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Review

Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*

Robert C. Dickson *, Chiranthani Sumanasekera, Robert L. Lester

Department of Molecular and Cellular Biochemistry and the Lucille Markey Cancer Center, University of Kentucky College of Medicine, 741 S. Limestone St., BBSRB, B173, Lexington, KY 40536-0509, USA

Abstract

We describe recent advances in understanding sphingolipid functions and metabolism in the baker's yeast *Saccharomyces cerevisiae*. One milestone has been reached in yeast sphingolipid research with the complete or nearly complete identification of genes involved in sphingolipid synthesis and breakdown. Other advances include roles for sphingolipid long-chain bases as signaling molecules that regulate growth, responses to heat stress, cell wall synthesis and repair, endocytosis and dynamics of the actin cytoskeleton. We touch briefly on other sphingolipid functions so that readers unfamiliar with the field will gain a broader view of sphingolipid research. These functions include roles in protein trafficking/exocytosis, lipid rafts or microdomains, calcium homeostasis, longevity and cellular aging, nutrient uptake, cross-talk with other lipids and the interaction of sphingolipids and antifungal drugs.

Contents

* Corresponding author. Tel.: + 1 859 323 6052; fax: + 1 859 323 1037.
E-mail address: bobd@uky.edu (R.C. Dickson).

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Nomenclature

DHS	dihydrosphingosine
DHSP	dihydrosphingosine-1-phosphate
ER	endoplasmic reticulum
GDP	guanosine diphosphate
GEF	guanosine nucleotide exchange factor
GFP	green fluorescent protein
LCB	(sphingolipid) long-chain base
LCBP	long-chain base phosphate
IPC	inositol-phosphoceramide
MAPK	mitogen-activated protein kinase
MIPC	mannose-inositol-phosphoceramide
$M(IP)_2C$	mannose-(inositol-P)$_2$-ceramide
PDK1	phosphorylation site in the activation loop of a protein kinase
PDK2	phosphorylation site in the hydrophobic motif of a protein kinase
PH	pleckstrin homology domain
PHS	phytosphingosine
PHSP	phytosphingosine-1-phosphate
PI	phosphatidylinositol
PI4P	phosphatidylinositol 4-phosphate
PI4,5P	phosphatidylinositol 4,5-bisphosphate
SPT	serine palmitoyltransferase
TOR	target of rapamycin

1. Introduction

Sphingolipids along with glycerophospholipids and sterols are the primary lipids comprising membranes in eucaryotic cells, and like the other lipids, sphingolipids play additional roles as second messengers for regulating signal transduction pathways. Decades of research on mammalian cells have revealed a complexity in the polar head groups and types of fatty acids found in sphingolipids that is only now beginning to be appreciated and understood [1]. Simpler model organisms have been used with great success to better understand the complexity of mammalian cells and the common baker's yeast *Saccharomyces cerevisiae* has proven especially useful in understanding specific aspects of sphingolipid biology, especially the identification of genes that encode sphingolipid metabolic enzymes [2,3]. This review focuses on selected recent advances in sphingolipid metabolism and functions in yeast including the genes and proteins necessary for sphingolipid metabolism, of which we now have a nearly complete catalog, and the role of sphingolipid long-chain bases as signaling molecules for regulating growth, responses to heat stress, cell wall synthesis and repair, endocytosis and dynamics of the actin cytoskeleton in response to stresses. Other roles for sphingolipids will be mentioned briefly and previous reviews can be consulted for more detailed information [2–6]. These functions include roles in protein trafficking/exocytosis, lipid rafts or microdomains, calcium homeostasis, longevity and cellular aging, nutrient uptake, cross-talk with sterols and the action of some antifungal agents.

2. Sphingolipid metabolism

2.1. Synthesis of long-chain bases and ceramides

The components of all sphingolipids are a long-chain base (LCB), a fatty acid and a polar head group. The two types of LCBs in yeast are dihydrosphingosine (DHS, official IUPAC nomenclature is sphinganine) and

its 4-hydroxy derivative, phytosphingosine (PHS). Further complexity arises because of differences in chain length with DHS containing 16, 18 or 20 carbons and PHS containing 18 or 20 carbons [7]. The fatty acid in mammalian sphingolipids can vary in chain length, degree of saturation and hydroxylation whereas the fatty acids in *Saccharomyces* sphingolipids are primarily 26 carbons long and are unsaturated. They can, however, contain 0, 1 or 2 hydroxyls [2]. This section presents a basic outline of yeast sphingolipid metabolism with emphasis of recent results. Several reviews of yeast sphingolipid synthesis have appeared recently and these should be consulted for more detailed information about each enzymatic step [2,3,5,6].

Sphingolipid synthesis begins in the endoplasmic reticulum (ER) where serine palmitoyltransferase (SPT) catalyses the condensation of serine with fatty acyl-CoA to yield 3-ketodihydrosphingosine (ketosphinganine) and CO_2 (Fig. 1). SPT is a heterodimer composed of the Lcb1 and Lcb2 subunits (reviewed in [8]). Lcb1 appears to span the ER membrane three times with the active site located at the interface between Lcb1 and Lcb2 where it faces the cytosol [9]. Homologs of Lcb1 and Lcb2 have been found in all organisms that make sphingolipids. The fatty acyl-CoA most likely is presented to the enzyme by the acyl-CoA binding protein, Acb1, which is required for ceramide synthesis and formation of normal vacuoles [10]. A third, small hydrophobic protein, Tsc3, is necessary for optimal SPT activity and is essential for activity at elevated temperatures, but its function remains unknown [11]. The temperature-sensitivity of a *tsc3* mutant can be suppressed by point mutations in *LCB2*, suggesting that Tsc3 influences Lcb2 rather than Lcb1 in the known Tsc3-Lcb2-Lcb1 protein complex [12]. No mammalian homologs of Tsc3 have been detected.

Point mutations in the human homolog of *LCB1* cause hereditary sensory neuropathy, type I (HSN1) [13,14]. HSN1, an autosomal dominant progressive degeneration of dorsal root ganglia and motor neurons, is the most common hereditary disorder of peripheral sensory neurons. Mutations in yeast *LCB1* corresponding to the human mutations that produce HSN1, generate a form of Lcb1 that is dominant to the wild type and that reduces SPT activity thereby providing a mechanism for how HSN1 is initiated [15,16].

Tsc10 catalyzes the second step in sphingolipid synthesis in which 3-ketodihydrosphingosine is reduced to DHS [17]. DHS is then attached to a C_{26} fatty acid via an amide linkage by either of two ceramide synthases,

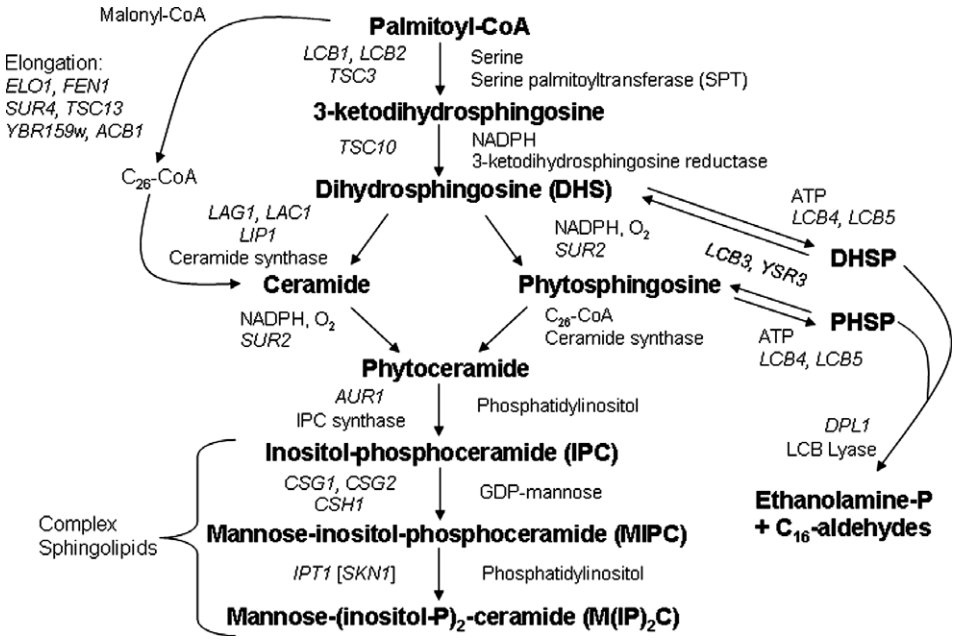

Fig. 1. Diagram of sphingolipid metabolism in *Saccharomyces cerevisiae*. Metabolites are shown in bold, genes are in italics and enzyme names are in regular lettering. Structures of the metabolites can be found in previous publications [2,5]. The cellular locations of these reactions are discussed in the text and in a recent discussion of intracellular sphingolipid trafficking [1]. Gene designations are the preferred ones listed at the Saccharomyces Genome Database (www.yeastgenome.org) and common aliases are listed in the text. When grown aerobically the fatty acid in complex sphingolipids is often hydroxylated at C2 and sometimes at C3, a reaction that requires Scs7 (not shown). Ceramides can be hydrolyzed by two ceramidases, Ydc1 and Ypc1, to yield a fatty acid and an LCB (not shown) likewise, the polar head group on complex sphingolipids can be removed by the action of Isc1 to yield ceramide (not shown).

