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# Distribution and Functions of Sterols and Sphingolipids

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Sterols and sphingolipids are considered mainly eukaryotic lipids even though both are present in some prokaryotes, with sphingolipids being more widespread than sterols. Both sterols and sphingolipids differ in their structural features in vertebrates, plants, and fungi. Interestingly, some invertebrates cannot synthesize sterols *de novo* and seem to have a reduced dependence on sterols. Sphingolipids and sterols are found in the plasma membrane, but we do not have a clear picture of their precise intracellular localization. Advances in lipidomics and subcellular fractionation should help to improve this situation. Genetic approaches have provided insights into the diversity of sterol and sphingolipid functions in eukaryotes providing evidence that these two lipid classes function together. Intermediates in sphingolipid biosynthesis and degradation are involved in signaling pathways, whereas sterol structures are converted to hormones. Both lipids have been implicated in regulating membrane trafficking.

Typical examples of eukaryotic lipids, sterols, and sphingolipids can both be found in membranes from simple unicellular fungi and protists to multicellular animals and plants. Their versatile use as structural elements but also as signaling molecules has probably played an important role during the evolution of this large and diverse group of organisms. There are also many eukaryotes that have lost the ability to synthesize sterols *de novo* including nematodes, insects, and marine invertebrates, which have to take up sterols with their diet. Sterol biosynthesis has also been reported in a number of

bacteria. Sphingolipids are more widely spread among prokaryotes than sterols and also show a greater variety of structures among the different eukaryotes.

In this short review we will first give an overview about the diversity of sterol and sphingolipid structures and their distribution in nature. Then we will discuss their subcellular distribution. A brief technical section will add some information on the separation and detection of these lipid molecules. Subsequently, we will summarize different genetic approaches to study the functions of sterols and sphingolipids, and finally, we will discuss the functional and possible physical interactions of the two lipid classes within the cell. Far from being

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<sup>1</sup>These authors contributed equally to this article.

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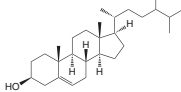
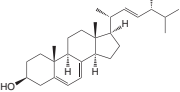
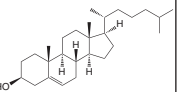

comprehensive, we will focus only on a few interesting aspects and try to give new view points, which are less frequently discussed.

### STRUCTURES AND DISTRIBUTION AMONG SPECIES

Sterol biosynthesis probably evolved after the emergence of oxygen about 2.5 billion years ago (Galea and Brown 2009). The first dedicated step, which is common to all sterol biosynthetic pathways, is the mono-oxygenation of squalene catalyzed by Squalene Epoxidase/Erg1p and uses molecular oxygen. Even at this early step in the pathway, the cyclization of the linear precursor, which gives rise to the polycyclic sterol ring structure, shows a diversification between kingdoms, with cycloartenol as the product in most algae and plants and lanosterol in fungi and animals (Volkman 2003). Recently, it was found that some plants also have the ability to produce lanosterol, which can contribute

to phytosterol biosynthesis (Ohyama et al. 2009). Both cycloartenol synthase and lanosterol synthase are closely related to each other and to the bacterial squalene-hopene cyclases, which produce the bacterial polycyclic hopenoids without a requirement for molecular oxygen and which are most likely the evolutionary ancestors of the sterol biosynthetic enzymes. Cycloartenol and lanosterol are further metabolized via (de-) methylations and double-bond modifications to give rise to the final sterol products: several C-24 alkylated phytosterols, C<sub>28</sub> campesterol, C<sub>29</sub> sitosterol, and stigmasterol in plants (Benveniste 1986), a single C-24 methylated C<sub>28</sub> ergosterol in most fungi (Weete et al. 2010), and the single nonmethylated C<sub>27</sub> cholesterol in animals (Gaylor 2002) (Table 1). Most invertebrates including the important genetic model organisms *Drosophila melanogaster* and *Caenorhabditis elegans* have lost the ability to synthesize sterols (Clayton 1964; Kurzchalia and Ward 2003). They are

