). The plate was removed from the TLC tank, allowed to air dry, and returned to the tank and redeveloped a second time to maximize separation of spots. The plate was air dried and sprayed with 10% copper in 8% phosphoric acid to wetness. After the plate dried it was charred in a 180°F oven for 20-40 min until the background began to turn brown.

### Results

LHYa53 (tsc11-1,  $csg2\Delta$ ) was isolated as the single member of the TSC11 complementation group.

Cell viability assay. Growth rates of the tsc11-1, csg2D mutant strain were measured at 26°C and 37°C. Overnight cultures of wild type  $csg2\Delta$ , and tsc11-1, csg2D mutant cells were diluted and incubated at 26°C and 37°C. At hourly intervals the OD<sub>600</sub> was



Figure 19. Tsc11-1, csg2D cell viability assay. (Shaded area represents colony counts)

measured and at 2 hour intervals, aliquots were plated onto YPD medium and incubated at 26°C for colony counts. The results show that the tsc11-1,  $csg2\Delta$  mutant cells remain viable for up to 3 hours after shifting to  $37^{\circ}$  C. The colony counts indicate that an increase in cell number occurs after the initial shift to  $37^{\circ}$  C for up to four hours, but the cell number does not double (Figure 19). This result suggests that mutations in tsc11 may cause cell cycle arrest, however microscopic evaluation of tsc11 mutants after a shift to  $37^{\circ}$ C does not show a uniform cell morphology.

*Identifying TSC11 coding sequence.* LHYa53 (*tsc11-1, csg2* $\Delta$ ) was transformed with a plasmid-based genomic library and complementing plasmids were obtained as outlined in the methods section. The complementing plasmid (p3-4) was hybridized to the ATCC yeast genomic array and found to contain sequence from chromosome V. Restriction enzyme mapping indicated the plasmid insert contained 5 complete open reading frames ranging from YER091C to YER094C.<sup>1</sup> Subclones of the p3-4 complementing plasmid insert indicate that YER093C is required for complementation of *tsc11* (Figure 18). *YER093C disruption/Linkage to tsc11*. To determine if the gene product of YER093C is essential for spore germination the coding sequence was disrupted and integrated into a heterozygous diploid (*csg2* $\Delta$ /*tsc11*). The diploid was sporulated and dissected to evaluate viability of tryptophan prototrophic spores and the segregation of lethality/TRP<sup>+</sup> to the *tsc11* phenotype. Assuming YER093C is alleleic to *TSC11*,

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A small open reading frame adjacent to YER093C is referred to as YER093C(a) accounting for the apparent numeric discrepancy.

integration in the heterozygous diploid could occur at either the wild type allele or at the tsc11-1,  $csg2\Delta$  mutant allele. In the diploid chosen to sporulate, the integration occurred on the wild type TSC11 allele, because all the tetrads (48/48 tested) exhibited a 2:2 lethal phenotype with all viable spores being  $trp1^{-}$  auxotrophs and exhibiting a tsc11-1,  $csg2\Delta$  growth phenotype. This experiment indicates that YER093C is allelic to TSC11because of the lack of crossover between TRP1 and ts and that the gene is essential for spore germination because all disrupted  $(TRP1^{+})$  spores were inviable.

To determine if YER093C is required for vegetative growth, a  $csg2\Delta$  diploid was co-transformed with the linearized YER093C knockout construct and a wild type YER093C on a centromeric plasmid possessing the *URA3* gene. This plasmid covers the requirement for YER093C in spore germination. Transformants were selected on media lacking both tryptophan and uracil indicating that both the disrupting allele of YER093C and the rescuing plasmid were taken up by the strain. The diploids were sporulated and dissected and TRP<sup>+</sup>/URA<sup>+</sup> prototrophic spores were selected. Spore viability was consistent with previous experiments (see above) in that TRP<sup>+</sup> spores (YER093C disruptants) that did not co-inherit the URA<sup>+</sup> YER093C covering plasmid were not recovered. URA<sup>+</sup>/TRP<sup>+</sup> spores were plated on FOA media to test whether they are able to lose the *URA3* containing wild type YER093C covering plasmid. No growth was seen on the FOA plate indicating that the YER093C:::*TRP1* strain cannot grow without the wild type YER093C covering plasmid. These data indicate that YER093C is essential for vegetative growth as well as for spore germination.