Lag1 and Lac1 [18,19], to yield *N*-acylsphinganine (dihydroceramide) which is then hydroxylated at C-4 by Sur2/Syr2 to give phytoceramide. Alternatively, DHS can be hydroxylated by Sur2 to form PHS which is then amide linked to a C_{26} fatty acid to yield phytoceramide [20,21]. Ceramide synthases in yeast but not mammals contains another subunit, Lip1, whose function requires elucidation [22]. It is assumed that the active site of ceramide synthase faces the cytosol, but this remains to be determined experimentally. The C_{26} fatty acid moiety of phytoceramide can contain 0, 1 OH (at C-2) or 2 OHs (second at C-3 [23]) fatty acids and their addition requires the Scs7/Fah1 protein [20,24,25].

2.2. Synthesis of C_{26}-fatty acids

Elongation of fatty acids from C_{14}–C_{18} to C_{26} occurs in the ER and requires several genes including *ELO1* [26], *FEN1* (*ELO2*), *SUR4* (*ELO3*) [27], *TSC13* [28], *YBR159w* [29] and *ACP1* [30]. The elongation cycle is composed of four reactions. First, malonyl-CoA is condensed with an acyl-CoA to form a 3-ketoacyl-CoA. Second, the 3-ketoacyl-CoA is reduced to 3-hydroxy acyl-CoA. Third, 3-hydroxy acyl-CoA is dehydrated to an enol intermediate and, finally, the enol is reduced to yield an acyl-CoA that is 2 carbons longer than the starting compound. Elo1 is necessary for elongation of C_{14} and C_{16} fatty acids and probably catalyzes the condensation reaction. Fen1 probably carries out the condensation reaction starting with C_{20} and C_{22} fatty acyl-CoAs while Sur4 probably does the same for C_{22} and C_{24} fatty acyl-CoAs [31]. Ybr159w probably catalyzes the second reaction at least for acyl-CoAs greater than 18 carbons [29,32]. The enzyme that dehydrates the 3-hydroxy intermediate to give an enol is unknown. Reduction of the enol is catalyzed by Tsc13 to complete the elongation cycle. Finally, Acp1 encodes an acyl-CoA binding protein that is thought to be necessary for delivery of C_{18}-CoA to the elongation system. None of the steps in the elongation cycle have been performed with purified components, so there are likely to be additions and corrections to the schema described here. Historical details of how these genes were isolated and the various phenotypes produced by mutant alleles can be found in previous reviews [2,5].

Mutants defective in synthesis of the C_{26}-fatty acid show interesting phenotypes that could reveal new functions for sphingolipids. For example, *fen1* mutants have reduced 1,3-β-glucan synthase activity and accumulate PHS which was shown to be a non-competitive inhibitor of the enzyme in vitro [33]. Thus, PHS or DHS may regulate synthesis of 1,3-β-glucan, the major polysaccharide in yeast cells. This observation may be related to the observation that inositol starvation promptly decreases mannan and glucan synthesis [34]. Examination of *sur4* mutants showed that the cytosolic component of the vacuolar ATPase (the V_1 component) assembled and bound to the V_0 component on the vacuolar membrane but the ATPase was not active, indicating that sphingolipids with a C_{26}-fatty acid are required for vacuolar ATPase activity [35].

2.3. Synthesis of complex sphingolipids in the golgi apparatus

Once made, ceramides must be transported from the ER to the Golgi apparatus where the polar head groups are added. There is evidence for both vesicle transport of ceramide and a non-vesicle transport that does not require ATP and appears to require direct contact between ER and Golgi membranes [36]. A novel mechanism of non-vesicle ceramide transport in mammals has recently been uncovered with the identification of the CERT protein that appears to extract ceramides from the lumenal surface of the ER and then deposit them on the outer leaflet of the Golgi membrane [37,38]. There is no yeast sequence homolog of mammalian CERT so it is unclear whether yeast uses another method of non-vesicle ceramide transport or whether there is a functional yeast homolog of CERT which lacks sequence similarity.

Ceramide is probably delivered to the outer leaflet of the Golgi apparatus bilayer. It must then be flipped to the inner leaflet in order for inositol phosphate to be transferred from phosphatidylinositol onto the C_1 OH of ceramide to form the first complex sphingolipid, inositol phosphoceramide (IPC). This transfer is catalyzed by inositol phosphorylceramide synthase (IPC synthase), encoded by the *AUR1* gene [39], whose active site appears to be located in the lumen of the Golgi apparatus [40]. It is not known how ceramide is flipped from the outer to the inner leaflet of the lipid bilayer. The Aur1 protein is a multi-membrane spanning protein and it may be that it also is able to move or flip ceramide or IPC from one leaflet of the ER membrane to the other.

The reaction catalyzed by IPC synthase has been suggested to play a role in the transition from the G1 to the S phase of the cell cycle because the reaction produces diacylglycerol [41]. It is argued that the diacylglycerol activates Pkc1 which is involved in the G1 to S transition, both in yeast and in mammals [42]. This is an appealing model that requires further substantiation.

The second complex sphingolipid is mannose-inositol-phosphoceramide (MIPC), formed by transfer of mannose from GDP-mannose onto the inositol 2-OH moiety of IPC. This reaction requires three proteins, Csg1 (Sur1) and Csg2 [43] and Csh1 [44,45]. These proteins have been proposed to form two distinct inositol phosphoceramide mannosyltransferase complexes, Csg1–Csg2 and Csh1–Csg2, with the Csg1 and Csh1 subunits performing catalysis and Csg2 performing a regulatory function [44]. The *CSG1* and *CSG2* genes were isolated because when mutated they prevent cells from growing in the presence of a high concentration of calcium [46]. The proteins may function in calcium homeostasis but the mechanism is unclear. The calcium-sensitive phenotype of *csg1* and *csg2* mutants has proven to be an extremely valuable approach for identifying genes involved in sphingolipid metabolism [17]. Proteins involved in transporting mannose into the lumen of the Golgi also affect sphingolipid synthesis including Vrg4 [47] and Gda1 [48].

The third and most complex sphingolipid in yeast, mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C), is made by transfer of a second inositol phosphate from phosphatidylinositol to MIPC, a reaction that requires the *IPT1* gene [49]. Another gene, *SKN1*, has been shown to be required for M(IP)$_2$C synthesis [50]. Skn1 may work by regulating Ipt1 activity. Alternatively or during nutrient poor growth conditions, Skn1 may be a functional homolog of Ipt1, since it is known that *ipt1*-deleted cells make a low level of M(IP)$_2$C when grown in half-strength potato dextrose medium [51]. A more detailed discussion of ceramide transport and sphingolipid metabolism in the Golgi apparatus can be found in a previous review [5].

Although sphingolipids in wild type *S. cerevisiae* contain only PHS or DHS as their LCBs, it has been shown recently that cells defective in *lcb2* will use sphingosine supplied in the medium if the *lcb4* gene is mutated [52]. The *lcb4* mutation probably blocks breakdown of sphingosine via the Dpl1 lyase pathway and forces cells to incorporate it into sphingolipids (Fig. 1). Cells with sphingosine as their LCB have various abnormalities including failure of the proton ATPase, Pma1, to associate with lipid rafts and sensitivity to calcium ions and hygromycin B. It is quite impressive that yeast cells can adapt to the bend in sphingosine caused by the 4,5-double bond and survive.

Finally, a mathematical model describing sphingolipid metabolism in *S. cerevisiae* has recently been presented [53]. Predictions of the model were examined experimentally and found to be valid, indicating that the model should be helpful in understanding sphingolipid metabolism in greater detail including which reactions regulate the level of a specific sphingolipid and what variables influence such key reactions.

