Metabolism and Roles of Sphingolipids in Yeast *Saccharomyces cerevisiae*

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Abstract

Sphingolipids are major membrane components of all eukaryotic cells. They are also important bioactive molecules involved in a plethora of essential cellular processes that are implicated in various human diseases. Most bioactive sphingolipids also serve as intermediate products of the sphingolipid metabolic network. It is thus critical to understand sphingolipid metabolic pathways in order to dissect sphingolipid function at single-species level. Here, we review in detail the biosynthetic and degradation pathways of sphingolipids in the yeast *Saccharomyces cerevisiae*, a model organism whose sphingolipid composition is rather simple but with conserved metabolic pathways and regulation mechanisms as higher eukaryotes. The functions of yeast sphingoid bases, ceramides,

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and complex sphingolipids are also discussed. Together, this knowledge provides a foundation to understand sphingolipid metabolism, their physiological roles, their potential as therapeutic targets for human diseases, and the major challenges the field is facing.

1 Introduction

Sphingolipids were first discovered as the major component of brain extract in 1865, and their chemical composition was revealed decades later (Carter et al. 1947; Kanfer and Hakomori 1983). Until the mid-1980s, sphingolipids had been regarded as mere structural components of eukaryotic cell membranes. The discovery of sphingosine as a specific inhibitor of protein kinase C suggested the possible role of sphingolipids as second messengers and as bioactive molecules (Hannun et al. 1986; Merrill et al. 1986; Wilson et al. 1986). These studies marked the start of a new era where the investigation of the physiological relevance of sphingolipid metabolism in cell took the center stage. Sphingolipids are now appreciated as important bioactive molecules involved in a plethora of essential biological processes such as cell growth, apoptosis, differentiation, migration, angiogenesis, and inflammation (Hannun and Obeid 2008).

In addition to the sphingolipidoses, inherited disorders caused by defective sphingolipid degradation in the lysosome, altered sphingolipid metabolism is also associated with a variety of other prevalent human diseases including type 2 diabetes, cancer, inflammation, and neurological disorders such as Alzheimer's disease and hereditary sensory and autonomic neuropathy type I (HSAN1) (Bejaoui et al. 2002; Kolter and Sandhoff 2006; Pralhada Rao et al. 2013).

Despite the rapid progress in deciphering the role of sphingolipids at both the cellular and organism levels, major challenges still exist. One of these challenges relates to the fact that individual bioactive sphingolipids are connected by complex metabolic pathways, and it is difficult to alter one species without affecting the level of its precursors or downstream products, which makes it hard to pinpoint the function of any specific species. This problem is further compounded by the compartmentalization of sphingolipid metabolism such that there are recognized complements of sphingolipid enzymes that reside in each of the major subcellular compartments (nucleus, endoplasmic reticulum (ER), mitochondria, Golgi, lysosomes, plasma membrane, and extracellularly). Understanding sphingolipids is complicated even further by the diverse classes of sphingolipid derivatives. The blueprint of the structure of sphingolipids is fairly simple (Fig. 1). It is composed of three moieties: a long-chain base (LCB) containing hydroxyl and amino groups, a long-acyl-chain amide linked to the LCB amino group, and a head group attached to the hydroxyl group at C1 position of the LCB. It is the modifications to this basic structure that give rises to diverse species with variations in head groups, acyl-chain length, position and number of hydroxyl groups, and degree of unsaturation of both the LCB and the acyl-chain. Sphingolipids contain several chiral centers, and among all the possible stereoisomers, natural sphingolipids only occur in certain configuration

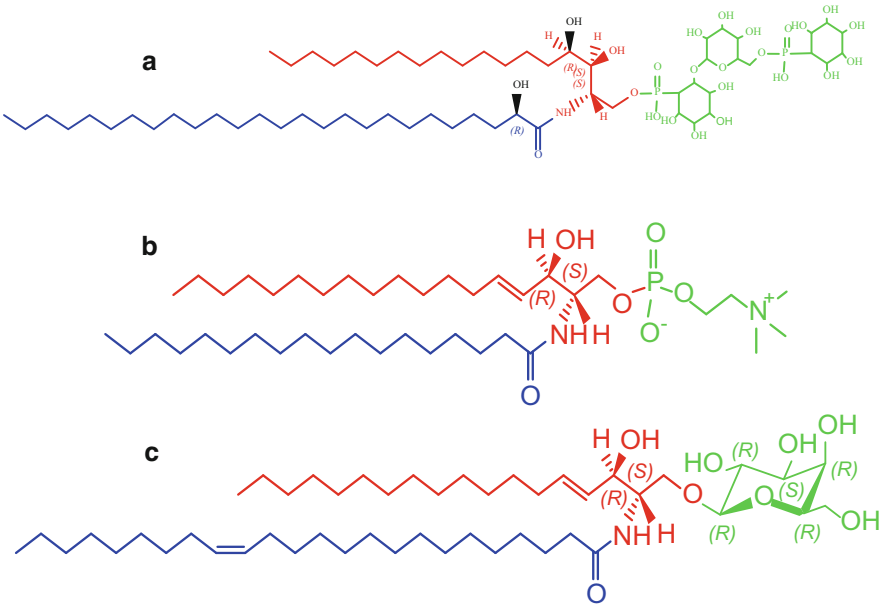

Fig. 1 Sphingolipid structure. The long chain base (*LCB*) backbone is shown in *red*. Acylation of LCB forms ceramide; the amide-linked acyl chain to the LCB amino group is shown in *blue*. The addition of head group to ceramide forms complex sphingolipids; the head group attached to the C1 hydroxyl group is shown in *green*. Hydroxyl groups that decorate both the LCB backbone and the acyl chain are shown in *black*. Examples of different sphingolipids are shown in (**a**) *S. cerevisiae* α-OH-C26-M(IP)2C (Mannose-(inositol-P)$_2$-ceramide); (**b**) structure of porcine brain sphingomyelin and (**c**) C24:1 Galactosyl (β) ceramide

as shown in Fig. 1. Unique biological properties have been found to associate with each stereoisomer.

