Published in final edited form as:
Biochim Biophys Acta. 2007 March ; 1771(3): 421–431.

Yeast Sphingolipids: Recent developments in understanding biosynthesis, regulation, and function

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Summary

Sphingolipids function as required membrane components of virtually all eukaryotic cells. Data indicate that members of the sphingolipid family of lipids, including sphingoid bases, sphingoid base phosphates, ceramides, and complex sphingolipids, serve vital functions in cell biology by both direct mechanisms (*e.g*., binding to G-protein coupled receptors to transduce an extracellular signal) and indirect mechanisms (*e.g*., facilitating correct intracellular protein transport). Because of the diverse roles these lipids play in cell biology, it is important to understand not only their biosynthetic pathways and regulation of sphingolipid synthesis, but also the mechanisms by which some sphingolipid species with specific functions are modified or converted to other sphingolipid species with alternate functions. Due to many factors including ease of culture and genetic modification, and conservation of major sphingolipid metabolic pathways, Saccharomyces cerevisiae has served as an ideal model system with which to identify enzymes of sphingolipid biosynthesis and to dissect sphingolipid function. Recent exciting developments in sphingolipid synthesis, transport, signaling, and overall biology continue to fuel vigorous investigation and inspire investigations in mammalian sphingolipid biology.

Keywords

sphingolipids; heat stress response; endocytosis; sphingoid bases; ceramide; sphingosine-1-phosphate

I. Introduction

Sphingolipids have emerged over the last several decades as a family of key signaling molecules including sphingosine, ceramide, and sphingosine-1-phosphate. Data indicate that these lipids regulate fundamental and diverse cell processes such as differentiation, migration, and apoptosis. Moreover, on the organismal level, sphingolipids play roles in higher order physiological processes including inflammation [1,2] and vasculogenesis [3]. Most importantly, however, recent studies implicate sphingolipid involvement in many of the most common and currently relevant human diseases including diabetes [4–6], a range of cancers [7,8], infection by microorganisms [9–12], Alzheimer's disease [13,14], diseases of the cardiovascular and respiratory systems including heart disease [15], an array of neurological syndromes [16,17], and many others. Thus, it is increasingly important to understand the routes

and regulation of sphingolipid metabolism, to elucidate the signaling targets for these lipids, to identify cell processes that they regulate, and to determine the sphingolipid-dependent component(s) of cellular, physiological, and disease processes.

Due to its relative simplicity as a model system, the yeast *Saccharomyces cerevisiae* has provided numerous opportunities for understanding fundamental cell processes in fields including cell cycle control [18], aging [19], and stress responses [20]. Moreover, the conservation of sphingolipid metabolic pathways between all eukaryotes has enabled utilization of yeast for studying the formation and function of these important lipid mediators. In fact, cloning of many of the mammalian genes of sphingolipid metabolism was possible based on their homology to yeast counterparts [21]. In general, yeast studies have provided key insights into sphingolipid biosynthesis, regulation, and function [22]. Though major developments in sphingolipid biology have already arisen from studies in yeast, recent and exciting data emphasize the need for continued vigorous investigation and hint at the depth and complexity of sphingolipid biology that awaits discovery.

II. Sphingolipid metabolism in yeast

a. *De novo* synthesis

De novo sphingolipid biosynthesis begins with the condensation of fatty acyl-coA (most commonly palmitoyl- and stearoyl coA, yielding C18 and C20 products, respectively) and serine via the serine palmitoyltransferase (SPT) complex (Scheme 1)[23]. This enzyme, which consists of two major subunits, encoded by *LCB1* and *LCB2* (long chain base), and a minor subunit, encoded by *TSC3* (temperature-sensitive suppressor of *csg2Δ*, discussed below)[24], requires pyridoxal-5′-phosphate and bears sequence and functional homology to α-oxoamine synthases [25]. *In vitro* and *in vivo* data unequivocally indicate that both Lcb1p and Lcb2p are required for SPT activity [23], and deletion of either gene renders cells inviable unless they are supplied with exogenous sphingoid bases [26–28]. The precise nature of the interaction of these two subunits has yet to be determined; however, modeling studies utilizing alignments to a bacterial α-oxoamine synthase with known crystal structure indicated that the subunits form a heterodimer in a 1:1 stoichiometry [29]. An interesting observation is that Lcb2p requires interaction with Lcb1p for stability, though Lcb1p is relatively stable without Lcb2p [30].

Though Lcb1p and Lcb2p are sufficient for SPT activity, several reports indicate that a small peptide encoded by *TSC3* interacts with the complex and stimulates activity [24]. Data suggest that this increased activity is required under certain stress conditions [24], however, the mechanism of interaction of Tsc3p with SPT as well as factors regulating the interaction remain to be determined. Interestingly, however, several point mutants in *LCB2* increased SPT activity in the absence of *TSC3*, suggesting that Tsc3p mediates SPT activation possibly *via* interaction with the Lcb2p subunit [31].