2.4. Turnover of sphingolipids

Turnover of sphingolipids in multicellular eucaryotes is vital for survival and has been extensively characterized because many human diseases, sphingolipidoses, are due to the lack of an enzyme essential for turnover of one or more sphingolipids [54]. Nearly the exact opposite picture exists in yeast because it has been difficult to detect turnover biochemically. Complex yeast sphingolipids comprise about 30% of the phosphorylated membrane lipids and nearly 7% of the mass of the plasma membrane [55]. Thus, it would be experimentally difficult to detect turnover of a small fraction of complex sphingolipids (IPC, MIPC and M(IP)$_2$C) which is one reason why turnover in yeast has been difficult to detect. There is biochemical evidence for the existence of an enzyme(s) with phospholipase C-type activity, similar to mammalian sphingomyelinases, that hydrolyses the polar head group from IPC, MIPC and M(IP)$_2$C to yield ceramide [56] as well as an activity that acts like a mammalian sphingomyelinase to release ceramide from yeast sphingolipids [57]. These discoveries coupled with the identification of yeast genes encoding homologs of mammalian sphingolipid degradative enzymes demonstrate the potential of sphingolipid turnover in yeast.

Sequence similarity led to the identification of *ISC1* which encodes the phospholipase C-type and sphingomyelinase activity in *S. cerevisiae* [58]. That Isc1 plays a role in stress tolerance was suggested by the observation that sodium and lithium ions inhibit growth of *isc1*-deleted cells [59]. The biochemical role of Isc1 in ion tolerance remains to be determined. Haploid mutant *isc1* cells grow slower than wild type cells and do not grow to as high a density when they are fermenting glucose [60]. On a non-fermentable carbon source

isc1 mutant cells barely grow, suggesting a role in respiration/mitochondria [61]. These phenotypes are probably related to the fact that localization of Isc1 changes from the endoplasmic reticulum to mitochondria as cells go from fermentative to respiratory growth during the diauxic shift [60]. Also during this transition from fermentative (early log phase) to respiratory growth (early stationary phase) the specific activity of the enzyme increases 3–5-fold and phytoceramide increases 4-fold in wild type but not in *isc1* mutant cells. The growth behavior of the *isc1* mutant resembles that of a *pgs1* mutant, defective in phosphatidylglycerolphosphate synthase, which fails to make phosphatidylglycerol and cardiolipin. Closer examination of these similarities revealed that Isc1 requires phosphatidylglycerol and cardiolipin for activation but not for localization to mitochondria [61]. The *pgs1* mutant also blocks the Isc1-dependent generation of phytoceramide during the diauxic shift, suggesting that phosphatidylglycerol and cardiolipin are upstream regulators of Isc1. Like a *pgs1* mutant, the *isc1* mutant has a reduced level of the mitochondrial cytochrome *c* oxidase subunits Cox3 and Cox4. These data establish Isc1 as a vital component in the diauxic shift during which time yeast switch from fermentation, which does not require functional mitochondria, to respiration, which requires functional mitochondria. It remains to be determined whether the phytoceramide generated by Isc1 acts as a lipid stimulator or whether its generation reduces the concentration of a complex sphingolipids that acts a lipid inhibitor. Complex sphingolipids have not been detected in yeast mitochondria [62,63], but this possibility should be examined more thoroughly.

Isc1 has recently been shown to be responsible for part of the increase in ceramide [56,64] that occurs when yeast are heat shocked [65]. During a heat shock Isc1 preferentially generates dihydroceramide with a C_{16}, $C_{24:1}$, C_{24} or C_{26}-fatty acyl group and phytoceramide with a C_{16}, $C_{26:1}$ and C_{26}-fatty acyl group. This is the first convincing indication that complex sphingolipids are turned over. Another indication that Isc1 can turnover a significant fraction of complex sphingolipids is the finding that an *isc1* mutation is synthetically lethal with the *lcb1-100* mutation [65]. *lcb1-100* cells are known to have reduced levels of LCBs and complex sphingolipids even when grown at a permissive temperature [66,67]. The finding of synthetic lethality implies that *lcb1-100* cells are so impaired in de novo synthesis of ceramides that they can only survive by turning over and reusing the LCBs in complex sphingolipids. It is not clear why turnover is required for survival when one could imagine that *lcb1-100* cells might just reduce their growth rate in order to produce enough sphingolipids to survive. Perhaps turnover is necessary to coordinate or balance the level of one or more sphingolipids with other lipids or vital metabolite.

Analysis of gene expression in *isc1* mutant cells shortly after a heat stress indicated that expression of 57 genes was abnormal with 35% involved in sexual reproduction, 21% involved in fermentation, 9% in carbon utilization and the remainder in known and unknown processes [65]. Following up on the gene expression data, *isc1* mutants were found to sporulate 50% less efficiently than wild type cells. The most straightforward interpretation of these results is that ceramides generated from turnover of complex sphingolipids are playing roles in several cellular processes and they may be acting as signaling molecules. Thus, this is the first convincing indication that turnover of complex sphingolipids has been conserved across the evolutionary time scale from yeast to mammals [68] as a way of regulating cellular processes.

The *Schizosaccharomyces pombe* homolog of Isc1, Css1, seems to perform quite different functions than does Isc1. Css1 localizes both to the plasma membrane and to the secretory pathway (endoplasmic reticulum and Golgi apparatus). *CSS1* is an essential gene and is some how involved in coordination of cell wall formation and cell division [69].

Ceramide in mammals is a very bioactive lipid with critical roles in apoptosis and stress responses [68]. Its concentration is controlled by de novo synthesis, breakdown of complex sphingolipids such as sphingomyelin and by ceramidases that hydrolyze the amide bond between sphingosine and the fatty acid. Deficiency of acid ceramidase in humans causes ceramide accumulation and the lysosomal storage condition known as Farber's disease [70]. Two ceramidases, Ydc1 [71] and Ypc1 [71], have been identified in *S. cerevisiae*. Cells lacking Ydc1 activity have increased sensitivity to heat stress but no other phenotypes whereas cells lacking Ypc1 activity show no obvious phenotypes. Thus, the biological role of these enzymes is unknown. Ydc1 is particularly intriguing because it localizes to the nucleus [72]. Since most research in yeast is done with log phase haploid cells, it may be that ceramide and the ceramidases perform functions that are needed for other growth phases including stationary phase, spore formation or germination or filamentous growth. Recently is has been demonstrated that diploid cells, but not commonly used haploid, auxotrophic yeast cells, grown under

continuous culture conditions have at least 3 distinct phases of metabolism that occur on a 4–5-h cycle [73]. Perhaps ceramidases and ceramide or other sphingolipids play roles in such cycles. The fact that Ypc1 and Ydc1 are highly conserved in other fungi including pathogenic fungi argues that they have important cellular roles.

PHS and DHS derived from turnover of ceramide as well as that derived from de novo synthesis can be phosphorylated by two LCB kinases, Lcb4 and Lcb5 [74] to yield PHS-1-phosphate (PHSP) and DHS-1-phosphate (DHSP). Lcb5 activity is a small fraction of total LCB kinase activity and its physiological functions, except for a role in induced thermotolerance [75], are unknown. Considerably more is known about Lcb4 which accounts for 97% of LCB kinase activity. About two-thirds of its activity is membrane-bound [74] similar to mammalian homologs SK1 and SK2 [76]. Membrane-binding is due to palmitoylation at Cys34 and Cys36 by the Akr1 acyltransferase [77]. Lcb4 is also modified by phosphorylation by the cyclin-dependent protein kinase Pho85 [78]. Phosphorylation does not appear to regulate LCB kinase activity, rather it increases the rate of Lcb4 turnover which is mediated by a ubiquitin-dependent pathway that requires the yeast vacuole [78]. As cells enter stationary phase (during the diauxic shift) the concentration of Lcb4 decreases, suggesting that it, or more likely its products PHSP and DHSP, have a role in the diauxic shift or true stationary phase which comes after the diauxic shift. Palmitoylation is required for complete phosphorylation of Lcb4 and for turnover in stationary phase. Native Lcb4 localizes to the plasma membrane [77], whereas Lcb4 with a C-terminal epitope tag is found in the ER and Golgi [79] and in endosomes [80].

Lcb4 has also been shown to be necessary for rapid but not slow incorporation of LCBs from the culture medium into sphingolipids [79]. To explain this result it has been proposed that LCBs taken up by cells from the culture medium are phosphorylated by Lcb4 and then dephosphorylated by Lcb3 in the endoplasmic reticulum before being *N*-acylated by ceramide synthase to form ceramide. This is in contrast to the simpler possibility that LCBs derived from outside cells can serve directly as a substrates for ceramide synthase. The physiological reason for this Lcb4/Lcb3 pathway is not clear [1].