The sphingolipid composition of yeast *Saccharomyces cerevisiae* is much simpler than that of humans and thus serves as an excellent model to study sphingolipid metabolism and their cellular roles. The unicellular nature, fast growth cycle, and ease of genetic manipulations make the yeast *S. cerevisiae* a powerful working model in investigating sphingolipid metabolism and function. Indeed, many of the enzymes in sphingolipid metabolic pathway were first discovered in yeast followed by the cloning of their counterparts in other fungi, plant, and animal kingdoms. Studies in *S. cerevisiae* have not only helped elucidate the function of individual genes in the sphingolipid metabolic pathways but have also pioneered in the use of systems biology in dissecting specific sphingolipid functions (Cowart et al. 2010b; Montefusco et al. 2013). The goal of this review is to provide students and researchers who are interested in the sphingolipid field with an introduction to the major sphingolipid metabolic pathways in *Saccharomyces cerevisiae*, their functions, and their potential connections to higher organisms.

2 Sphingolipid Metabolism in Yeast

2.1 Sphingolipid Biosynthesis

De novo sphingolipid synthesis begins at the cytosolic leaflet of the ER where serine palmitoyl transferase (SPT) catalyzes the first and rate-limiting step of condensation of serine with palmitoyl-CoA to produce 3-ketodihydrosphingosine (Dickson and Lester 1999) (Fig. 2). Yeast SPT belongs to a subfamily of pyridoxal 5′-phosphate enzymes known as α-oxoamine synthases, and it is composed of the catalytic subunit – a heterodimer of two homologous subunits Lcb1 and Lcb2 – and a small hydrophobic subunit, Tsc3, which is required for optimal enzyme activity (Gable et al. 2000; Nagiec et al. 1994). Homologues of LCB1/2 are found in all organisms that synthesize sphingolipids including mammals (Hanada et al. 1997). Mutations in the gene encoding the human SPT Lcb1b subunit (SPTLC1) are associated with HSAN1 (Bejaoui et al. 2002). In these cases, the mutant SPT shows substrate preference for alanine and glycine over serine, resulting in the formation of alternative (noncanonical) sphingolipids, and this altered amino acid selectivity of SPT is proposed to cause the neuropathy (Gable et al. 2010; Penno et al. 2010). Two low molecular weight proteins, ssSPTa and ssSPTb, have been identified in mammalian cells. Although they share no sequence homology to Tsc3, they are regarded as its functional homologue since they interact with hLCB1/hLCB2 and stimulate SPT activity (Han et al. 2009).

Yeast studies have also disclosed additional layers of regulation of sphingolipid synthesis through modulating SPT activity. Orm1/2 are homologous ER proteins functioning as negative regulators of yeast SPT, which were purified in the same SPT complex (Han et al. 2010). The inhibitory effect of Orm1/2 on SPT activity correlates with their phosphorylation/dephosphorylation status, which is controlled by Pkh-Ypk and Cdc55-PP2A pathways (Roelants et al. 2011; Sun et al. 2012). Pkh1/2 can be activated by TORC2 (target of rapamycin signaling complex 2) in an Slm1-/2-dependent manner (Berchtold et al. 2012). Slm1/2 are homologous plasma membrane (PM) proteins that bind both PI(4,5)P2 and TORC2 (Tabuchi et al. 2006). Slm1/2 was proposed to facilitate Pkh1 phosphorylation by TORC2 through recruiting Pkh1 to PM (Niles et al. 2012; Niles and Powers 2012). SPT activity can also be upregulated through inhibition of TORC1 (target of rapamycin signaling complex 1) which leads to Orm1 phosphorylation by Npr1 (Shimobayashi et al. 2013). ORM1/2 are conserved in humans (ORMDL1/2/3), and their human homologues show the same interaction and inhibitory effect on human SPT. Their inhibitory effect on SPT is also controlled by their phosphorylation status. The study of ORMs is of significant clinical interest since polymorphism of ORMDL3 is associated with childhood asthma (Breslow et al. 2010). Yeast cells with lack of Orm1/2 show increased unfolded protein response, and this defect can be corrected by inhibition of sphingolipid biosynthesis, suggesting the role of sphingolipids in protein quality control in the ER (Han et al. 2010).