The reaction of SPT produces 3-ketodihydrosphingosine, a short lived metabolite that is rapidly converted to dihydrosphingosine in an NADPH-dependent manner by the 3-ketodihydrospihgosine reductase encoded by *TSC10* (temperature-sensitive suppressor of *csg2Δ*, discussed below) [32]. TLC methods routinely used to quantify sphingoid bases do not detect accumulation of 3-ketodihydrosphingosine, suggesting that it is not a biologically active species, but a transient intermediate in *de novo* sphingolipid synthesis [32]. Thus, the first biologically relevant sphingolipid produced in the *de novo* pathway is probably dihydrosphingosine. *TSC10* was identified in a screen for suppressors of a mutation that caused the aberrant accumulation of a complex sphingolipid species, imparting a calcium-sensitive phenotype [32]. Thus, isolation of mutants that could override this defect revealed many genes that prevented accumulation of the complex lipid, and thus, genes likely to catalyze prior steps

of sphingolipid synthesis, many of which are discussed below (and are similarly named 'TSC'). This clever screen exemplified the usage of yeast genetics for discovery of biochemical pathways. Moreover, the screen identified several complementation groups that await characterization.

The product of Tsc10p, dihydrosphingosine, can be converted to phytosphingosine by the Sur2p/Syr2p hydroxylase (suppressor of rsv161/syringomycin resistance, discussed below) [33](Scheme 1). Deletion of this enzyme leads to an accumulation of dihydrosphingosine, but mutant cells grow and divide at normal rates and have otherwise normal complex sphingolipids, indicating that dihydrosphingosine probably serves as a suitable substrate for all subsequent sphingolipid synthesis necessary for growth under typical laboratory conditions [33]. Together, phyto- and dihydrosphingosine are termed 'sphingoid bases', and to date, no known functions unequivocally distinguish one from the other in yeast (though some activities of the bases, as discussed below, are mediated by lower concentrations of phytosphingosine). Furthermore, each sphingoid base can potentially undergo either phosphorylation at C1 or N-acylation, as described below.

The sphingoid bases can be phosphorylated to form phyto- or dihydrosphingosine phosphate by the sphingoid base kinases encoded by *LCB4* and *LCB5* [34]. Data indicate significant overlap in biochemical function of these enzymes, though by far the greatest source of sphingoid base kinase activity comes from Lcb4p, which contributes over 90% of total activity [34]. The role of Lcb5p remains unknown, but perhaps it mediates sphingoid base phosphorylation under specific conditions or in specific intracellular compartments. For most experimental purposes, depletion of sphingoid base kinase activity requires deletion of both isoforms. Interestingly, deletion of *LCB4* together with *LCB5* causes tremendous accumulation of sphingoid bases, indicating that the sphingoid base kinase pathway may function as a 'bottleneck' in the overall biochemical pathway[35]. The double deletion mutant has normal growth rates and few detectable phenotypes despite the aberrant sphingoid base content, indicating that, sphingoid bases do not inhibit yeast growth.

The sphingoid base phosphate lyase, Dpl1p, cleaves phyto- and dihydrosphingosine-1-phosphate, generating fatty aldehydes and ethanolamine phosphate [36]. Importantly, this pathway remains the only know biochemical route by which sphingolipids are converted to non-sphingolipid molecules, and thus, is the only route by which sphingolipids may 'exit' the pathway. Therefore, this degradation pathway holds a unique position in overall sphingolipid biology, as it may function as a key node to regulate overall sphingolipid levels. On the other hand, some data suggest that it may also serve to produce ethanolamine phosphate for phosphatidylethanolamine biosynthesis through the Kennedy pathway [37]. The relative importance of each of these possibilities for the lyase pathway remains to be elucidated, and a possibility exists that both functions, *i.e*., degradation of sphingolipids and production of ethanolamine phosphate, are important.

Interestingly, a deletion mutant lacking the *DPL1* gene accumulated extremely high levels of sphingoid base phosphates, which were inhibitory for yeast growth [38]. This phenotype enabled a screen for suppressors of sphingoid base phosphate toxicity, which revealed the existence of an ATP-dependent sphingiod base transporter, Rsb1p [39]. Rsb1p may pump excess sphingoid bases outside the cell to preserve normal intracellular functions including correct membrane composition [39]. It is possible that this transporter is upregulated and/or posttranscriptionally modified to provide cells with a biologically viable solution to increased sphingoid base phosphates. In an exciting recent study, this pump was shown to be regulated by transcription factors known to mediate the retrograde and pleiotropic drug resistance responses, raising intriguing possibilities that sphingoid bases participate in these transcriptional programs [37].