PHSP and DHSP have two fates in yeast (Fig. 1). They can be dephosphorylated by either of two phosphatases, Lcb3 (Ysr2) or Ysr3 (Lbp2) [81–83] or they can be cleaved by an LCB phosphate lyase, Dpl1 [84], to yield ethanolamine phosphate and a C_{16} aldehyde (Fig. 1). Lcb3 spans the endoplasmic reticulum membrane multiple times and the active site probably faces the lumen [85]: the location of the Ysr3 and Dpl1 active site is unknown. It is now well established that accumulation of LCBPs, such as occurs when the lyase gene *DPL1* and the phosphatase gene *LCB3* are both inactivated, is toxic to yeast cells [86,87]. Suppressor gene analysis has been used to try and determine the mechanism of toxicity. Besides mutations in *LCB4*, which block formation of LCBPs, and mutations in *PDR5*, which enhance LCB export from cells [88], four genes (*HEM14*, *HMG1*, *KES1* and *ERG5*) that play roles in heme and ergosterol synthesis, were also isolated [89]. These mutations all perturbed Lcb4 so that the level of LCBPs was reduced and it was suggested that changes in sterols alter the location of Lcb4 which alters its phosphorylation. Mutations in four other suppressor genes, *PBP1*, *UFD4*, *TPS1* and *WHI2*, may play roles in LCBP signaling, but this requires experimental substantiation.

Recent attempts to identify functions for essential yeast genes with no known function suggest that the open-reading frame *YPL097w*, renamed *PHS1*, plays a role in LCB or LCBP metabolism [90]. This conclusion is based upon the observation that deletion of *LCB3* strongly reduces growth of mutant cells producing a reduced level of Phs1 and, the observation that a *phs1* and a *dpl1* mutant show a similar pattern of epistatic interactions to a collection of yeast deletion mutants. In addition, mutant cells with a low level of Phs1 are more resistant to PHS or DHS in the culture medium, much like an *lcb4* deletion mutant. Analysis of LCBs and LCBPs in cells with a low level of Phs1 showed an increase in C_{20}-PHS and C_{18}-DHSP. While these results do not explain what Phs1 does, they do support the idea that Phs1 plays a role in sphingolipid metabolism or signaling. Phs1 is highly conserved in mammals and is most closely related to protein tyrosine phosphatase-like proteins that could be anti-phosphatases.

Finally, the Rsb1 protein has been shown to transport LCBs out of yeast cells into the culture medium in an ATP-dependent process. Rsb1 is either a transporter or a flippase that moves LCBs from the inner to the outer leaflet of the plasma membrane [85] or it may be involved in adjusting the lipid composition of the inner or outer leaflet of the plasma membrane. Another protein implicated in sphingolipid trafficking is Ncr1, an ortholog of the mammalian protein NP-C whose loss in activity results in the fatal human neu-

rodegenerative disorder known as Niemann Pick type C disease. It has been proposed that a primary function of Ncr1 is to recycle sphingolipids between cellular membranous compartments and that defects in recycling give rise to cholesterol and sphingolipid accumulation in lysosomes, as seen in Niemann Pick C patients [91].

3. Heat stress

One similarity in response to heat stress shared by yeasts and mammals is the generation of sphingolipid signals. LCBs are the best characterized heat-induced sphingolipid signaling molecules in yeast whereas in mammals it is ceramide which signals cells to undergo apoptosis during a severe heat stress [92]. There is no known role for LCBs or other sphingolipid in yeast apoptosis [93], although they could be playing unidentified roles.

Sphingolipids were first suspected of playing roles in heat stress because heat, osmotic stress and low pH stress inhibited growth of mutant strains lacking LCBs [94]. LCBs are normally required for viability, but these LCB-lacking strains were viable due to synthesis of a compensatory set of glycerol-based lipids that mimicked some functions of sphingolipids [95,96]. The next indication that sphingolipids are necessary for surviving heat stress was the isolation of a temperature-sensitive mutant, *end8* (now called *lcb1-100*) [97], which does not grow at 37 °C and which is allelic to *LCB1*, encoding a subunit of SPT (Fig. 1). Because sphingolipid synthesis is severely depressed at the restrictive temperature, it could not be determined which sphingolipid(s) was required for growth during heat stress or whether any were acting as signaling molecules.

The first indication that LCBs could be signaling molecules came from heat shock experiments in which cells were shifted from 25 to 37 or 39 °C. This shift caused a 2–3-fold increase in C_{18}-DHS and C_{18}-PHS, and a more than 100-fold increase in C_{20}-DHS and C_{20}-PHS. These increases occurred rapidly and peaked around 5–10 min after the temperature shift and then returned to normal even though the cells remained at an elevated temperature [64,98]. Heat stressing yeast cells induces accumulation of the disaccharide trehalose [99], a well known thermoprotectant [100], and LCBs are required for accumulation [98,101]. The temporary increase in LCBs induced by heat stress also plays a role in the transient arrest of cells at the G1 phase of the cell cycle where LCBs act through an unknown pathway that regulates the Cln3 cyclin [102].

Other studies have shown that PHS plays a role in heat stress-induced turnover of the uracil transporter, Fur4, a plasma membrane protein that is endocytosed and degraded in the vacuole by a ubiquitin-dependent process [103]. Heat shocked *lcb1-100* cells do not turnover Fur4 like wild type cells, but adding PHS to the culture medium restores turnover, suggesting that PHS or a sphingolipid derived from it plays a role in turnover [104]. Further study of Fur4 in *lcb1-100* cells supports the idea that that LCBs or some other sphingolipid are limiting in cells grown at the restrictive temperature and are, therefore, needed for Fur4 turnover [67]. The exact role for LCBs/sphingolipids in turnover remains to be determined. In contrast to these results, Fur4 with a C-terminal GFP tag appears to be endocytosed at a normal rate in *lcb1-100* cells grown at the restrictive temperature [105]. Differences in culture media may be responsible for these opposite results. When *lcb1-100* cells are grown in complex medium containing yeast extract and peptone, endocytosis of Fur4 (no epitope tag) is blocked at the restrictive temperature [104] whereas when cells are grown in defined medium, Fur4-GFP is endocytosed [105]. Other differences that could underlie the opposing results are the GFP epitope tag and the use of cycloheximide to inhibit protein synthesis [105].

Heat stress also induces a transient increase in LCBPs [106]. For example, in the JK9-3d strain background the concentration of all LCBPs increases: C_{16}-DHSP (2.4-fold), C_{18}-DHSP (23-fold), C_{20}-DHSP (8-fold), C_{18}-PHSP (9-fold) and C_{20}-PHSP (30-fold) [75]. The peak increase occurs about 10 min after switching cells from 25 to 37 °C. It is believed that these increases and those in LCBs are due to the de novo synthesis pathway (Fig. 1) rather than to turnover or a reduction in flux through the Dpl1 lyase reaction, but the mechanism is unknown.

The transient increase in LCBPs could be serving as a signal to regulate cellular processes that mediate resistance to heat stress. But this possibility remains controversial and unproven [2,4]. All studies have utilized mutant strains deleted for *dpl1*, *lcb3*, *ysr3*, *lcb4* and *lcb5* or combinations of these genes [75,83,106–108]. The rationale for experiments in this area is that LCBPs should go up if the breakdown pathways are blocked by mutating *dpl1*, *lcb3* or *ysr3* or some combinations of these genes. This expectation is fulfilled since both the

basal (25 °C) and heat stressed level of LCBPs goes higher in these mutants than in the wild type (see for example [75]). The quandary in interpreting phenotypes like increased heat stress resistance in *dpl1*, *lcb3* or *ysr3* mutants is that LCBs also increase in concert with the LCBPs. Some have attempted, with limited success, to overcome this quandary by using mutations in the LCB kinase genes, *lcb4* and *lcb5*, to block synthesis of LCBPs [75]. The *lcb5* and the *lcb4 lcb5* double mutant strains were about 2-fold less resistant to induced thermotolerance, suggesting that LCBPs are involved in this specialized type of heat stress resistance. No difference in stress resistance was seen when log phase or stationary phase cells were heat shocked. One explanation that would reconcile the different results obtained by various laboratories is that it is the relative amounts of LCBs and LCBPs that are important in regulating heat stress resistance. As suggested previously [2], the most convincing way to demonstrate a role for LCBPs in heat stress is to show that they bind to or regulate the activity of a protein having a well established role in heat stress resistance.