**Table 1.** Sterol and sphingolipid structures and distribution among species.

Organism	Plants	Fungi	Vertebrates	Auxotrophic invertebrates	Bacteria
<b>Main sterol</b>	Campesterol (R = methyl) Sitosterol (R = ethyl) Stigmasterol (R = ethyl, Δ22) 	Ergosterol, some exceptions 	Cholesterol 	Modifications of dietary sterols 	Generally absent, very few exceptions
<b>Main sphingoid base</b>	Several bases including: d18:1 <sup>Δ8*</sup> d18:2 <sup>Δ4,Δ8*</sup> t18:1 <sup>Δ8*</sup>	Variety of structures <i>S. cerevisiae</i> : sphinganine (d18:0*) phytosphingosine (t18:0*) <i>P. pastoris</i> : 9-methylsphinga-4,8,-dienine	Sphingosine (d18:1 <sup>Δ4*</sup> ), several others present	<i>D. melanogaster</i> : C <sub>14</sub> sphingosine <i>C. elegans</i> : C <sub>17</sub> iso-sphingosine	Mostly absent, some exceptions
<b>Higher sphingolipids</b>	-Inositolphosphoryl glycolipids -Glycosphingolipids (mainly glucosyl-, some mannosylceramide series)	-Inositolphosphoryl glycolipids -Glycosphingolipids <i>S. cerevisiae</i> : only IPC <sup>†</sup> , MIPC <sup>†</sup> , M(IP) <sub>2</sub> C <sup>†</sup>	-Sphingomyelin -Glycosphingolipids (mainly glucosyl-, some galactosylceramide series)	-Sphingomyelin ( <i>C. elegans</i> ) -Phosphoethanolamine ceramide ( <i>D. melanogaster</i> ) -Glucosyl ceramide series	Mostly absent, some exceptions with glucosyl ceramide series
<b>SREBP</b>	Not found	Oxygen sensor, but not found in <i>S. cerevisiae</i>	SREBP1a/c regulate fatty acid & fat metabolism SREBP2 regulates sterol metabolism	Regulates fatty acid and fat metabolism	Not found

\*Shorthand nomenclature for sphingoid bases “aXX:YΔZ”: a-number of hydroxylgroups ( $d = 2, t = 3$ ); XX-number of carbon atoms; Y-double bonds, ΔZ-position of double bonds.

<sup>†</sup>IPC-inositolphosphorylceramide, MIPC-mannosylinositolphosphorylceramide, M(IP)<sub>2</sub>C-mannosyldiinositolphosphorylceramide.



dependent on external sterol sources and use some of the remaining conserved sterol biosynthetic enzymes for sterol modifications, including steroid hormone production (Vinci et al. 2008) and regulation (Hannich et al. 2009).

Another very important feature of sterol biosynthesis in vertebrates is its regulation by the membrane bound basic helix-loop-helix (bHLH) transcription factor SREBP (Osborne and Espenshade 2009). The vertebrate genome contains two copies encoding three isoforms with SREBP-2 regulating mainly cholesterol biosynthesis and SREBP-1a and c regulating mainly fatty acid and phospholipid metabolism as well as fat storage. Invertebrates have one copy of SREBP, which has been shown to regulate fatty acid and phospholipid metabolism in flies and fatty acid metabolism and fat accumulation in worms (see Ye and Debose-Boyd 2011). Although no SREBP homolog has yet been found in *Saccharomyces cerevisiae*, other fungi like *Schizosaccharomyces pombe* also express a copy that serves as a sterol-dependent oxygen sensor that regulates oxygen-dependent steps in ergosterol, heme, and sphingolipid metabolism (Todd et al. 2006). So far no SREBP homolog has been described in plants even though a number of membrane-bound bHLH transcription factors still await further characterization.

A detailed description of sphingolipid biosynthesis can be found in recent reviews (Hannun and Obeid 2008; Futerman and Riezman 2005). Sphingolipids are a very diverse class of lipids, which is predicted to include tens of thousands or maybe more distinct structures (Merrill et al. 2009). This diversity is due to combinations of structural varieties of sphingoid bases, the N-acylated fatty acid, and the polar head group. The basic structure of the central building block, the sphingoid/long-chain base (LCB), depends on the substrate specificity of its biosynthetic enzymes.