Sphingoid base phosphates can be hydrolyzed back to sphingoid bases by the phosphatases encoded by *YSR2* and *YSR3* [40,41]. Though enzymes encoded by both of these genes participate in similar reactions, Ysr3p, but not Ysr2p, was determined to facilitate utilization of exogenous sphingoid bases from media [40,42]. This study and further studies with the Lcb4p sphingoid base kinase indicated that exogenous sphingoid bases taken up from media are likely phosphorylated (*i.e*., by Lcb4p) and then dephosphorylated by Ysr3p prior to their incorporation into cell sphingolipid pools [43].

In addition to conversion to phosphorylated derivatives, an alternative fate of sphingoid bases is their N-acylation to form phyto- or dihydroceramides. Like the SPT reaction, this reaction also requires acyl CoA as an acyl chain donor, but the preferred substrates are C24–C26 in length [44]. The lengthened acyl CoAs derive from the fatty acid elongases Elo2p and Elo3p [45], Tsc13p, which co-immunoprecipitated with Elo2p and Elo3p, and the protein encoded by the YBR159w gene [46,47], which co-immunoprecipitated with Elo3p and Tsc13p, suggesting these proteins are subunits of a fatty acid elongation complex [47]. Deletion of these enzymes caused accumulation of short chain fatty acids as well as ceramides containing N-acyl groups much shorter than in parental strains [47]. The N-acylation of sphingoid bases to form ceramides requires the gene products encoded by *LAG1* or *LAC1* [48]. These genes are highly homologous and overlap in function, since both must be deleted to attenuate ceramide synthesis. Until very recently, whether these peptides actually catalyzed the ceramide synthase reaction had not been biochemically demonstrated; however, a recent study confirmed that the enzyme a mammalian homologue of *LAG1* and *LAC1*, LASS5, is a *bona fide* ceramide synthase [49]. The LASS genes comprise a family of six members which catalyze ceramide synthesis in mammalian cells [50], and which are partially distinguished by fatty-acyl chain length specificity [51,52]. In yeast, purification of Lac1p and Lag1p subunits, followed by electrophoretic resolution of immunoprecipitated complexes, revealed a novel component of the ceramide synthase complex, termed Lip1p [53]. In yeast and mammals, ceramide synthesis is inhibitable by the fungal toxin Fumonisin B1 [54,55], making fumonisin quite a useful experimental tool for dissecting the biological relevance of various branches of sphingolipid synthesis.

The very long chain fatty acids incorporated into phytoceramide by Lag1p/Lac1p/Lip1p can undergo hydroxylation after their incorporation. This activity is encoded by *SCS7/FAH1* [25, 56,57]. The biological function of this hydroxylation remains unclear; however, similar to the *syr2* deletion, a *scs7* deletion strain is less sensitive to certain fungicides that function by causing pore formation in the plasma membrane [33,57–59]. This suggests that both the hydroxylations mediated by Syr2p and Fah1p affect lipid packing in membranes. Potentially supporting this notion, the mammalian homologue of *FAH1*, FA2H, hydroxylates lipids that are highly abundant in the myelin sheath of the nervous system, perhaps enhancing structural stability of the membrane [60]. Interestingly, a deletion in *scs7* suppressed the calcium sensitive phenotype of the *csg2* mutant strain, indicating that part of the toxicity observed in this mutant may derive from accumulation of hydroxylated ceramides, thus suggesting specific functions for that subclass of ceramides [57].

After sphingoid bases convert to ceramides, they can be cleaved back into bases and free fatty acid by the ceramidases Ypc1p and Ydc1p [61,62]. The roles for these enzymes in yeast remain unknown; however, their mammalian counterparts are key players in sphingolipid signaling, as they are required to generate sphingosine, the precursor for sphingosine-1-phosphate (sphingosine has also been reported to have biological activity in some situations). Interestingly, however, Ypc1p, which is specific for phytoceramide, catalyzed the reverse ceramidase reaction, or ceramide synthesis from free fatty acid (as opposed to fatty acyl-coA, which is required for Lag1p/Lac1p/Lip1p mediated ceramide synthesis) and phytosphingosine, and was not inhibitable by fumonisin B1 [62]. Thus, in experimental situations where ceramide

synthase activity is detected in the presence of fumonisin and/or using free fatty acid as substrate, one should consider the likelihood of reverse ceramidase activity.

In yeast, ceramides serve as substrates for the synthesis of complex lipids that comprise up to 10% of total membrane lipids. The inositolphosphorylceramide (IPC) synthase Aur1p attaches a phosphoinositol headgroup to ceramide, forming IPC [63], which can then undergo mannosylation by the enzymes encoded by *CSG1*, *CSG2*, and *CSH1* [64,65]. Current evidence supports a model in which two enzyme complexes exist for inositol phosphosphingolipid mannosylation, Csg1p/Csg2p and Csg2p/Csh1p [65]. The relative importance of each complex, as well as the biological significance of having two complexes, remains obscure. Deletion of *CSG1* and 2, however, imparted a calcium-sensitive phenotype to cells, suggesting that sphingolipids function to regulate calcium sensitivity, and thus, these genes also served as tools for identification of many genes involved in sphingolipid metabolism in a screen for suppressors of their defect, as described above [25] After mannosylation, the enzyme Ipt1p can then catalyze the addition of another inositol phosphate group, forming mannosyl $(inositol)_2$phosphorylceramide [66]. Complex sphingolipids in yeast not only play important structural roles, but they also may function as a 'sink' for ceramide, since enzymes exist that catalyze the reverse of many of the reactions thus far described, producing sphingolipids through catabolic pathways.