DNA microarray technology has also been used to try and uncover new roles for sphingolipids in yeast. Messenger RNA abundance in parental cells was compared with mutant *lcb1-100* cells following a 15 min heat shock at 37 °C. These strains and growth conditions were chosen because heat-induced increases in LCB, LCBPs and ceramides reach there maximum between 10 and 60 min after a heat shock [56,64,75,98,102] and sphingolipid synthesis is rapidly reduced in *lcb1-100* cells [66,67,102,104]. Seventy genes showed a difference in transcript level with 41 having a higher expression level in the *lcb1-100* cells than in the wild type, suggesting that sphingolipids play a role in downregulating their transcription. Transcription of the other 29 genes was reduced in *lcb1-100* cells compared to the wild type. These 70 genes fell into several categories including amino acid metabolism, stress response, cell cycle control, protein synthesis and cell wall synthesis. Data were also analyzed by hierarchical clustering which provides a way to analyze the data over time (15, 30, 45 and 60 min) rather than depending upon one time point. This analysis identified 206 genes whose transcription was influenced by sphingolipids. Included in this data set are genes encoding tRNAs, not previously thought to be dependent upon sphingolipids. Another cluster involved genes in carbon/carbohydrate metabolism, suggesting that sphingolipids play roles in carbon sensing and signaling. The target of rapamycin (TOR) protein kinases, Tor1 and Tor2, are most prominent in such sensing and signaling [109,110] and these microarray data suggest a connection between TOR signaling and sphingolipids (see below).

Further understanding of sphingolipid functions in yeast during heat stress has also made use of *lcb1-100* cells. One study screened for multiple copies suppressor genes that enabled *lcb1-100* cells to grow at a restrictive temperature (37 °C) and identified *UBI4* as a suppressor gene [101]. *UBI4* encodes ubiquitin and further analysis indicated that induction of heat shock proteins, a universal response of organisms to a sudden increase in temperature [111], was defective in *lcb1-100* cells when shifted to a restrictive temperature. This response showed that accumulation of misfolded or aggregated proteins is toxic to yeast cells [112]. Two stress response pathways in yeast, one requiring the heat shock transcription factor (Hsf1) and the other requiring the Msn2/4 transcription factors, failed to operate correctly in *lcb1-100* cells during heat stress. Thus, one important function of sphingolipids during heat stress is the induction of heat shock proteins which prevent the accumulation of toxic misfolded and aggregated proteins.

The transcriptional profiling studies mentioned above found that induction of mRNAs for heat shock proteins was normal in *lcb1-100* cells [113]. Thus, since the mRNAs are made in *lcb1-100* cells following a heat shock, but heat shock proteins are not [101], there must be a defect in export of mRNAs from the nucleus, translation of mRNAs or stability of the heat shock proteins in *lcb1-100* cells during heat stress. Detailed analysis of *lcb1-100* cells following heat stress has recently shown that they cannot initiate translation of CAP-dependent mRNA translation [114]. Experiments indicated that LCBs but not ceramides, LCBPs or complex sphingolipids were the lipids required for translation initiation. Since LCBs regulate the activity of Pkh1/2 (Fig. 2A), a mutant defective in Pkh1/2 was examined and shown to have a defect in production of heat shock proteins, although the defect is not as severe as in *lcb1-100* cells, indicating that LCBs may control other signaling pathways that play roles in translation initiation. Because Ypk1/2 act downstream of Pkh1/2, at least in some cases, *ypk1/2* mutant cells were examined for defects in the induction of heat shock protein synthesis and found to behave differently from *pkh1/2* mutants. *ypk1/2* mutant cells make heat shock proteins immediately after being heat shocked, but upon prolonged heat stress they fail to upregulate protein synthesis like wild type cells. Thus, while LCBs regulate the activity of Ypk1/2 and Pkh1/2 during heat stress, the kinases appear to regulate different aspect of the heat stress response.

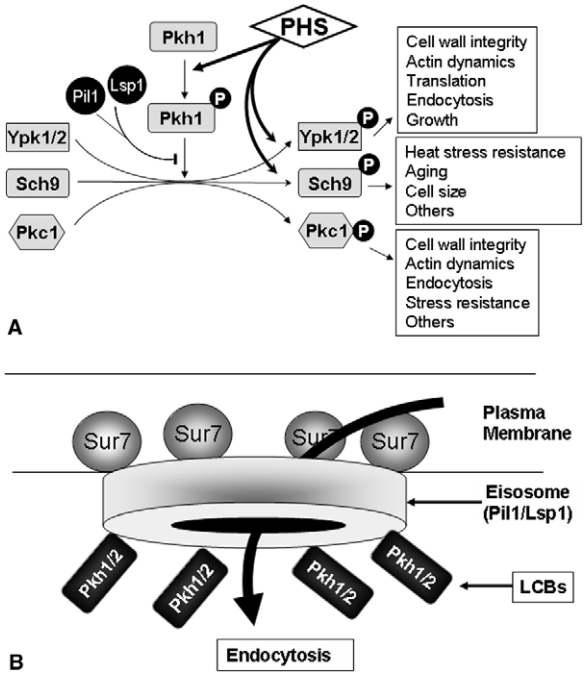

Fig. 2. Signaling pathways and cellular pathways regulated in LCBs. (A) The bold arrows leading away from PHS indicate autophosphorylation reactions that are stimulated by PHS which increase the activity of Pkh1/2, Ypk1/2 and Sch9. PHS stimulation of Pkc1 activity has not been examined. Pkh1/2 stimulates the phosphorylation and activation of Ypk1/2, Sch9 and Pkc1 as described in the text and highest activity requires a combination of both Pkh1/2 and PHS or other LCB. Phosphorylation is thought to occur in the activation loop at the PDK1 site of Ypk1/2, Sch9 and Pkc1. Pil1 and Lsp1 are indicated as downregulators of signaling pathways regulated by Pkh1/2. (B) Pil1, Lsp1 and Sur7 form eisosomes which mediate endocytosis of lipids and proteins from the plasma membrane. Eisosomes are estimated [140] to contain 2000–5000 copies of both Pil1 and Lsp1 with about 400–800 copies of Sur7 based on the estimated abundance of these proteins [192]. The actin cytoskeleton (not shown) plays an essential role in endocytosis. Panel A was redrawn from Liu et al. [125] with permission.

Future work should reveal the substrates of the two types of kinases that regulate the initiation of CAP-dependent protein synthesis during heat shock.

4. Cellular processes regulated by or that require LCBs

4.1. Growth control

Initial evidence for a signal transduction pathway regulated by LCBs was uncovered during attempts to identify yeast genes that could evade growth inhibition by a low concentration of myriocin [115]. Myriocin inhibits SPT and at the low concentration used in these experiments it most likely inhibited growth by limiting sphingolipid synthesis [116]. One of the more interesting genes identified in this screen was *YPK1*, which encodes a protein kinase involved in maintaining the integrity of the cell wall and in regulating the actin cytoskeleton [117,118], endocytosis [119] and translation during nitrogen starvation and nutrient sensing [120]. Ypk1 and its paralog Ypk2 are structural and functional homologs of mammalian serum and glucocorticoid-inducible kinase (SGK) [121]. Little is known about how Ypk1/2 functions in cells because no substrates have been identified.

Since Ypk1 was known to lie downstream of the protein kinase Pkh1 and to be phosphorylated by it [121], Sun et al. [115] tested and found that multiple copies of *PKH1* also promoted growth in the presence of myriocin. Pkh1 is a homolog of mammalian phosphoinositide-dependent protein kinase 1 (PDK1), well known for being activated by 3-phosphoinositides via binding to a pleckstrin homology (PH) domain. Pkh1 has no PH domain and in vitro experiments showed that it was not activated by phosphoinositides, leaving open the question of what the upstream activation signal was [121]. Experiments of Sun et al. [115] suggested that

sphingolipids were an upstream activation signal. For example, they showed that myriocin-treated cells lacked a phosphorylated form of Ypk1. This species reappeared in vivo even in the presence of myriocin when PHS was added to the culture medium, suggesting that a sphingolipid regulated phosphorylation. These seminal experiments could not establish whether PHS or some other sphingolipid was the signaling lipid or whether the lipid was directly or indirectly regulating Ypk1 via activation of Pkh1, which would then phosphorylate and activate Ypk1.

4.2. Endocytosis, the actin cytoskeleton and cell wall integrity

The next advance in understanding LCB-regulated signaling pathways came from studies of endocytosis using *lcb1-100* cells in which endocytosis is blocked at the restrictive temperature. These studies found that multiple copies of the protein kinase genes *PKC1* or *YCK2* restored endocytosis at a restrictive temperature [122]. Preceding work had shown that Pkc1 lies downstream of and is activated by Pkh1 [123], a relationship that was suggested by others [121]. These results implied that multiple copies of *PKH1* or a close relative, *PKH2*, should also suppress the endocytosis defect in *lcb1-100* cells and this proved to be correct [124]. Most importantly, PHS was shown to stimulate Pkc1 phosphorylation in vitro by Pkh1 or Pkh2. Others showed by using *ypk1/2* and *pkh1/2* mutants that these protein kinases are required for endocytosis of a membrane-bound receptor protein (Ste2) [119]. Together these data support the idea that constitutive synthesis of PHS and DHS under non-stress conditions and the transient increase produced by heat shock serve to activate Pkh1 and Pkh2 causing them to autophosphorylate in their activation loop at the so-called PDK1 site [121]. Once activated, they phosphorylate and activate the downstream kinases Ypk1, Ypk2 and Pkc1 (Fig. 2A). Recent work shows that LCBs also directly help to activate Ypk1/2, but that full activation requires both Pkh1/2 and an LCB [125]. The way in which Ypk1, Pkc1 and Yck2 are involved in endocytosis remains to be determined.