The main vertebrate LCB is D-erythro-sphingosine (short d18:1Δ4E for (2S,3R,4E)-2-amino-octadec-4-ene-1,3-diol; see Table 1 legend for an explanation of the shorthand nomenclature) but in addition also substantial amounts of C<sub>20</sub> and minor amounts of C<sub>12</sub> to

C<sub>22</sub> (Han and Jiang 2009) as well as 1-deoxy and 1-deoxymethyl long chain bases can be found (Penno et al. 2010). Differences in double bonds and base hydroxylation additionally give rise to sphinganine (d18:0), phytosphinganine (t18:0), and their analogues. Also in plants C<sub>18</sub> sphingoid bases, especially with Δ8 and Δ4Δ8 double bonds, predominate (Pata et al. 2010). Many fungi additionally methylate the alkyl chain at C-9, giving rise to C<sub>19</sub> bases (Ternes et al. 2006), but the baker's yeast *S. cerevisiae* produces only sphinganine and phytosphinganine (mainly d18:0 and t18:0, some d20:0, and t20:0). In invertebrates other chain lengths are abundant. Flies produce mainly C<sub>14</sub> and some C<sub>16</sub> straight chain bases (Acharya and Acharya 2005), whereas nematodes build their sphingolipids from C<sub>17</sub> iso-branched sphingoid bases (Chitwood et al. 1995). Free LCBs exist only at very low levels in the cell. Most of these can be phosphorylated to form sphingosine-1-phosphate and other phosphorylated sphingoid bases. The majority of sphinganine is transformed into dihydroceramide (likewise for other sphingoid bases) by N-acylation with fatty acids ranging from C<sub>14</sub> to C<sub>26</sub> or perhaps even up to C<sub>36</sub> in rare cases. The majority of incorporated fatty acids are saturated or monounsaturated and can be modified with an α and one additional hydroxylation. Great diversity between species is created by different polar head groups that are attached to the C-1 hydroxyl group of the ceramide backbone to create higher sphingolipids, including phosphosphingolipids, glycosylinositolphosphorylceramides, and glycosphingolipids. Phosphorylation leads to ceramide-phosphate but also the phosphobases phosphocholine and phosphoethanolamine can be attached. These give rise to sphingomyelin, which is abundant in vertebrates and some invertebrates like *C. elegans*, and phosphoethanolamine ceramide, which is only produced in very low amounts in mammalian cells (Vacaru et al. 2009) but replaces sphingomyelin in *D. melanogaster* (Acharya and Acharya 2005). Plants and fungi have a series of glycolipids that are derived from inositolphosphoryl ceramide (IPC), which can have a diverse range of more or less complex (modified) sugar chains

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attached to it (Pata et al. 2010). Plants, fungi, invertebrates, and vertebrates all also transfer sugars directly onto the ceramide moiety to form glycosphingolipids. In plants, glucosylceramide predominates and serves as a basic structure for series of up to tetraglycosylceramides but also some mannosylceramide series are produced (Sperling and Heinz 2003). Glucosyl- and to a lesser degree galactosylceramides are also widely spread among fungi, even though *S. cerevisiae* does not show any glycosylceramides. In invertebrates and vertebrates, hundreds of different head group structures are formed by adding different chains of sugar molecules and their derivatives to the first sugar moiety, which is mainly glucose. In addition, vertebrates also show a series of galactosylceramide-based glycolipids. In *S. cerevisiae* the higher sphingolipids are inositolphosphoryl ceramide (IPC), and its derivatives mannosyl inositolphosphoryl ceramide (MIPC), and mannosyl di-inositolphosphoryl ceramide (M(IP)<sub>2</sub>C) (Dickson and Lester 1999).

Very few studies have shown unequivocally the presence of sterols in prokaryotes, but both biochemical and genomic analysis have identified a fairly elaborated sterol biosynthetic pathway in *Methylococcus capsulatus* producing also demethylated sterols (Bird et al. 1971; Lamb et al. 2007) and a very basic pathway in *Gemmata obscuriglobus* that only produces cycloartenol and its isomer parkeol (Pearson et al. 2003). Most likely the last eukaryotic common ancestor (LECA) produced an elaborate set of sterols via the cycloartenol pathway and differences with modern day eukaryotes are characterized by small changes, including side chain methylations (Desmond and Gribaldo 2009). Although these rare observations of bacterial sterol biosynthesis were initially explained by horizontal gene transfer from an early eukaryote, the dynamic endo-membrane system of *G. obscuriglobus* and the recent discovery of its membrane coat-like proteins, both additional key eukaryotic features, have tempted others to suggest a close relationship of these bacteria to the origin of eukaryogenesis (Santarella-Mellwig et al. 2010). To date no side-chain C-24 alkylation has been observed in prokaryotes and this

metabolic feature is therefore used as a signature for different eukaryotic traces in paleogeology (Summons et al. 1988).