After the initial condensation reaction catalyzed by SPT, 3-ketodihydrosphingosine is reduced to dihydrosphingosine (DHS or sphinganine) by the enzyme 3-ketodihydrosphingosine reductase, Tsc10 (Beeler et al. 1998). Like LCB1/2, TSC10 is an essential

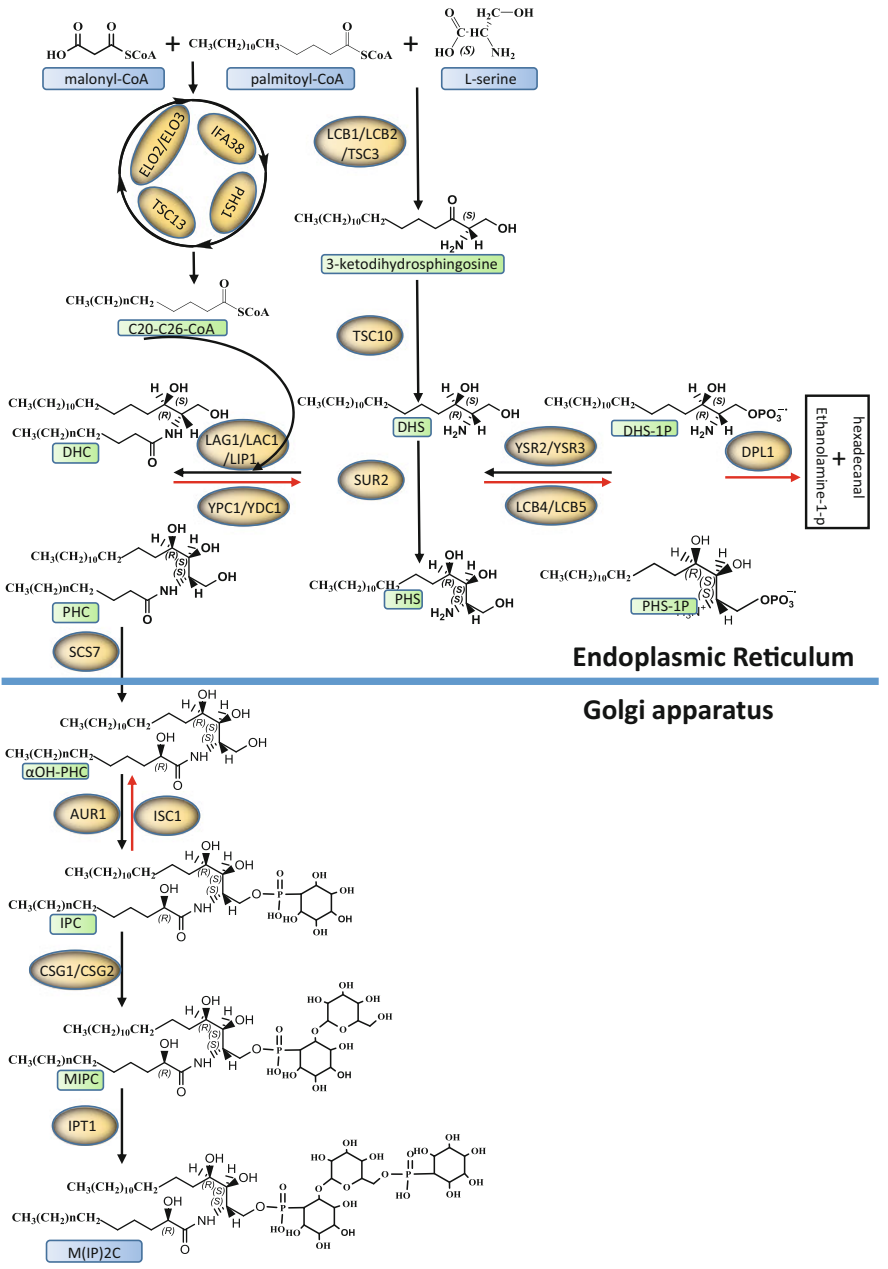

Fig. 2 Overview of yeast sphingolipid metabolism. The initial substrates and final products of the sphingolipid biosynthetic pathway are highlighted with *blue rectangles*. The intermediate products are highlighted in *green rectangles*. Genes catalyzing each step are indicated in *gold capsules*. *Black arrows* show the steps in the biosynthetic pathway and the *red arrows* points to the reactions that lead to sphingolipid catabolism

gene, and its deletion causes cell death unless the growth medium is supplemented with dihydrosphingosine or phytosphingosine. TSC10 homologues in both human (hFVT-1) and mouse (mFVT-1) have been cloned and can functionally substitute for yeast TSC10 (Kihara and Igarashi 2004). A point mutation in bovine 3-ketodihydrosphingosine reductase, *FVT1*, was proposed as the candidate causal mutation for bovine spinal muscular atrophy (Krebs et al. 2007).

The C4 position of DHS can be hydroxylated by yeast hydroxylase, Sur2p, to form phytosphingosine (PHS) (Grilley et al. 1998). SUR2 is not an essential gene, and both DHS and PHS exist in yeast although the functional difference between the two has yet to be defined. However, some studies suggest that PHS is the more biologically active LCBs in yeast and have reported that PHS shows specific inhibition of yeast growth and nutrient uptake (Chung et al. 2001).