b. Sphingolipid catabolism

The headgroups of complex sphingolipids are hydrolyzed by the enzyme encoded by the *ISC1* gene to yield both phyto- and dihydroceramides [67]. Isc1p bears significant structural and functional homology to mammalian neutral sphingomyelinases, a fact that facilitated its cloning based on mammalian enzyme sequences [67]. Interestingly, this enzyme bears activity not only to yeast complex sphingolipids but also to the mammalian counterpart to yeast complex sphingolipids, sphingomyelin, though sphingomyelin is not present in yeast [67]. Whether this sphingomyelinases activity serves a biological role or is merely an evolutionary anomaly remains to be determined; however, recent studies in pathogenic strains of *S. cerevisiae* indicate that in order for yeast to survive in a host, it must adapt to low glucose concentrations *in vivo* [68], a role that Isc1 plays in yeast batch cultures, as discussed below. Thus, the possibility exists that Isc1 or its homologues in more virulent pathogenic yeast, such as *Candida albicans*, may act on host sphingomyelin during infection. In a similar vein, the IPC synthase enzyme encoded by *AUR1* in *Cryptococcus neoformans* was recently demonstrated to function in pathogenesis of that virulent fungus [11].

Ceramides produced either *de novo* through Lag1p/Lac1p/Lip1p, or from complex sphingolipid hydrolysis through Isc1p, can undergo cleavage back to sphingoid bases by the ceramidases encoded by the *YPC1* and *YDC1* genes, as discussed above. The sphingoid bases produced by these enzymes can theoretically undergo all reactions that *de novo* produced lipids undergo, including phosphorylation and, interestingly, N-acylation back to ceramide. This process has been termed 'recycling', and seems to plays major roles in mammalian sphingolipid biology [69,70]. Though re-acylation of a recently cleaved ceramide seems inefficient, evidence suggests that this process functions to alter N-acyl chain lengths of ceramides, and topology (*i.e.*, compartmentalization of ceramide production) may also be a consideration in the recycling reaction.

II. Regulation of sphingolipid metabolism

For such an important biochemical pathway, puzzlingly little is understood about its regulation. In fact, in several situations where increased production of one or more of the products occurs, no evidence exists for changes in enzyme levels [71]. The advance of microarray technology has enabled the production of many yeast gene expression data sets under a myriad of

conditions [20]; perplexingly, however, across a wide variety of conditions, little transcriptional regulation is observed for the key enzymes in the sphingolipid metabolic pathway (Cowart LA, unpublished observations). There are, however, a few situations where data suggest that regulation could occur by enzyme translocation, posttranslational modification, or transcriptional regulation.

The requirement of yeast for both Lcb1p and Lcb2p allowed isolation of suppressor strains that generated novel glycerophospholipids [72]. These lipids presumably serve some of the same functions as sphingolipids; however, not all functions were complemented by the novel lipids, as the suppressor strains displayed sensitivity to growth at low pH or high temperature [73]. Notably, however, the isolation of these suppressor strains inspired studies that demonstrated a flux through *de novo* sphingolipid synthesis after heat stress. This flux is characterized by an initial rise in sphingoid bases and their phosphorylated derivatives, followed by a decrease in these species as their downstream metabolites, ceramides, accumulate [35,71,74–77]. The driving force for this flux remains unknown; however, the generation of 3-ketodihydrosphingosine by serine palmitoyltransferase is the first and rate-limiting step of *de novo* sphingolipid synthesis and thus, studies into its mechanisms of regulation deserve special emphasis, as regulation at this initial step potentially regulates all anabolic pathways of sphingolipid biosynthesis.

Previous data indicate that sphingoid base phosphates are inhibitory for cell growth and thus, that regulation of their levels mediates cell cycling and/or viability [38]. Interestingly, a relatively recent study identified that the Lcb4p sphingoid base kinase is phosphorylated and utilized a kinase mutant yeast collection to identify the Pho85 cyclins Pcl1p and 2p as required for this modification [78]. Moreover, data in this study indicate that phosphorylation mediated the degradation of phosphorylated Lcb4p in the vacuole after its sorting through the mutli-vesicular body, thus indicating that those factors that regulate Pho85p, including nutrient deprivation, may also regulate sphingoid base kinase levels *via* degradation of the Lcb4p kinase. This is supported by the observation that Lcb4p levels significantly decreased during stationary phase, with a concomitant decrease in sphingoid base phosphate levels and a slowing of the cell cycle [78]. Moreover, in the *dpl1Δ* mutant, where sphingoid base phosphate levels are elevated, cell cycle arrest does not occur as cells approach stationary phase [79]. These findings support the notion that phosphorylation of Lcb4p regulates cell cycle arrest mediated by decreasing sphingoid base phosphates. Phosphorylation of Lcb4p is apparently related to its localization at the plasma membrane due to palmitoylation by Akr1p [80], as strain expressing Lcb4p mutated at its palmitoylation sites showed aberrant cytosolic localization of Lcb4p and also failed to downregulate Lcb4p during stationary phase. The mechanism by which phosphorylation initiates the degradation of Lcb4p remains unknown.