Caffeine sensitivity. A previous study to assess the phenotypic effects of Tyl transposon

disruptions of open reading frames throughout the genome of S. cerevisiae indicated that transposition into YER093C resulted in a caffeine sensitive phenotype.<sup>67</sup> Testing of tsc11 indicated that it too showed a sensitivity to caffeine at 6 mM not exhibited by wild type cells (Figure 20). Several other *tsc* strains were tested on varying concentrations of caffeine as a possible secondary phenotype useful in cloning. All strains tested, including wild type were sensitive to 18 mM caffeine and none exhibited sensitivity to 1 mM caffeine. Several tsc strains exhibited sensitivity to 6 mM caffeine. We noted that all of the sensitive strains were *ade2*. Subsequent testing indicated that the caffeine sensitivity is linked to the *ade2* auxotrophy but the biochemical mechanism is not known. It may be that structurally adenine and caffeine are very similar and that caffeine may competitively inhibit transport of exogenous adenine into the *ade2*- cell. The *tsc11* strain was the only strain tested which exhibited caffeine sensitivity in both ade2 and  $ADE2^+$  strains supporting our conclusion that *tsc11* possesses a mutation in YER093C. Long chain base and ceramide analysis. The potential role of TSC11 in sphingolipid synthesis was tested. The tsc11-1,  $csg2\Delta$  mutant is not rescued by exogenous long chain base suggesting that it is not defective in formation of phytosphingosine. The gene coding for ceramide synthase has not yet been identified and falls after the formation of long chain base but prior to the mannosylation step defective in the  $csg2\Delta$  mutant that is suppressed by the tsc11-1,  $csg2\Delta$  mutation. To test if YER093C encodes a ceramide synthase we performed a temperature shift assay and extracted long chain base and ceramides from a tsc11-1,  $csg2\Delta$  mutant. Our hypothesis suggests that if ceramide synthase activity is lost in the tscl1-l,  $csg2\Delta$  mutant there will be an accumulation of

# Caffeine Sensitivity of the *tsc* Mutants, 26 deg C



Figure 20. The effect of caffeine in the media on the growth of *tsc* mutants at 26° C.

long chain base and a depletion of ceramides upon shift to the nonpermissive condition. The results of this analysis indicated no obvious loss of ceramide or accumulation of long chain base after shift to 37° C for up to 5 hours (data not shown). These results do not support Tsc11p being the ceramide synthase but the possibility cannot be ruled out. If cells become static with regard to sphingolipid biosynthesis upon temperature shift the flux in ceramide would not be seen. We do not know what biochemical activity is ongoing at the nonpermissive condition or for how long.

*Homology searches with YER093C sequence*. Protein sequence databases were searched with the YER093C amino acid sequence in an attempt to identify homologous proteins or to identify functional domains within the YER093C sequence. The amino terminus possesses sequence consistent with the presence of a coiled coil domain suggesting that Tsc11p may bind to another protein or form dimers. However, sequence homology that would suggest a function for Tsc11p has not been identified. The gene possess homology to one other nucleotide sequence of unknown function in *Saccharomyces pombe*. Future steps to identify Tsc11p function include isolating suppressors to *tsc11* and identifying them by complementation and pursuing the similar caffeine sensitivity phenotype of other mutants in the *tsc* collection.

### Discussion

The *tsc11* mutant possesses a defect in an uncharacterized open reading frame designated YER093C located on the right arm of chromosome V. It encodes an essential protein of 1433 amino acids with no homology to any known protein or domain.

Exogenous phytosphingosine added to the media fails to rescue the ts phenotype of tsc11 suggesting that the defect is downstream of the formation of long chain base or is in a peripheral pathway which influences sphingolipid biosynthesis. Transposon disruption of YER093C in a previous study indicated a sensitivity to caffeine.<sup>67</sup> Caffeine sensitivity is also seen in tsc15/tor2 a PI kinase responsible for forming phosphatidylinositol-4phosphate by phosphorylating phosphatidylinositol and tsc14/mss4, a PI-4-P 5-kinase responsible for the phosphorylation of phosphatidylinositol-4-phosphate (PI-4-P) to phosphatidylinosisitol-4,5-diphosphate (PI-4,5-P2).68,69,70 The common sensitivity of tsc11, tsc14 and tsc15 to caffeine suggests they may function in the same or related pathways. PIP<sub>2</sub> is the source of IP<sub>3</sub> and diacylglycerol which initiate separate signal transduction pathways by opening intracellular Ca<sup>2+</sup> channels and activating protien kinase C (PKC) respectively.<sup>73</sup> Caffeine sensitivity has been noted previously in yeast mutants that possess defects in the PKC signal transduction pathway.<sup>74</sup> It is tempting to speculate that this pathway may in some way influence the response to  $Ca^{2+}$  by  $csg2\Delta$ mutants.