The next step in yeast sphingolipid synthesis is the *N*-acylation of LCBs to form dihydro- and phytoceramide. The reaction is catalyzed by Lag1 and Lac1, two apparently redundant homologues that comprise the major ceramide synthase (or also called sphingoid base *N*-acyl transferase) activity (Guillas et al. 2001; Schorling et al. 2001). Homologous genes of LAG1/LAC1 have been identified in almost all eukaryotes. LASSs/CerSs are the mammalian homologue of LAG1/LAC1, and they are *bona fide* ceramide synthases (Levy and Futerman 2010). Six members have been identified in the CerS/LASS family, and they are distinguished from each other by their preference to acyl-chains of different length. A third subunit, Lip1, is found to co-purify with Lag1p/Lac1 in yeast and is required for the yeast ceramide synthase activity (Vallee and Riezman 2005); however no mammalian homologue of Lip1 has been identified, and overexpression of Lip1 cannot regulate ceramide synthesis in mammals (Levy and Futerman 2010). Production of ceramide shares similar regulatory mechanism as LCB biosynthesis. Ypk1 was mentioned before to promote LCB production by alleviating negative regulation of SPT by Orm1/2. The activity of Lag1/Lac1 is also regulated by TORC2-dependent protein kinase Ypk1 phosphorylation and dephosphorylation by Ca2+/calmodulin-dependent protein phosphatase (PP2B) (Muir et al. 2014). Both Slm1/2, PH domain-containing proteins, and TORC2 subunit Avo1 have been suggested to recruit Ypk1/2 to TORC2 complex upon stress response (Berchtold et al. 2012; Liao and Chen 2012; Niles et al. 2012).

Notably, while ceramide in mammalian cells shows great diversity due to acyl-chains of different length linked to the amino group of LCB backbone as well as variations in hydroxylation and unsaturation, there is less diversity of ceramide species in yeast. One of the distinguishing features of yeast ceramides is the preponderant incorporation of very long-chain fatty acids (VLCFAs) into ceramide, such that yeast sphingolipids contain a significant amount of VLCFA of 26 carbons. The elongation from long-chain fatty acid (LCFA) of C14–C18 acyl-CoA to VLCFA of C22–C26 is catalyzed by the VLCFA synthase complex consisting of fatty acid elongase Elo2/Elo3(Oh et al. 1997), beta-keto reductase Ifa38p (Han et al. 2002), 3-hydroxyacyl-CoA dehydratase Phs1p (Denic and Weissman 2007; Schuldiner et al. 2005), and enoyl-reductase Tsc13p (Kohlwein et al. 2001). In this reaction, two carbons are added to the starting acyl-CoA after each cycle of elongation. In

contrast, in mammalian cells there are seven elongases, and ELOVL1-7 and VLCFA are incorporated into both sphingolipids and glycerophospholipids (Kihara 2012). Another unique feature of yeast sphingolipids is the abundance of hydroxylated products. Other than the Sur2p-catalyzed formation of C4-hydroxyl on the sphingoid base of phytoceramide, the C2 position of the fatty acid chain can be hydroxylated by the yeast hydroxylase Scs7p to form α-hydroxy- dihydro-(phyto)ceramide (Dale Haak 1997). Mammalian homologues of these hydroxylases do exist, and hydroxylated sphingolipids are also detected especially in the nervous system, although at present their function is largely unknown (Hama 2010). Finally, in addition to the hydroxyl status, the other major difference between yeast and mammalian ceramide is the formation of a double bond between sphingoid base carbon C4 and C5 by human DEGS1 desaturase. In contrast, no dihydroceramide desaturase has been identified in *S. cerevisiae.*

Once dihydro-/phytoceramides are synthesized, they are transported from the ER to the Golgi network where they can be further processed to form complex sphingolipids. This is mediated through vesicular membrane transport as well as in a non-vesicular way through direct contact between ER and Golgi networks in yeast (Funato and Riezman 2001). In mammalian cells ceramide can also be transported by ceramide transfer (CERT) protein (Hanada et al. 2003; Kumagai et al. 2005). Complex sphingolipids are formed from ceramide through modification of the 1-OH head group of the sphingoid base. In mammals this head group can be modified with either sugar residues to form glycosphingolipids or with phosphocholine to form sphingomyelin. In contrast, the final sphingolipid product in yeast is mannose-(inositol-P)$_2$-ceramide (M(IP)2C), which is formed in three steps involving four enzymes. These genes do not exist in mammals and thus present great potential as antifungal drug targets. Aur1p catalyzes the formation of inositol phosphoryl-ceramide (IPC) by transferring the phosphoinositol head group from phosphatidylinositol to dihydro-/phytoceramides (Nagiec et al. 1997). Notably, the by-product of this reaction is DAG, itself an important lipid signaling molecular that functions at the interface of glycerophospholipid metabolism and membrane trafficking. Phosphatidylinositol, the donor of the head group, is also a key signaling molecule. IPC is further mannosylated to mannose-inositol-phosphoceramide (MIPC) by the MIPC synthase complex containing the catalytic subunit, Csg1p, and the regulatory subunit, Csg2p (Beeler et al. 1997). Lastly M(IP)$_2$C is formed by the addition of a second phosphoinositol moiety by inositolphospho transferase, Ipt1p(Dickson et al. 1997b). Once synthesized, M(IP)2C is then transported to the plasma membrane, and it is the most abundant sphingolipid in yeast.

2.2 Sphingolipid Breakdown

The de novo synthesis of yeast sphingolipids starts in the ER by the condensation of serine and palmitoyl-CoA and ends at the plasma membrane where M(IP)2C predominantly resides. The multi-step biosynthesis pathway also generates important intermediate products such as LCBs and ceramides that have their own functions

in the cell. In reverse of the biosynthetic pathway, complex sphingolipids can be broken down to ultimately generate non-sphingolipid catabolites: fatty aldehydes and ethanolamine phosphate. These reactions are highlighted by red arrows in Fig. 2.