Since actin plays an important role in the internalization step of endocytosis, the organization of actin cables and cortical patches was examined in *lcb1-100* cells to determine if LCBs and sphingolipid synthesis are required for proper actin organization. At the permissive temperature (25 °C) actin patches were highly localized to sites of new cell growth while actin cables were directed towards new sites of growth just as in wild type cells [66]. However, at the restrictive temperature (37 °C) actin was not localized to patches, but if LCBs were included in the medium, cortical patches were restored at new sites of growth, indicating that synthesis of new LCBs was necessary for patch formation. It was then shown that multiple copies of *PKC1* [122] and *PKH1* or *PKH2* restored the actin organizational defect in *lcb1-100* cells [124]. Taken together, these results support the premise that LCBs activate Pkh1/2 which then phosphorylate downstream kinases including Pkc1 and Ypk1/2 that somehow regulate the actin cytoskeleton (Fig. 2A).

Pkc1 plays roles in many cellular processes [126,127] and only those known to depend upon LCBs will be addressed here. The first function of Pkc1 to be uncovered was its role in maintaining the yeast cell wall (called 'cell wall integrity') so as to prevent cells from lysing. Pkc1 maintains cell wall integrity primarily by regulating a mitogen-activated protein (MAP) kinase cascade whose terminal protein kinase Slt2/Mpk1 (the MAP kinase in the cascade) regulates transcription of genes necessary for maintaining cell wall integrity [128] (Fig. 3). Many results support the idea that LCBs play a role in maintaining cell wall integrity and that they do so by regulating the Pkc1-MAPK cascade as summarized in Fig. 3. As mentioned above, Pkc1 also plays a role in endocytosis. For example, Pkc1, but not the MAP kinase cascade, is necessary for actin depolarization which occurs when the cell wall is stressed by heat or high osmolarity [129]. Recovery from stress requires repolarization of the actin cytoskeleton which is controlled by the Pkc1-MAPK cascade [129].

The LCB-Pkh1/2 signaling pathway is by no means the only signaling pathway that regulates the Pkc1-MAPK cascade (Fig. 3) and it is only the most recent pathway to be uncovered. At least three other signaling pathways are needed to activate Pkc1 [126] and two of these require sphingolipids for activation. One signaling pathway, with no identified sphingolipid requirement, emanates from proteins (Wsc1-3, Mid2 and Mtl1) in the plasma membrane that sense membrane stretch caused by heat, high osmolarity or damage to the cell wall. These sensors transmit a signal to Rom1 or Rom2, two guanosine nucleotide exchange factors (GEFs) that regulate the activity of the small G-protein called Rho1 (or Rho2, Fig. 3), a GTPase that activates Pkc1 [127] (see below). The second signaling pathway requires synthesis of phosphatidylinositol (4,5)-bisphosphate

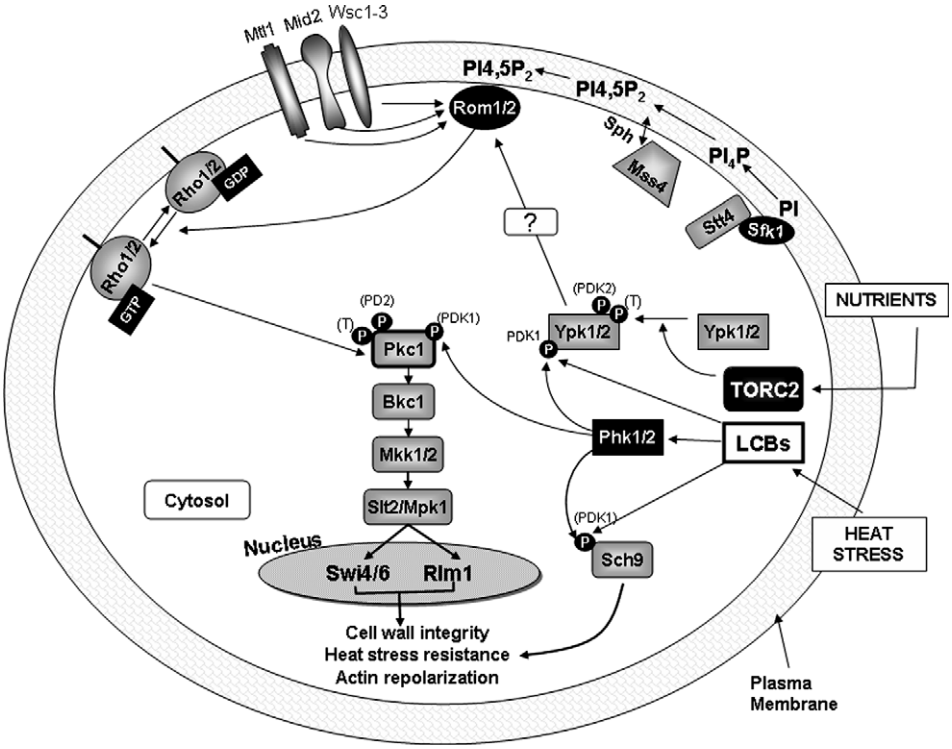

Fig. 3. Role of LCBs and other sphingolipids in regulating the Pkc1-MAPK cascade. The Pkc1-MAPK cascade begins with Pkc1 being activated in an unknown manner by the small G proteins Rho1/2. Pkc1 activation also requires phosphorylation at the PDK1 site in the activation loop which is mediated by Pkh1/2. Available evidence suggests that LCBs stimulate both basal and heat stress-induced activation of Pkh1/2. Full activation of Pkc1 likely requires phosphorylation at the PDK2 site in the hydrophobic motif and phosphorylation at the turn (T) motif: the protein kinases that catalyzed these reactions are unknown. Activated Pkc1 then initiates a cascade of phosphorylation events that culminate in activation of Slt2/Mpk1, the protein kinase that phosphorylates and activates the Swi4/6 and Rim1 transcription factors which upregulate expression genes necessary to maintain cell wall integrity. Rho1/2 are converted to their active GTP-bound form by the guanosine nucleotide exchange factors Rom1/2 which are activated by membrane-bound sensors Mtl1, Mid2 and Wsc1-3. In order to be activated, Rom1/2 must be bound to the plasma membrane via interaction with $PI4,5P_2$, which is generated from PI by two lipid kinases, Stt4 and Mss4. Mss4 requires sphingolipids (Sph), probably MIPC, in order to bind the plasma membrane and access its substrate PI4P. TORC2 senses nutrients and when they are adequate it phosphorylates the PDK2 and turn motifs of Ypk2 and probably Ypk1. Full activation of Ypk1/2 also requires phosphorylation by Pkh1/2 and perhaps LCBs. Ypk1/2 then helps to activate Rom1/2 in an unknown manner. Many factors known to regulate Rom1/2, Rho1/2 and the Pkc1-MAPK cascade are not shown [126].

($PI4,5P_2$) in the plasma membrane. Synthesis initiates with Stt4, a phosphatidyl 4-kinase, essential for growth, that catalyzes phosphorylation of phosphatidylinositol to phosphatidylinositol 4-phosphate (PI4P) [130]. Stt4 is anchored to the plasma membrane by binding to Sfk1 [131]. PI4P is phosphorylated by Mss4, a phosphatidylinositol 4-phosphate 5-kinase [130,132], to yield $PI4,5P_2$, which serves to bind Rom2 (or Rom1), via a pleckstrin homology (PH) domain, to the plasma membrane [131]. Rom1/2 must localize to the plasma membrane in order to activate Rho1/2 thereby turning on the Pkc1-MAPK cascade and initiating the process of repairing the cell wall.