Though factors including heat stimulate ceramide synthesis in yeast and mammalian systems, mechanisms that potentially regulate the enzymes have been elusive. A very interesting study presented data that ceramide synthase is subject to regulation by casein kinase, as a deletion of a subunit of that complex encoded by CKA2 was demonstrated to have 70–75% reduction in ceramide synthase activity in isolated membranes, as well as an accumulation of ceramide precursors in intact cells [81]. The mechanism by which Cka2p regulates ceramide synthase could be direct, *i.e.*, a casein kinase-mediated phosphorylation event is required to activate the enzyme, or indirect, *e.g.*, regulation of transcription of a component of the ceramide synthase complex. In case of the former, that mechanism would represent a major development of the regulation of *de novo* synthesis. Importantly, these findings may relate to mammalian situations where factors including chemotherapy mediate the production of ceramide, which conducts many of their downstream actions including cell death (reviewed in [8,82]).

In another set of exciting studies, it was found that transcriptional mediators of the pleiotropic drug response (PDR), Pdr1p and Pdr3p, bind to a functional PDR response element (PDRE) in the promoter of *LAC1* and activate its transcription [83]. Interestingly, in this study, the promoter of the *LAG1* gene did not contain a PDRE, perhaps providing some insight as to the apparent biochemical redundancy of these two enzymes. Perplexingly, hyperactivation of the PDR caused overall changes in sphingolipid levels that were not dependent on *LAG1* or *LAC1*, and subsequently, the promoters of other sphingolipid metabolic enzymes were examined for PDREs. Indeed, active PDREs were found in the promoters of both the *LCB2* and *SUR2* genes, suggesting the aberrant sphingolipid profiles resulted from enzymes required for sphingoid base production and underscoring the importance of this regulatory node for the control of overall sphingolipid levels [83]. It should be taken into account, however, these specific transcriptional changes are not yet linked to major changes in sphingolipid profiles, perhaps reflecting the complex nature of sphingolipid biosynthesis. Despite this deficiency, however, involvement of the PDR in mediating multidrug resistance could potentially indicate a role for sphingolipids in this important pathway.

The above described regulatory routes for sphingolipid metabolism pertain primarily to *de novo* synthesis. However, several recent studies suggest regulation of the hydrolysis of complex lipids by the inositol phosphosphingolipid phospholipase C, Isc1p. Levels of this enzyme increased in mitochondria after the diauxic shift based on fractionation of yeast cells expressing a FLAG-tagged construct [84]. Moreover, Isc1p was demonstrated to require phosphatidylglycerol or cardiolipin as a lipid activator for optimum activity [85], and these lipids are synthesized in mitochondria by phosphatidylglycerol synthase Pgs1p. Supporting that Pgs1p activity is required to activate Isc1p, phenotypes of the *pgs1Δ* strain were highly overlapping those of the *isc1Δ* strain, and neither strain effectively utilizes non fermentable carbon sources, an activity requiring mitochondria [86].

These recent studies have shed some light on mechanisms of regulation of sphingolipid metabolism. All in all, however, many key issues remain unanswered, including the regulation of serine palmitoyltransferase in response to heat stress, which conditions signal alterations in sphingolipid metabolism, and the overall mechanisms by which the pathway shifts emphasis from one branch, for example, production of complex lipids to another, for example, hydrolysis of complex lipids to form ceramides.

III. Functions of yeast sphingolipids

a. Roles in Heat Stress

The finding that sphingolipid synthesis increases upon heat stress (above) laid ground for a flurry of studies of these lipids regarding their roles in stress responses. An observation that further fueled the field of yeast sphingolipids was the isolation of a temperature-sensitive mutant in endocytosis, *end-8* [87], which was later mapped to the *LCB1* locus [88]. The temperature sensitive allele of *LCB1* (*lcb1-100*) conferred a heat sensitive phenotype, and thus, the *lcb1-100* mutant strain has been used extensively to demonstrate sphingolipid-mediated regulation of nearly every major event of the yeast heat stress response (discussed below).