The first enzyme involved in sphingolipid breakdown is ISC1, which encodes a phospholipase C type activity that cleaves the polar head groups from yeast sphingolipids (Sawai et al. 2000). Isc1p is able to hydrolyze all three of the complex sphingolipids IPC, MIPC, and M(IP)2C back into dihydro-/phytoceramides, and it is the only enzyme known thus far in yeast that can do this. Isc1 normally resides in the ER but is predominantly located in mitochondria during the diauxic shift when cells change from fermentative to respiratory growth (Vaena de Avalos et al. 2004).The mammalian homologues of Isc1 are nSMase1 and nSMase2 (neutral sphingomyelinase), which catalyze the breakdown of sphingomyelin into ceramide. The role of nSMase1 in mammalian sphingomyelin metabolism however is questionable as its preferred substrates are lysoPAF and lysoPC. nSMase2 is activated by a host of extracellular signals to produce ceramide which functions as bioactive regulatory molecule in processes like inflammation signaling and apoptosis (Chatterjee 1999). nSMase2 thus plays important roles in human pathologies such as cancer metastasis and atherosclerosis (Hwang et al. 2015). Mammalian systems also have acid sphingomyelinase, and mutations in human acid sphingomyelinase, SMPD1, cause type A and type B Niemann-Pick disease (Schuchman 2010).

Yeast ceramides are in turn broken down to LCBs by Ypc1p or Ydc1p. Ypc1p preferentially hydrolyzes phytoceramide to generate phytosphingosine, whereas Ydc1p shows preference for dihydroceramide to generate dihydrosphingosine (Mao et al. 2000a, b). Significant ceramide synthase activity has also been detected for Ypc1 but no Ydc1. Ypc1 and Ydc1 also show functional differences with the deletion of only YDC1 being able to reduce yeast thermotolerance. Biochemically, both Ypc1 and Ydc1 function optimally at alkaline pH, and their mammalian homologues, alkaline ceramidases (ACERS 1–3), have been cloned and studied. They demonstrate similar functions to break down mammalian ceramides into LCBs (Mao et al. 2001). A mutation in ACER3 has been identified in patients with leukodystrophy (Edvardson et al. 2016). The loss of ACER3 activity and the accompanied abnormal accumulation of sphingolipids were proposed to be the underlining mechanism behind a certain form of leukodystrophy. Neutral and acid ceramidase are also present in mammalian cells, with mutations in human acid ceramidase associated with lipid storage disorder, Farber disease (Park and Schuchman 2006).

Yeast LCBs can be phosphorylated at the 1-OH group to form LCB phosphates by cytosolic LCB kinases Lcb4/5, products of two paralogs that arose from whole-genome duplication (Nagiec et al. 1998). The LCB4/5 are not essential genes in yeast, but their mammalian counterparts, SK1 and SK2, are key regulators of cell proliferation and angiogenesis through generation of sphingosine-1-phosphate (Hla et al. 2001). Mammalian sphingosine-1-phosphate exerts its extracellular signaling roles by binding to the G-protein-coupled Edg receptor family (S1PR 1–5). Intracellular targets of sphingosine-1-phosphate have also been suggested (Strub et al. 2010). There is limited information on the function of dihydro-/phyto-sphingosine-

1-phosphate in yeast other than its possible role in regulating genes required for mitochondrial respiration through the HAP complex transcription factor (Cowart et al. 2010b). Also no receptors of LCB phosphate have been discovered in yeast. LCB phosphate in yeast can be dephosphorylated by phosphatases encoded by YSR2 and YSR3 (Mao et al. 1997; Qie et al. 1997). Mammalian sphingosine-1-phosphate phosphatases (SPPases) are the functional homologues of yeast Ysr2p (Mandala et al. 2000). The phosphatase controls the balance between LCB and LCB-1-phosphate.

Dihydro-/phyto-sphingosine-1-phosphate can be cleaved by sphingosine-1-phosphate lyase, Dpl1p, to generate hexadecanal/hydroxy-hexadecanal and ethanolamine-1-phosphate, which are the end products of the sphingolipid catabolism pathway (Percy et al. 1984). This is an irreversible process and the only exit point from the sphingolipid metabolic network. The end products can be reused by the cell through various metabolic pathways to synthesize complex metabolites including phosphatidylethanolamine (Saba et al. 1997). Misregulation of mammalian sphingosine-1-P lyase, SPL, has been identified in metastatic tumor tissues including colon and ovarian cancer specimen of human patients (Fyrst and Saba 2008; Ikeda et al. 2004).

3 Functions of Yeast Sphingolipids

Sphingolipids, along with glycerophospholipids and cholesterol, are major components of cell membranes. Beyond a structural role, sphingolipids have specific functions in several fundamental cellular processes that in turn are integral players in human diseases such as cancer, diabetes, inflammation, and neural degenerate diseases. The bioactive sphingolipids include the end products of the sphingolipid biosynthetic pathway, the complex sphingolipids, as well as major intermediate products such as ceramide, LCBs, and their phosphorylated derivatives. Ceramides and sphingosine-1-P are the best known and studied bioactive sphingolipids, with ceramide largely thought to function predominantly as an antiproliferative signal, while sphingosine-1-P acts as a pro-growth signal (Hannun and Obeid 2008). The signaling pathways mediated through ceramide were first demonstrated in mammalian cells and later proved to be conserved in the yeast *S. cerevisiae*. Both complex sphingolipids and LCBs in yeast have been implicated in specific functions which will be described below. In contrast, no DHS-1P or PHS-1-P receptors have been identified, and there is so far limited information about the definitive functions of these lipids in yeast.