Mss4 function has recently been shown to depend upon a normal complement of sphingolipids, revealing another way in which sphingolipids are required for functioning of the cell wall integrity pathway [116]. The Mss4 sphingolipid connection was discovered during the screen, mentioned above, for multicopy genes that overcame growth inhibition by a low concentration of myriocin, used to inhibit SPT (Fig. 1). *MSS4* was found to be a suppressor gene in addition to *YPK1* and *PKH1* and suppression (resistance to myriocin) required Mss4 kinase activity, suggesting that $PI4,5P_2$ played a role in the suppression mechanism. Mss4 kinase activity was reduced by 60% in myriocin-treated cells and the protein failed to localize to the plasma membrane, suggesting that sphingolipids are required for membrane binding. But which sphingolipid is required? Cells lacking Csg2 cannot make MIPC and $M(IP)_2C$ (Fig. 1) and their growth is strongly inhibited by low

concentrations of myriocin whereas cells lacking Ipt1 make MIPC but not $M(IP)_2C$ and their growth in not inhibited by myriocin. Thus, it seems that MIPC is the sphingolipid necessary for binding of Mss4 to the plasma membrane. Other data showed that like *MSS4*, *ROM2*, *RHO2* and *PKC1*, but not *RHO1* or *SLT2*, were multicopy suppressors of myriocin sensitivity and that localization of Rom2 to areas of active cell wall growth was blocked by myriocin treatment or by deletion of *CSG2*, indicating that failure of Mss4 to bind the plasma membrane was preventing Rho1/2 activation. Finally, myriocin treatment was shown to disrupt actin cables, which is expected because the Mss4 pathway is known to regulate actin dynamics [132,133]. These data build a strong case for MIPC involvement in binding of Mss4 to the plasma membrane. MIPC has been assumed to be localized in the outer leaflet of the plasma membrane. In this case it is hard to imagine how it would play a role in Mss4 membrane binding. If, however, some MIPC were on the cytoplasmic leaflet of the plasma membrane, it might be directly bound by Mss4. Further experiments should reveal how MIPC facilitates Mss4 binding to the plasma membrane.

The third signaling pathway requires Tor2 as part of the TORC2 complex [110]. Tor2 has recently been shown to phosphorylate Ypk2 (and probably Ypk1, see below) at the PDK2 site in the hydrophobic motif and in the nearby turn motif [134]. Phosphorylation of Ypk1/2 by Tor2 had previously been predicted [135]. Phosphorylation at these two sites is hypothesized to be necessary in order for the PDK1 site to be phosphorylated in the activation loop. In the fourth and final signaling pathway, LCBs stimulate Pkh1/2 to phosphorylate Ypk1/2 at the PDK1 site to fully activate the enzymes which probably act upstream of Rom1/2 [135] (Fig. 3), since TOR signaling is known to act through Rom1/2 to activate Pkc1 in a mechanistically uncharacterized manner [136,137]. The LCB-Pkh1/2 pathway also acts to phosphorylate the PDK1 site in Pkc1 and thereby contribute to its activation [121,124].

Two additional substrates of Pkh1 and Pkh2, Pil1 and Lsp1, have been identified and they provide the missing link to explain why LCBs and Pkh1/2 are necessary for endocytosis (Fig. 2B). Pil1 and Lsp1 first caught attention because they complexed with Pkh1/2 [138], suggesting that they might be substrates. They proved to be substrates in vitro whose phosphorylation is regulated oppositely by LCBs. PHS stimulates Pkh1/2 to phosphorylate Lsp1 whereas it weakly inhibits phosphorylation of Pil1 by Pkh1 and strongly inhibits phosphorylation of Pil1 by Pkh2 [139]. This opposite response to LCBs indicates that Pil1 and Lsp1 have different functions in cells even though there are about 70% identical. Their sequence is highly conserved in all other fungi whose genomes have been sequenced, but because they lack characterized domains, there cellular function has been elusive.

Since Pkh1/2 regulate the Pkc1-MAPK cascade and since this pathway is necessary for surviving heat stress, a role for *PIL1* and *LSP1* in heat stress was examined. Deletion of either gene reduced heat stress resistance and caused hyper-activation of Slt2, suggesting that Pil1 and Lsp1 either act directly to downregulate the activity of Pkh1/2 or act indirectly on downstream signaling components [139]. Other data indicated that Pil1 and Lsp1 were also downregulating Ypk1/2-mediated functions necessary for growth.

The key to unlocking the function of Pil1 and Lsp1 was their cellular location. Fluorescent microscopy of cells expressing Pil1 or Lsp1 with a C-terminal green fluorescent protein (GFP) co-localized them to the cytoplasmic face of the plasma membrane in large, stable structures, 20–45 per cell, that were immobile [140]. Since these sites were shown to be where endocytosis of both lipids and proteins was occurring, they were named eisosomes (from the Greek '*eis*', meaning into or portal, and '*soma*' meaning body). Pil1 and Lsp1 are necessary for a normal rate of endocytosis.

Eisosomes were found to co-localize with the Sur7 protein which had been shown previously to have a cellular distribution [141] that looked very much like that found for Pil1-GFP and Lsp1-GFP [72]. Sphingolipids were analyzed in *sur7* cells because a *sur7* mutation was first identified as a suppressor of mutations in *rvs161* and *rvs167* and other suppressors including *sur1* (*CSG1*), *sur2* and *sur4* had been found to play roles in sphingolipid synthesis (Fig. 1). Only small changes in various species of IPC were found in *sur7* cells [141] and it remains to be determined how these changes suppress defects in *rvs161* and *rvs167* mutants. These genetic interactions further support a role or roles for sphingolipids in endocytosis [142] and actin dynamics [143,144] since Rvs161 and Rvs167 are critical for these processes. These roles may utilize sphingolipids other than LCBs and could include interactions between sphingolipids and proteins in the plasma membrane or they might involve sphingolipids that aid in membrane curvature, a necessity for endocytosis.

Pil1 appears to play a more central role than Lsp1 or Sur7 in organizing, maintaining and localizing eisosomes based upon the observation that deletion of *PIL1* caused Lsp1-GFP to mislocalize into a few large structures on the plasma membrane and diffusely throughout the cytoplasm. In contrast, deletion of *LSP1* or *SUR7* did not perturb Pil1-GFP localization to eisosomes [140]. These data further emphasize the differential functions of Pil1 and Lsp1, despite their high sequence similarity.

While it appears that Pil1 in particular plays a structural role in eisosomes and that both Pil1 and Lsp1 are necessary for endocytosis, they probably play additional roles in cells. As mentioned above, deletion of either gene decreases heat stress resistance and causes phosphorylation and activation of Slt2 and the cell wall integrity pathway. Endocytosis is not known to play roles in these processes. Likewise, *PIL1* and *LSP1* show genetic interactions with *YPK1/2* and Pil1 and Lsp1 are found in complexes with other proteins that function in processes that have no known connection to endocytosis [138,145] including Slt2.

5. Other signaling pathways regulated by LCBs and by Pkh1/2

The amino acid sequence around the PDK1 site in the activation loop of Pkc1 that is phosphorylated by Pkh1/2 shows similarity to other AGC kinase family members including Ypk1, Ypk2 and Sch9 [121]. This similarity argues that LCBs stimulate Pkh1/2 to phosphorylate these kinases at their PDK1 site thereby partially activating them. Recently PHS has been shown in vitro to stimulate Pkh1 to phosphorylate Ypk1, Ypk2 and Sch9 (Fig. 2A) [125]. Further experiments showed that about half of the total stimulation in activity occurred when Ypk1, Ypk2 or Sch9 were treated with PHS, indicating that PHS partially activates these kinases. Maximal activation in vitro requires both Pkh1 and PHS. It has not been determined if PHS acts directly on Pkc1 to stimulate activity.

Until this experimental evidence, there was no known upstream activator of Sch9, which is most closely related to mammalian protein kinase B/Akts [121]. Sch9 plays many interesting but mechanistically unclear roles in cells including heat stress resistance [146,147], chronological aging [148], Ty1 transposition [149], cell size [150], entry into [151] and exit from stationary phase [152], homologous recombination in ribosomal gene hot spots [153] and adaptation to changes in nutrients [154]. Because these cellular processes are so diverse and since LCBs are only known to increase during heat stress, it seems likely that other upstream activators will be found to regulate Sch9.

It is not known how LCBs stimulate autophosphorylation of Pkh1/2, Ypk1/2 and Sch9 nor has the site(s) of phosphorylation been identified experimentally. However, the LCB-stimulated phosphorylation site is most likely to be the PDK1 site in the activation loop, since this site is necessary for enzyme activity and can be autophosphorylated by these protein kinases [121,125,155]. Full activation of mammalian homologs of Ypk1, Ypk2 and Sch9 require phosphorylation at other sites including the PDK2 site in the C-terminal hydrophobic motif [121] and the turn motif between the activation loop and the hydrophobic motif [156]. Thus, it is likely that the corresponding sites in yeast homologs must also be phosphorylated for full protein kinase activity. As mentioned above, the Tor2 protein kinase phosphorylates the PDK2 site and the turn motif in Ypk2 [134]. Phosphorylation of these sites is physiologically important because without phosphorylation actin patches do not form in small and medium sized daughter cells as they bud from mother cells and this blocks budding. High amino acid similarity in the turn and hydrophobic motif of Ypk2 and Ypk1 suggests that Tor2 phosphorylates Ypk1 also, but this needs to be determined experimentally. There is less conservation of these regions in Sch9 and Tor2 may not be responsible for phosphorylation of the PDK2 and turn sites in Sch9. The mammalian homolog of Tor2, mTOR, is known to phosphorylate the hydrophobic motif in the p70 S6 protein kinase which is closely related to Ypk1/2 [157]. Such phosphorylation plays a critical role in fostering CAP-dependent translation initiation [158]. Ypk1 may also play a role in translation initiation since *ypk1*Δ cells fail to make large polysomes and show other defects in the translation initiation machinery during nitrogen starvation [120] or heat stress [114].