The role of Tsc11p in sphingolipid metabolism is not yet understood but planned studies including isolation and identification of suppressors to the ts phenotype of *tsc11* and dose-dependent suppressor studies with genes in the phosphatidyl-inositol pathway (*MSS4, TOR2*) as well as by looking for synthetic lethal phenotypes of *tsc11* mutants in combination with mutants in the *MSS4/TOR2* pathway.

## <u>Chapter 8</u> <u>Discussion and Conclusions</u>

The purpose of this research is to identify and characterize genes and proteins that function in sphingolipid homeostasis in a yeast model system. We have taken a genetic approach, isolating mutants defective in sphingolipid synthesis. We then identify a second site, suppressing mutation, which bypasses the initial sphingolipid defect. The genes mutated to bypass the initial defect are in the same or a related pathway and so their identification expands our knowledge of sphingolipid synthesis. The sphingolipid synthesis mutant used in our screen was  $csg2\Delta$ , which fails to mannosylate sphingolipids, thereby accumulating the precursor to mannosylation, inositol phosphorylceramide (IPC-C), which is toxic to yeast grown in the presence of Ca<sup>2+</sup> for an unknown reason.<sup>32</sup> Suppressors of the Ca<sup>2+</sup> sensitivity of the  $csg2\Delta$  mutant possess mutations that decrease synthesis or alter the structure of IPC-C.<sup>19, 32, 35, 50, 75</sup> The  $csg2\Delta$  suppressor mutants isolated in this study exhibit a temperature sensitive conditional lethal phenotype at 37°C and are therefore referred to as *tsc* mutants or **T**emperature §ensitive suppressors of the **C**alcium sensitivity of  $csg2\Delta$  mutants.

Tsc1p (Lcb2p) and Tsc2p (Lcb1p) are subunits of serine palmitoyltransferase (SPT), an essential enzyme which catalyzes the first committed step of sphingolipid biosynthesis in yeast (condensing serine with palmitoyl CoA forming 3ketosphinganine).<sup>31</sup> Tsc3p is also required for SPT activity but its role is not known (see below). When grown at 26°C and assayed at 37°C, membranes isolated from *tsc1*, *tsc2* and *tsc3* mutants exhibit no measurable SPT activity. At 26°C these mutants tolerate Ca<sup>2+</sup> concentrations toxic to  $csg2\Delta$  mutants lacking the defect in SPT. This suppression of the Ca<sup>2+</sup> sensitivity phenotype of  $csg2\Delta$  mutants by mutations in SPT is due to a decreased flux through the sphingolipid biosynthetic pathway leading to decreased IPC-C levels in the cell.<sup>32</sup> The primary structure of the SPT subunits suggest that Lcb2p may be a catalytic domain of SPT while Lcb1p may have evolved into a regulatory domain.<sup>32</sup> One or both of these domains may be activated by Ca<sup>2+</sup> either directly or though a Ca<sup>2+</sup> activated cascade. A mutation in either domain may affect the response to Ca<sup>2+</sup> leading to decreased SPT activity and rescue of the  $csg2\Delta$  mutant.

A role for  $Ca^{2+}$  in the activation of SPT is consistent with the results seen in our laboratory and with known functions of  $Ca^{2+}$  as an intracellular messenger.<sup>76</sup>  $Ca^{2+}$  is an extremely potent intracellular messenger because normally cytosolic concentrations are kept extemely low, in the nanomolar range.<sup>73</sup> The cytosolic  $Ca^{2+}$  concentration can then be abruptly raised by the transient opening of  $Ca^{2+}$  channels in the plasma membrane or in intracellular membranes.<sup>76</sup>  $Ca^{2+}$  can impose dramatic conformational changes to proteins by ionic interaction with oxygen containing amino acid residues.<sup>76</sup> This cross-linking may expose catalytic or binding sites on a  $Ca^{2+}$  binding protein that are not available in the absence of  $Ca^{2+}$ . An example of the effect of increased cytoplasmic  $Ca^{2+}$  is the activation of calmodulin, a  $Ca^{2+}$  binding protein involved in signal transduction. Calmodulin possesses 4 EF hand  $Ca^{2+}$  binding domains. When free cytosolic  $Ca^{2+}$ concentrations increase (100 to 500 nM), an activated  $Ca^{2+}$ -calmodulin complex is formed.  $Ca^{2+}$ -calmodulin stimulates a wide variety of proteins including CaM kinase II which phosphorylates target proteins involved ionic permeability.<sup>76</sup>  $Ca^{2+}$ -calmodulin is also involved in restoring the cytosolic  $Ca^{2+}$  concentrations to basal levels by activating the  $Ca^{2+}$ -ATPase pump.<sup>73</sup>