3.1 Function of Yeast Ceramides

The establishment of ceramide as a bioactive molecule in mammalian cells stemmed from the following observations: (1) activation of various cell types such as HL-60 leukemic cells and MCF7 breast cancer cells by a number of extracellular reagents

such as Vitamin D3, TNFα, and α-interferon results in early accumulation of ceramide; (2) exogenously added ceramide analogs specifically induce similar cell responses to those caused by ligands (Dressler et al. 1992; Hannun et al. 1993; Kim et al. 1991; Kolesnick 1991; Obeid et al. 1993); (3) moreover, interfering with ceramide formation often obliterates or diminishes the respective action of those extracellular agents. Subsequent to these observations, considerable research has identified ceramide-mediated cell responses including the cell cycle arrest, induction of differentiation, and initiation of apoptosis (Hannun 1994). The immediate ceramide effectors are proposed to include both the ceramide-activated protein kinases (CAPK) and ceramide-activated protein phosphatase (CAPP). The presumptive CAPKs include JNK, PKCζ, and C-Raf, and the CAPPs include PP2A and PP1 (Hannun and Obeid 2008; Huwiler et al. 1996; Kolesnick and Golde 1994; Kolesnick 1991; Liu et al. 1994). In addition, ceramide has been shown to activate the protease cathepsin D. Most evidence favors a key role for CAPPs in mediating the actions of ceramides on a number of targets including Akt, PKC, ß-catenin, Bcl2, and many others. Yet more questions remain including the detailed mechanisms such as ceramide production upon specific stimuli, how ceramide interacts with its immediate downstream effectors, and what is the nature of the signaling pathways downstream of ceramide effectors. The yeast *S. cerevisiae* is a useful model system to study the mechanism of ceramide function for three major reasons. Firstly, the generation of ceramide through both the biosynthetic pathway and the hydrolytic pathway from complex sphingolipid degradation is conserved between yeast and mammalian systems. Secondly, various ceramide effectors, including both CAPK and CAPP, have been found in yeast. Finally, many of the major signaling pathways related to ceramide functions are conserved between yeast and mammalian systems.

The role of ceramide in yeast was first explored by adding C2-ceramide, a cell-permeable analogue of ceramide to the yeast culture where it was found to inhibit yeast cell growth. Importantly, a ceramide-dependent serine/threonine phosphatase activity in the yeast lysate was demonstrated and found to be required for the growth inhibition (Dobrowsky and Hannun 1993; Fishbein et al. 1993). Later it was shown that exogenously added ceramide induces a G1 arrest in yeast, and this effect was mediated by Sit4, a yeast phosphatase belonging to the PP2A family (Nickels and Broach 1996). This PP2A phosphatase complex is composed of one catalytic subunit, Sit4, and two regulatory subunits, Cdc55 and Tpd3. Notably, the inhibitory effect of ceramide on yeast cell growth was greatly reduced in mutants of any subunit suggesting that Sit4 is a direct downstream target of ceramide in mediating its inhibitory effect on cell growth.

Additionally, the observation that activation of the RAS pathway is synthetically lethal with reduced Sit4 activity suggests the possible antagonism of ceramide and RAS pathway. Yeast pheromone-induced cell cycle arrest at G1/S is also mediated through ceramide, which induces MAP kinase transcription (Villasmil et al. 2016).

Ceramide has also been shown to associate with the yeast genotoxic response. Yeast mutants defective in ceramide synthesis (*lag1Δlac1Δ*) or salvage (*isc1Δ*) show sensitivity to DNA-damaging reagents methyl methane sulfonate (MMS) and

hydroxyurea (HU). This sensitivity reveals a new role of yeast ceramide in G2/M regulation through its effect on Swe1 and the G2/M checkpoint cyclin-dependent kinase Cdc28. As with the G1 arrest above, this process also depends on the PP2A phosphatase Sit4. Importantly, C18:1-phytoceramide was subsequently identified as the specific ceramide species that mediates HU resistance in yeast (Matmati et al. 2009, 2013). The existence of a sphingolipid-PP2A-Swe1-Cdc28 pathway is also supported by analysis of cell cycle delay in tgl3Δtgl4Δ, a mutant defective in yeast neutral lipid lipolysis. Supplementation of PHS rescues the cell cycle delay in tgl3Δtgl4Δ mutant through Swe1 phosphorylation of Cdc28 in a PP2A- and Cdc55-dependent manner (Chauhan et al. 2015).

Yeast ceramides are also important regulators of mitochondria function as supported by the following evidence: (1) the change in location of ISC1 from ER to mitochondria during the switch from fermentation to respiration at diauxic shift (Kitagaki et al. 2007; Vaena de Avalos et al. 2004); (2) yeast lacking ISC1 demonstrates a failure of growth at diauxic shift, oxidative stress, shortened chronical life span, and impaired mitochondrial dynamics (Almeida et al. 2008; Kitagaki et al. 2009); and (3) these phenotypes are mediated by ceramide signaling through Sit4 and Hog1, known as ceramide-responsive phosphatases and MAP kinase (Teixeira et al. 2015).