The Tor2 results help to clarify how Ypk1/2 and Tor2 (as part of the TORC2 protein complex) regulate actin polarization and endocytosis and how they function upstream of Rho1 [119,135,137]. One set of experiments suggested that that Ypk1/2 functioned upstream of the Pkc1-MAPK cascade to regulate Slt2 [117]. In contrast, other experiments suggested a role for Ypk1/2 in a pathway parallel to the Pkc1-MAPK cascade, but they did not rule out the possibility of Ypk1 working upstream of Pkc1 [118]. The new data are most consistent with

Tor2 phosphorylating Ypk2 (and probably Ypk1) at the PDK2 site in the hydrophobic motif as well as in the turn motif thereby generating a substrate that can be phosphorylated at the PDK1 site by Pkh1/2, just as in the case for p70 S6 kinase [159]. Activated Ypk2 then turns on Rho1 in an unknown manner followed by Rho1 activation of Pkc1 and the MAP kinase cascade which control actin polarization [129] (Fig. 3).

Finally, while it is clear that LCBs stimulate the activity of Pkh1/2, Ypk1/2 and Sch9 it is not clear how they do so. The thought is that LCBs, similar to phosphoinositides, bind to a domain in protein kinases, but thus far no such domain has been identified.

6. Other roles for sphingolipids in *S. cerevisiae*

One of the roles described first for sphingolipids, specifically ceramide, was to promote transport of secretory vesicles from the ER to the Golgi apparatus [97,160,161]. Ceramide is also used to replace a large fraction of the diacylglycerol moiety in glycosylphosphatidylinositol-anchored proteins in *S. cerevisiae* [162–164] and ceramide or inositol-containing sphingolipids are required for stable association of glycosylphosphatidylinositol-anchored proteins to the plasma membrane in yeast [165].

Sphingolipids are an important lipid component of microdomains (lipid rafts) along with a sterol: cholesterol in mammals and ergosterol in fungi and plants. Rafts in yeast play vital roles for delivering and sorting membrane-bound proteins to their correct cellular destination and they are also necessary for fusion of cells during mating [45,166,167]. These membrane-bound proteins include Pma1 [168–172], Gas1 and Nce2 [170], Fus2, Fig. 1, Sho1, Ste1 and Prm1 [173], Fur4 [67,105], and Can1 [174]. Roles for sphingolipids in exocytosis based upon suppressor mutant analyses were reviewed previously [2]. One new observation is that cells lacking the v- and t-SNAREs involved in endocytosis are rescued by what appears to be activation of the so called ceramide-activated protein phosphatase, CAPP [175]. Addition of C_2-ceramide to the culture medium, deletion of *sur4*, which causes accumulation of LCBs and LCBPs and maybe ceramides, or overexpression of *SIT4*, encoding the catalytic subunit of CAPP, restored endocytosis and were proposed to work by increasing CAPP activity, which would dephosphorylate Tlg1/2 and restore SNARE function [176]. These results are interesting and unique, but need to be verified by in vitro experiments using purified systems to be sure that ceramide and not some other sphingolipid activates CAPP in vivo. Finally, a screen for genes that are necessary for protein sorting in the *trans*-Golgi network for delivery via exocytosis to the cell surface revealed a role for genes in sphingolipid metabolism (*SUR2*, *SUR4*, *YPC1* and *AYR1*) as well as for ergosterol synthesis [167].

In mammals, sphingosine-1-phosphate is involved in intracellular calcium signaling through an unknown mechanism [177]. Likewise, sphingolipids may regulate calcium fluxes and signaling pathways in yeast, but how this happens is unclear (reviewed in [2]).

The *LAG1* (Longevity-Assurance Gene) [178] was the first gene isolated in yeast that extends the replicative lifespan, that is, the number of times that a mother cell can produce daughter cells. A related gene, *LAC1*, also affects lifespan and, as discussed in section 2.1, they encode ceramide synthases. It is still not known how Lag1 and Lac1 regulate life-span and aging [2,179] and any new mechanistic insight would be a significant contribution to the longevity and aging field.

LCBs play roles in nutrient transport as first indicated when they were found to inhibit tryptophan uptake [180]. PHS, but not related LCBs, was then shown to inhibit uptake of tryptophan, leucine, histidine and uracil [181]. It is not entirely clear how LCBs are regulating nutrient transport, except in the case of uracil where PHS has been shown to be important for heat-induced, ubiquitin-mediated breakdown of Fur4, the uracil transporter [104]. Since LCBs have been shown to regulate the activity of Ypk1 and Sch9 both, directly and via activation of the upstream kinases Pkh1/2 (Fig. 2A) [125], and since Ypk1 and Sch9 are though to be involved in nutrient sensing [120,154,182], further research may reveal that LCBs work through signaling pathways regulated by Ypk1 or Sch9 to modulate the activity and/or turnover of nutrient transporters in the plasma membrane.

Cross-talk between sphingolipids and both ergosterol and glycerophospholipids has been suggested. In one set of experiments mutations in SUR4 were found to suppress the growth defect of ergosterol mutants [183]. Other experiments indicate that yeast cells have mechanisms for maintaining the asymmetry in the distribution of sphingolipids and glycerophospholipids in the two leaflets of the plasma membrane so that a change in lipid class is compensated by a change in the other [88].

Specific roles for complex yeast sphingolipids, IPCs, MIPCs and $M(IP)_2Cs$, have only begun to be identified. $M(IP)_2C$ is now known to be essential for the antifungal action of some plant defensins and syringomycin E. Several lines of evidence show that $M(IP)_2C$ is needed for high affinity binding of DmAMP1, a peptide defensin produced by *Dahlia merckii* [184]. A current model envisions that DmAMP1 interacts with $M(IP)_2C$-containing lipid rafts and induces membrane permeabilization [185].

Syringomycin E is an antifungal cyclic lipodepsinonapeptide that interacts with the plasma membrane and inhibits growth of *S. cerevisiae* cells by forming ion channels. Yeast mutants defective in *ipt1*, *fen1* or *sur4*, *scs7* and *sur2* are drug resistant, showing that $M(IP)_2C$ with a C_{26}-fatty acid and PHS but not DHS is essential for the antifungal action of syringomycin E [21,186,187].

7. Concluding remarks

Over the past fifteen years our knowledge of sphingolipid functions in yeast has advanced remarkably. Much of this advancement is due to the identification of most, if not all, of the genes necessary for sphingolipid metabolism in *S. cerevisiae* [3]. These genes are an extraordinary resource and will continue to provide a unique tool for studying sphingolipid functions in *S. cerevisiae*. The genes will also facilitate studies of sphingolipids in pathogenic fungi and may lead to the development of more efficacious antifungal drugs for which there is an urgent need [188].

Much of what we have learned so far about sphingolipids in yeast is descriptive or phenomenological, thus, a necessary goal for future studies is to identify the underlying molecular mechanisms by which sphingolipids exert their actions. Achieving this goal will be considerably more difficult than identifying genes. One important lesson from using mutant strains defective in sphingolipid metabolism is that care must be taken in interpreting cause and effect because a block, while ridding the cell of one or more sphingolipids, also tends to cause accumulation of other sphingolipids.

One challenge for the future is to understand how sphingolipid synthesis is regulated and coordinated with cell growth. There is evidence for transcriptional regulation by Pdr1 and Pdr3 of a few sphingolipid biosynthetic genes including *LAG1* and *LAC1*, which encode ceramide synthase [189]. However, this regulation has only a small effect on sphingolipid levels and cannot explain how sphingolipid synthesis is related to growth. There is evidence that the protein kinase Cka2, casein kinase 2, regulates ceramide synthase activity, but the mechanism has not been determined [190]. These two results suggest that ceramide is an important intermediate in yeast sphingolipid metabolism as is the case in mammals [68,191]. Transcriptional regulation of a few other sphingolipid genes has been observed during global surveys of gene expression under a variety of growth and stress conditions (summarized at the SGD and MIPS websites under ''Yeast Microarray Global Viewer''). Even with this information it is unclear how sphingolipid synthesis is tied to cell growth and it appears that translational and post-translational mechanisms probably mediate the necessary coordination of synthesis and growth.

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