When yeast are cultured at elevated temperature (i.e., 37°C–42°C), they induce an array of cellular programs, the ultimate result of which is increased thermotolerance, or ability to continue to divide at elevated temperature. These programs include major changes in gene transcription, regulation of translation (especially of heat shock proteins), degradation of nutrient permeases and denatured proteins, synthesis of trehalose, and others [89]. Moreover, yeast undergo a transient cell cycle arrest in G_0/G_1 while these programs progress. After the original observation that heat increased sphingoid base production through SPT and the fortuitous finding that the increase could be selectively blocked with a heat-sensitive allele for

LCB1, Jenkins *et al.* demonstrated a requirement for *de novo sphingoid* bases for the arrest. Whereas parental strains arrest by 1 hour of heat stress and then resume the cell cycle by 2 hours, *lcb1-100* cells failed to arrest and subsequently died [90]. This finding emphasizes the importance of sphingolipids in stress responses. Interestingly, whereas the *lcb4/5* double deletion strain, which accumulates high levels of sphingoid bases, showed a normal heat-induced arrest, it failed to re-enter the cell cycle, which prompted the speculation that degradation of bases *via* their conversion to phosphorylated derivatives was required to permit continuation of the cell cycle [90]. It is uncertain how these studies relate to those described above wherein buildup of sphingoid base phosphates inhibited cell cycle progression, thus, further study is required to dissect respective roles of sphingoid bases *vs.* their phosphates in the regulation of cell cycle progression. Moreover, another study demonstrated that ceramides increased at later time points in heat stress, after the initial rise in sphingoid bases and base phosphates [75]. Much of this ceramide increase was *via de novo* synthesis; however, a later study demonstrated an Isc1p-dependent component of ceramide production, and the Isc1p-dependent ceramides were predominantly long-chain (as would be expected from complex lipid hydrolysis) [91]. The relative importance and functions between *de novo* and catabolic ceramides remain to be fully elucidated.

In addition to their role in heat-induced cell cycle arrest, microarrays of the yeast genome were utilized to determine the degree of heat-mediated gene regulation that was dependent on sphingolipid metabolism. Interestingly, greater than 50% of all genes regulated during heat stress in a wild type strain were aberrantly regulated in the *lcb1-100* mutant [92]. There is no doubt that many of these genes display altered regulation as an indirect consequence of other failed heat stress subprograms, however, some of these genes may be direct targets for sphingolipids and thus, could serve as important tools to determine mechanisms of sphingolipid-dependent gene regulation.

A perplexing finding emerging from that study was the failure of sphingoid bases to mediate transcriptional regulation of most heat shock proteins, as these mRNAs showed essentially equivalent induction during heat stress in the *lcb1-100* mutant as in the parental strain. However, previous studies had demonstrated the *lcb1-100* mutant failing to synthesize heat shock proteins [93]. Upon further investigation, however, a translational defect was demonstrated in that mutant. Specifically, both polysome analysis and ^{35}S-methionine labeling showed a significantly lower rate of protein synthesis during heat stress in the *lcb1-100* mutant [94]. Particularly noteworthy is that sphingolipid-dependent regulation of translation proceeded in part through the Pkh1p kinase, discussed below.

b. Sphingoid base-mediated signaling

One mechanism that seems to act in sphingolipid-mediated signal transduction is the activation of the Pkh1p/Pkh2p kinases. These highly redundant kinases bear significant homology to mammalian phosphoinositide-dependent kinase 1, PDK1, and thus, any insights into how yeast signal through Pkh kinases could possibly have direct implications to mammalian sphingolipid signaling. Sphingoid bases directly activate these kinases *in vitro*, though specific binding sites remain to be identified [95–97]; However, overexpression of Pkh1p ameliorated many of the heat-related phenotypes of the *lcb1-100* strain, indicating a role for these kinases in mediating sphingoid base signaling during heat stress [97]. Functions identified for these kinases thus far include regulation of cell wall integrity signaling [98], translation initiation of heat shock proteins [94], and endocytic internalization [97]. Interestingly, sphingolipids have also been implicated in each of these processes.

Downstream signaling partners of the Pkh1/2 kinsases include members of the AGC kinase family (homologous to the SGK kinases in mammals) such as Pkc1p, Sch9p, and Ypk1p/Ypk2p. Of particular importance, Pkc1p as well may mediate many of the heat-induced

programs that require sphingolipids [97,99]. Furthermore, Sch9p, a kinase closely related to mammalian Akt/PKB, became phosphorylated *in vitro* in a sphingoid base and Pkh1p-dependent manner, though its roles in the heat stress response remain poorly defined [96]. Interestingly, a direct role for the Ypk1p/Ypk2p kinases during heat stress has not yet become apparent in any study to date, but it likely functions in regulating overall translation as it was shown to mediate levels of eIF4G [100], presenting the possibility that it does mediate some aspect of recovery from heat stress.