Ceramide is also required for stable membrane association of glycosylphosphatidylinositol (GPI)-anchored proteins to the ER and their transport from the ER to Golgi (Watanabe et al. 2002). This was demonstrated by the defective ER-to-Golgi transport of a GPI-anchored protein, Gap1, in lcb1–100, a yeast mutant with impaired sphingolipid biosynthesis. This transport defect can be rescued by exogenous PHS but not in the presence of ceramide synthesis inhibitor australifungin. It is possible that the transport defect is due to defective GPI anchor lipid remodeling process, by which the lipid moiety of the GPI anchor is replaced with ceramide, caused by the lack of ceramide in the mutant (Bosson et al. 2009).

Despite the significant progress that has been made, major challenges still exist in determining specific signaling roles of distinct ceramide species. These challenges emanate from the following factors: (1) ceramide comprises actually a class of related molecules with variations in the acyl-chain length, degree of unsaturation, and different hydroxylation modifications. The mechanisms that regulate the generation of each subspecies are not clear yet and thus impede to study their functions through manipulation of individual enzymes. (2) Most of the ceramide-dependent functions so far are delineated from manipulation of individual enzymes of ceramide metabolism with the assumption that the functional changes are caused by the direct substrate or product of that enzyme. Sphingolipid metabolism is in fact composed of a highly connected network, and the manipulation of one enzyme is associated with a broad change of sphingolipid metabolites, and their contribution to the functional change caused by disturbing individual enzyme is mostly neglected. This is an area where yeast genetics has been of great value. A few studies have actually aimed at "triangulating" the actual lipid mediator by performing studies in deletion mutants in the various enzymes of ceramide metabolism. For example, such an approach has allowed the implication of ceramide and not the other sphingolipids in the action of

HU. (3) Notwithstanding, only limited functions have been identified to be associated with individual ceramides from studies testing the role of individual genes in the sphingolipid metabolism pathway. Montefusco et al. proposed a systematic approach that integrates measuring lipidomics and transcriptomic response to systematically perturbed ceramide metabolism (Montefusco et al. 2013). The study revealed new roles of ceramide such as the association between long-chain dihydroceramides and ion metabolism and very long-chain dihydroceramide with oxidative stress response.

3.2 Function of Complex Sphingolipids

Complex sphingolipids in yeast include IPC, MIPC, and $M(IP)_2C$. They are the major components of the plasma membrane where they constitute approximately 30% of the total phospholipid content (Patton and Lester 1991). Surprisingly, however, none of the yeast complex sphingolipids have been found to be essential for cell survival as is demonstrated by the normal growth of csg1 and csg2 deletion mutants that do not synthesize MIPC or its downstream product $M(IP)_2C$. Indeed, while treatment of yeast with aureobasidin A, an inhibitor of the IPC synthase Aur1p, is able to cause cell death, this lethality is reversed by the deletion of both LAG1 and LAC1. This suggests that it is the accumulation of ceramide rather than lack of complex sphingolipids that caused cell death after aureobasidin A treatment (Schorling et al. 2001).

The functions of yeast complex sphingolipids have also been investigated using a yeast strain in which AUR1 was deleted (Epstein et al. 2012). The lethality caused by ceramide accumulation is this strain is overcome by replacing the endogenous yeast ceramide synthases, Lag1p and Lac1p, with cotton CerS, GhLag1. The strain produces predominately C18 instead of the presumably toxic C26 sphingolipids. The lack of complex sphingolipids in this strain causes defects in cytokinesis as well as accumulation of lipid droplets. However, the underlying mechanisms by which complex sphingolipids regulate these processes have yet to be defined.

Finally, accumulation of IPC is associated with regulation of cellular Ca^{2+} homeostasis. This is demonstrated by the failure of cgs1/cgs2 mutants to grow in medium with a high Ca^{2+} concentration and the identification of suppressor mutants that can reverse the Ca^{2+} sensitivity of cgs1/2 mutant by inhibiting the IPC synthesis (Beeler et al. 1994, 1997, 1998; Schneiter 1999; Zhao et al. 1994). However, the mechanism of how IPC regulates Ca^{2+} homeostasis in yeast remains unknown.

3.3 Function of Yeast LCBs

Long-chain sphingoid bases (LCBs), DHS and PHS in yeast, are required for proper actin organization, which is critical for the internalization step of endocytosis (Munn and Riezman 1994; Zanolari et al. 2000). This physiological function of LCB was first established using lcb1-100, a yeast mutant that is defective in the first step of sphingolipid biosynthesis at non-permissive temperatures. In this study, significant

defects in endocytosis were observed when sphingolipid synthesis was blocked. Moreover, the blocking of endocytosis in lcb1-100 was abrogated by supplementation of exogenous LCB, DHS, or PHS, even when LCB-1-P and ceramide synthesis are abolished. Subsequent studies established that the signaling pathway downstream of LCB was composed of Pkh1/2, two kinases that are homologous to mammalian 3-phosphoinositide dependent kinase-1 (PDK1), and their downstream effectors PKC1, Sch9, and YPK1, homologues of the mammalian serum- and glucocorticoid-induced kinase SGKp (deHart et al. 2002; Friant et al. 2000, 2001; Liu et al. 2005; Sun et al. 2000). Indeed, Pkh1/2 was shown to be activated by nanomolar concentration of sphingoid bases, and overexpression of Pkh1/2 as well as their downstream effectors, Pkc1 and Ypk1, was shown to suppress the LCB requirement for endocytosis.