Ca<sup>2+</sup> upregulation of SPT subunit transcription or activation of the SPT enzyme directly would lead to an accumulation of toxic levels of IPC-C in a  $csg2\Delta$  mutant cell. Levels of transcription of the three genes required for SPT activity in the presence and absence of Ca<sup>2+</sup> were determined using recombinant DNA techniques. No increase in *LCB1, LCB2* or *TSC3* transcription was observed in the presence of Ca<sup>2+</sup>. However, SPT activity has shown a modest 2 fold increase when 0.2  $\mu$ M Ca<sup>2+</sup> is added *in vitro* to a membrane activity assay.<sup>37</sup> The results have yet to be verified but suggest that Ca<sup>2+</sup> has a positive effect on SPT activity and that this effect appears to be post translational.

As previously stated, Tsc3p is also required for SPT activity. However, *TSC3* shares no homology with the *LCB1/LCB2* pyridoxal phosphate family of enzymes that catalyzes the transfer of an acyl group from an acyl CoA to the  $\alpha$ -carbon of an amino acid. Tsc3p possess a hydrophobic domain of 43 amino acids, sufficiently long to span a lipid bilayer twice (Figure 10B). A string of T's in the nucleotide sequence of the hydrophobic domain of Tsc3p is the site of mutation in 4 of the 5 *tsc3* mutant alleles. Long strings of like nucleotides are hotspots for mutations due to the increased probability of the DNA polymerase slipping on the template strand and missing or adding a base to the nascent strand. The result of mutations in the hydrophobic domain of Tsc3p may be a failure to localize properly to the membrane and interact with SPT subunits to assemble the intact SPT enzyme complex. Ongoing co-immunoprecipitation and yeast two hybrid system studies will determine if Tsc3p interacts with SPT subunits.

The tsc4 and tsc5 mutants possess defects in the  $\alpha$ -subunit of fatty acid synthese (FAS2). Fatty acid synthase produces palmitoyl CoA which is required for sphingolipid synthesis in both the formation of LCB and VLCFA. Limiting substrate to either SPT or VLCFA synthesis enzymes (Elo2p and Elo3p) would result in decreased IPC-C synthesis and potentially rescue  $csg2\Delta$  mutants on Ca<sup>2+</sup> containing media. When myristate (C<sub>14</sub>) is added exogenously to tsc4 or tsc5 mutants their ts phenotype is rescued suggesting that this shorter chain fatty acid can substitute for palmitoyl CoA in its essential function at 37°C. Myristate also rescues csg2∆ mutants on Ca<sup>2+</sup> containing media (0.5 M myristate, 1% tergitol, 20 mM Ca<sup>2+</sup> in YPD agar plates). A possible explanation for this finding is that if  $Ca^{2+}$  is increasing SPT activity producing toxic levels of IPC-C in the  $csg2\Delta$ mutant, myristate may be comparably decreasing IPC-C synthesis by acting as a feedback inhibitor to fatty acid synthase and thereby limiting substrate for the formation of LCB and VLCFA. The result is decreased sphingolipid synthesis and IPC-C levels in the cell and rescue of the  $csg2\Delta$  mutant Ca<sup>2+</sup>sensitivity. The combination of myristate and a mutation in SPT (tsc1 or tsc2) is lethal suggesting that together they result in sphingolipid levels dropping below a minimum threshold required for cell viability. These cells are rescued by the addition of Ca2+ to the myristate media supporting the claim that Ca2+ activates sphingolipid synthesis (Figure 6).

TSC7 was found to be allelic to SUR2 which codes for a long chain base hydroxylase required for the formation of phytosphingosine from dihydrosphingosine. The *tsc7* mutant offered a unique and interesting phenotype as a result of a combination of genotypes. Previous study of SUR2 in our laboratory had identified its role in