Perhaps one of the most interesting findings so far is the discovery that, while sphingoid bases positively regulate Pkh1p singaling through AGC kinases, two recently described proteins, Pil1p and Lsp1p, seem to allow negative regulation of Pkh1p-mediated signaling in a sphingolipid-dependent manner. Indeed, sphingoid bases reduced the phosphorylation of Pil1p and increased phosphorylation of Lsp1p *in vitro* by Pkh kinases [101]. Pil1p and Lsp1p then down-regulated Pkh activity including phosphorylation of Ypk1p [101]. This study provided new insights into signaling pathways regulated by sphingoid bases, and, moreover, gave scientists a glimpse of the complexity of sphingolipid-mediated signaling.

c. Endocytosis

In another set of studies, Pil1p and Lsp1p were identified as localizing to constitutive cellular points for initiation of endoycytosis, termed ‘eisosomes’, with Pil1p itself proposed to regulate formation of theses sites, and thus, to regulate spatial organization of endocytosis [102]. This finding returned focus to the original observation of a role for sphingoid bases in regulation of endocytosis due to mediation of cytoskeleton organization through actin dynamics. Interestingly, the endocytic defect observed in the *lcb1-100* strain was overcome by overexpression of the yeast kinases Pkc1p or Yck2p, or deletion of a protein phosphatase 2A isoform [99]. These alterations also restored normal organization of the actin cytoskeleton in that study, indicating that sphingolipids facilitate, at least in part, the intimate relationships between cytoskeletal organization and efficient endocytosis. These studies demonstrated a sphingoid base requirement for endocytosis of the mating factor receptor and centered on cytoskeletal dynamics; however, subsequent studies clarified roles for sphingoid bases in endocytosis of nutrient permeases and discovered subsequent roles for sphingolipids in ubiquitin-mediated proteolysis [103,104]. The role of permease degradation in the heat stress response remains to be determined; however, one possibility is that these proteins become damaged upon exposure to heat and must be cleared from the cell. A more interesting possibility is that degradation of these permeases sends an amino acid starvation signal to the cell. This hypothesis is particularly intriguing because many amino acid biosynthesis pathways were transcriptionally activated upon heat stress in a sphingolipid-dependent manner [92].

Taken together, these studies provide several insights into mechanisms by which sphingolipids mediate endocytosis including through modulation of signaling pathways, regulation of cytoskeletal dynamics, and regulation of the spatial organization of endocytosis *via* eisosome maintenance; however, many questions remain unanswered and thus, further investigation is needed to completely elucidate these roles.

d. Intracellular protein trafficking and assembly

Aside from these roles of sphingoid bases, many roles in protein trafficking, targeting to the plasma membrane, and assembly of protein complexes have previously been described and extensively reviewed. In brief, sphingolipids were identified as necessary for appropriate ER to golgi transport of GPI-anchored proteins [105,106]. Sphingolipids have also been demonstrated as necessary for appropriate topology, cell surface delivery, stability, and/or plasma membrane association of major proteins including the plasma membrane ATPase, Pma1p [107,108], the Vacuolar ATPase [109], and the uracil permease Fur4p[110]. It will be

interesting to see how specific these roles are to these specific proteins, or if these findings represent a more general role for sphingolipids in mediating protein trafficking and assembly. In the case of Fur4p, however, sphingolipids may be a component of lipid microdomains necessary for correct association with the plasma membrane. These microdomains are probably composed of sterols and sphingolipids [111]. Thus, an important route of investigation should involve teasing apart the structural *vs.* signaling roles of yeast sphingolipids; however, the two roles may be related in the sense that microdomains could serve as signaling platforms, *i.e.*, through the assembly of multi-protein signaling complexes.

IV. Conclusions

Though previous and current research continues to demonstrate the importance of sphingolipids for cell growth and responses to extracellular stressors, the mechanisms by which sphingolipid synthesis mediates programs of cell regulation remain largely undefined. The ability of these lipids to mediate both signaling through activation of protein kinases, protein trafficking and intracellular localization, and the structure of lipid microdomains and signaling platforms, suggest complex and integral roles for sphingolipids in cell regulation. Many of these sphingolipid functions may not necessarily proceed through previously established paradigms and thus, creativity may be required for progress in this area. Furthermore, several exciting new findings should inspire vigorous investigation into sphingolipid-mediated cell regulation, as many interesting roles for these key lipids likely await definition.

Acknowledgements

The authors would like to acknowledge Stefanka Spassieva for a thorough reading of the manuscript and helpful suggestions. This work was supported in part by a MREP award (to L.A.C.) and by a MERIT Award (to L.M.O.) by the Office of Research and Development, Department of Veterans Affairs, Ralph H. Johnson VA Medical Center, Charleston, South Carolina. L.M.O. would also like to acknowledge support from NIH Grants AG16583 and GM 62887.