Sphingoid bases from de novo synthesis have also been established as potential second messengers in the heat stress response, the ability of yeast to adapt to growth at elevated temperatures as high as 39 °C. Complex physiological changes occur during the yeast response to heat stress. These changes include gaining of thermotolerance through generation of trehalose, induction of heat shock proteins, decreased nutrient import, transient arrest of the cell cycle at G0/G1, and finally recovery of normal growth state even at the elevated temperature. Strikingly, a ~10-fold increase of C20-PHS and C20-DHS was detected immediately after the temperature shift from 30 °C to 39 °C (Jenkins et al. 1997), and subsequent studies implicated DHS/PHS in many relevant heat stress pathways. For example, DHS/PHS can cause trehalose accumulation through activation of TPS1, a gene encoding a subunit of trehalose synthase (Dickson et al. 1997a); inhibit amino acid transport via downregulation of nutrient permease, such as Fur4, through ubiquitin-mediated protein degradation (Chung et al. 2000; Skrzypek et al. 1998); and induce cell cycle arrest at G0/G1 through Gln3 (Jenkins and Hannun 2001). These roles of DHS/PHS are supported by two lines of evidence: (1) the response to heat stress is abolished when the sphingoid generation is impaired by genetically inactivating specific genes or pharmaceutically inhibiting the specific enzyme activity and (2) exogenous addition of DHS/PHS is able to cause similar effects as heat stress. However, despite the possible downstream effectors of sphingoid bases identified for each physiological change associated with the yeast heat stress response, there is still a lack of information about the direct downstream targets of sphingoid base and if and how the identified LCB responsive kinase Pkh1/2 coordinates these different processes.

Indeed, a more broader analysis found that the expression level of ~1,500 out of a total of ~6,000 yeast genes changes during the heat stress response as measured by microarray analysis (Gasch et al. 2000). Of those, ~200 genes showed different transcription responses to heat stress between wild-type yeast and the *lcb1–100*, which is unable to produce sphingolipids under heat stress. This suggests that the regulation of these genes is sphingolipid dependent, and thus they function as potential targets of bioactive sphingolipids in yeast. Strikingly, these genes function in diverse cellular pathways including cell cycle control, protein synthesis regulation, amino acid metabolism, and tRNA synthesis (Cowart et al. 2003). This approach was validated by detailed analyses of the genes identified from the

microarray experiment. CHA1, a gene encoding serine/threonine dehydratase, was identified as one of the genes expressed differently between wild-type and lcb1-100 strains upon heat stress. It was suggested that serine-induced CHA1 expression, a major mechanism to attenuate serine levels in yeast, is mediated through the synthesis of sphingoid bases DHS or PHS, thus revealing the primordial role of sphingolipids in regulating amino acid metabolism (Montefusco et al. 2012). Sphingoid bases have also been implicated in the regulation of cap-dependent translation initiation (Meier et al. 2006). Reduced synthesis of heat shock proteins was observed in lcb1-100 cells upon heat stress. This was mediated by depletion of eIF4G, the cap-dependent translation initiation factor. The eIF4G level was shown to be partially controlled by the sphingoid base-responsive Pkh-Ypk pathway (Meier et al. 2006). Increased levels of yeast sphingoid bases are also required for mRNA "P-body" formation in response to heat stress, and that implicates a role of sphingolipids in translation initiation (Cowart et al. 2010a).

Finally, sphingolipid metabolism has also been associated with yeast aging as shown by the phenotypic expression of changed chronical life span (CLS) in various mutants in the sphingolipid metabolism pathway. Most strikingly, the mutants *lag1Δ* and YPC1 overexpression in yeast show decreased CLS (D'Mello et al. 1994; Yi et al. 2016). However, as the levels of both ceramide and sphingoid base change simultaneously in the two mutants, it is difficult to dissect the functional difference between ceramide and sphingoid bases on yeast aging. Some studies suggest that lack of ceramide is the primary effect underlying yeast CLS because of the decreased CLS observed in isc1Δ, where the gene deletion directly caused decreased ceramide level due to failed hydrolysis from IPC (Almeida et al. 2008; Barbosa et al. 2016). However, other studies suggest the accumulation of sphingoid bases as the major cause of CLS in yeast as manipulation of sphingoid base levels in these cells independently of ceramide is able to cause similar defects in yeast CLS (Yi et al. 2016). Nonetheless the identification of the direct downstream sphingolipid target in yeast aging will ultimately pinpoint the functional sphingolipid species in the process.

4 Research Needs

Here, we have reviewed the basic sphingolipid metabolic pathway in yeast and detailed the known functions of bioactive sphingolipids in yeast biology. As can be seen, the metabolism of sphingolipids is tightly regulated and integrated with major yeast signaling pathways (Dickson 2008; Olson et al. 2016). It is worth noting that sphingolipids represent a vast family of lipids including all the metabolic intermediates and a large number of derivatives that arise from modification to the basic structures. Moreover, with the advance of new techniques such as high-resolution mass spectrometry, previously unknown and unappreciated sphingolipid species are being discovered, leading to further questions about the enzymes that generate these species, the mechanism of their regulation, and their cellular function.

Our current knowledge on roles of sphingolipids is primarily based on the identification of bioactive sphingolipid targets. Further investigation of sphingolipid

function using system biology coupled with yeast genetics and biochemical approaches will not only reveal more sphingolipid targets but also establish more specific mechanisms between sphingolipids and their targets and downstream responses at the single-species level.

Lastly, our understanding of sphingolipid function will advance greatly with more detailed characterization of the physical interaction between sphingolipids and their direct targets, including the identification and characterization of novel sphingolipid-interacting domains.

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