sphingolipid biosynthesis and a disruption construct, sur2::TRP1 was known to suppress the Ca<sup>2+</sup> sensitivity phenotype of  $csg2\Delta$  but it did not confer a temperature sensitive phenotype as seen in tsc7.<sup>19</sup> During the course of our investigation of tsc7 mutants we discovered that the ts phenotype was only exhibited in a tryptophan auxotroph on media where tryptophan was limiting. A previous study had determined that the high affinity tryptophan transporter encoded by TAT2 is sensitive to exogenous phytosphingosine.<sup>77</sup> The kinetics of inhibition indicate that phytosphingosine may down-regulate Tat2p activity by way of a signal transduction pathway.<sup>78</sup> Involvement of a tryptophan transporter in the ts phenotype of *tsc7* is consistent with the rescue of *tsc7* by either adding tryptophan to the media or providing the cell with a wild type copy of the TRP1 gene (Figure 17). Increasing the concentration of tryptophan in the media either compensates for a defective Tat2p transporter or provides a low affinity transporter sufficient tryptophan to satisfy the cells needs. Providing the TRP1 gene bypasses the need for transport by providing the means for the cell to synthesize tryptophan endogenously. The data supports a role for sphingolipids in the transport of tryptophan across the plasma membrane and indicates that mutations in sur2 are synthetically lethal in combination with  $csg2\Delta$  and trp1 mutations at 37°C. Characterization of the sur2mutant supports the increasing scientific data implicating sphingolipids in multiple cellular functions that are complex and intertwined. Failing to hydroxylate the long chain base moiety of sphingolipids impacts both the response of a  $csg2\Delta$  mutant to Ca<sup>2+</sup> and the ability of the cell to transport tryptophan when concentrations of the amino acid are limiting at 37°C.

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The TSC11 gene encodes an essential protein with unknown function. Recent clues as to the function of Tsc11p stem from its sensitivity to caffeine. Caffeine is a purine analog that affects many cellular processes. Caffeine sensitivity has been associated with defects in MAP kinase signal transduction pathways and inhibits mammalian cAMP phosphodiesterase.<sup>74</sup> We have isolated caffeine sensitive suppressors of the Ca<sup>2+</sup> sensitivity of  $csg2\Delta$  which possess defects in the

phosphatidylinositoldiphosphate (PIP<sub>2</sub>) synthesis pathway (*mss4/tsc15*, *tor2/tsc14*) (unpublished observations). This is especially interesting because caffeine sensitivity has been associated with mutations in the protein kinase-C (PKC) MAP kinase pathway in yeast.<sup>74</sup> PKC is activated by diacylglycerol (DAG) which is a product of the breakdown of PIP<sub>2</sub> by phospholipase C.<sup>73</sup> Once activated, PKC phosphorylates target proteins that result in upregulation of transcription of specific genes.<sup>73</sup> The breakdown of PIP<sub>2</sub> also yields inositol triphosphate (IP<sub>3</sub>) which releases Ca<sup>2+</sup> from internal stores by opening Ca<sup>2+</sup> channels.<sup>76</sup> In yeast IP<sub>3</sub> releases Ca<sup>2+</sup> from the vacuole.<sup>81</sup> Ca<sup>2+</sup>, as described earlier, is a potent intracellular secondary messenger. Mutations resulting in decreased PIP<sub>2</sub> substrate for phospholipase C would make available less IP<sub>3</sub> and DAG to activate Ca<sup>2+</sup> and PKC dependent pathways. Reduced IP<sub>3</sub> release of Ca<sup>2+</sup> from internal stores does not explain the increased tolerance of *csg2*Δ mutants to exogenous Ca<sup>2+</sup> in our suppressor mutants. Perhaps a member of the PKC pathway is the Ca<sup>2+</sup> target and reduced DAG formation is cause of the lack of a Ca<sup>2+</sup> response in a *csg2*Δ background.

The genetic approach taken to the study of sphingolipid homeostasis in the yeast Saccharomyces cerevisiae, has led to the isolation of the *tsc* mutants. Identification of the genes mutated in the *tsc* mutant collection has added greatly to our knowledge of sphingolipid synthesis. Our preliminary studies suggest that sphingolipids are involved in multiple cell processes and may be most instrumental in maintaining the integrity of membranes and integral membrane proteins. The role of Ca<sup>2+</sup> has yet to be completely explained but experimental evidence suggests that Ca<sup>2+</sup> may act as an upregulator of sphingolipid biosynthesis by activating the catalytic activity or the transcription of SPT subunits and that the response to Ca<sup>2+</sup> may be the key to suppression in the PIP<sub>2</sub> synthesis mutants (*tsc14*, *tsc15*) and in *tsc11*. We have also learned that there is tolerance range of IPC-C levels in the *csg2* $\Delta$  mutant. In the presence of Ca<sup>2+</sup> the tolerance level is exceeded but when sphingolipid synthesis capability is eliminated or dramatically reduced as seen in the combination of an SPT mutation and fatty acid synthase down regulation, the cells die. Therefore, the *tsc* mutant collection must include exquisite mutations which work in tandem with the parental *csg2* $\Delta$  defect to maintain sphingolipid levels within this tolerable range.

Because of work done in the isolation, identification, and characterization of the *tsc* mutant collection the biosynthetic pathway of sphingolipids in yeast is now more completely understood and a collection of conditional lethal mutants possessing defects at points throughout the biosynthetic pathway is available for future study.

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