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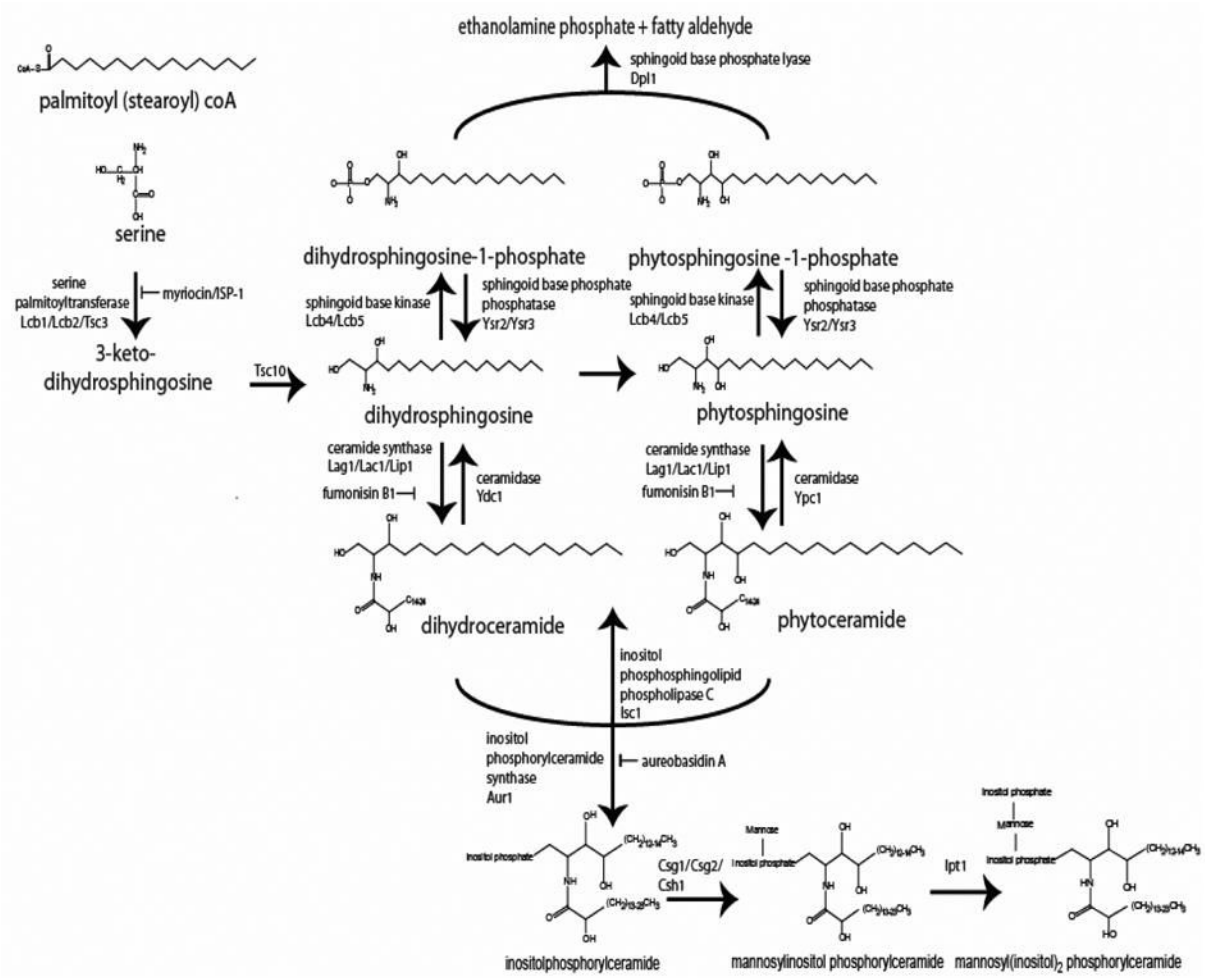

Scheme 1.
Major pathways of sphingolipid metapolism in *Saccharomyces cerevisiae*

Table 1

Major Functions of Yeast Sphingolipids

Function	Implicated Lipid(s)	Implicated Gene(s)	Reference(s)
Endocytosis	Sphingoid bases	*LCB1*	[88,97,99,104]
Pkh1 activation	Sphingoid bases	*LCB1/LCB2*	[96,97,101]
Heat Stress Response	Sphingoid bases, Sphingoid base phosphates, Phytoceramide	*LCB1/2, LCB4/5, LAG1/LAC1, ISC1*	[35,41,74–77,90–92,94, 104]
Regulation of Translation	Sphingoid bases	*LCB1/2*	[94]
Regulation of transcription	Unknown	*LCB1*	[92]
GPI-anchored protein trafficking	Very long chain Ceramides, complex sphingolipids	*LAG1/LAC1, ELO2, ELO3*	[44,105,106,112,113]
Calcium sensitivity	Complex sphingolipids	*TSC3, TSC10, TSC13, CSG1, CSG2*	[29,46,56,57,64,66,114–116]
Cell cycle regulation	Sphingoid bases, Sphingoid base phosphates	*LCB1/2, LCB4/5, DPL1, YSR2, YSR3*	[42,79,90]