Production of sphingolipids in bacteria is more widespread. The first crystal structure for a sphingolipid biosynthetic enzyme was obtained from a soluble bacterial serine palmitoyl transferase (SPT) (Yard et al. 2007). Recently, the sphingosine 1 phosphate lyase structure was determined from *Symbiobacterium thermophilum* (Bourquin et al. 2010). In some species like *Sphingomonas capsulata*, glycosphingolipids functionally replace lipopolysaccharide in the outer membrane (Geiger et al. 2010). It can be assumed that sphingolipid biosynthesis first evolved in prokaryotes from other members of the  $\alpha$ -oxoamine synthase family, which fuse organic acids to amino acids, and probably was already present in the LECA. So far, no prokaryotes with both sphingolipid and sterol synthesis have been discovered to our knowledge.

#### DISTRIBUTION WITHIN CELLS

It is a long-standing dogma that concentrations of sterols increase along the secretory pathway, being very low in the ER, higher in the Golgi, and highest in the plasma membrane. Sterols have also been shown to be enriched in early and recycling endosomes, but not late endosomes. In lipid storage diseases such as Niemann-Pick type C (NPC), sterol and sphingolipids are abnormally accumulated in late endocytic compartments (Mesmin and Maxfield 2009). In our opinion this dogma still needs to be confirmed by unequivocal experimental data. Many studies on sterol localization depend on binding to filipin, but we do not have sufficient molecular insights into how filipin binds cholesterol and other sterols as the structure of the complex has not been determined. There is also substantial experimental data suggesting that the evaluation of sterol concentration by filipin binding is dependent on membrane composition (see Jin et al. 2008, as an example). The sterol content of a specific organelle can also be measured by subcellular fractionation and lipid quantification, however many

of the previous studies, while using state of the art purification techniques, did not analyze pure fractions. As will be discussed later, current advances in mass spectrometry and organelle immunopurification should provide comprehensive information about the lipid composition of isolated organelles, thereby allowing us to address the reality of the dogma. Recently, highly purified ER membrane was prepared, and subsequent quantification by mass spectrometry showed a very delicate threshold of sterol regulatory element-binding protein (SREBP)-mediated cholesterol sensing (Radhakrishnan et al. 2008): the ER cholesterol concentration above or below 5 mol% is sharply distinguished. In addition, a quantitative shotgun lipidomics approach (Ejsing et al. 2009) showed that post-Golgi secretory vesicles are enriched in sterols and sphingolipids, consistent with the idea that lipid sorting occurs in the *trans*-Golgi network (Klemm et al. 2009). In this study, the vesicles were purified from a conditional yeast mutant. It is highly difficult to isolate organelles without contamination, for example separation of Golgi and endosomes is generally difficult and even immunoisolation techniques will have to rely on proteins other than the many that cycle between the two compartments. Due to the high sensitivity of mass spectrometry it is no longer necessary to isolate large amounts of purified organelles for analysis and therefore, more sophisticated and selective techniques can now be used. It is important to develop such techniques in order to gain insight into normal and abnormal states, such as normal and NPC endosomes.

In model membranes, under optimal conditions of lipid composition and temperature, sterols and sphingolipids are laterally segregated from other lipids to form different physical phases, including a liquid-ordered phase. The concept of “lipid raft” states that these phase separations also occur in biological membranes, incorporating specific proteins that preferentially interact with these two lipids. Currently, lipid rafts are considered to be highly dynamic nanoscale structures (Lingwood and Simons 2010).

As mentioned above, sterols have been localized by making use of sterol-binding

compounds, including fluorescent filipin, which has long been used for this purpose. In both yeast and mammalian cells, filipin stains the plasma membrane and intracellular compartments, probably the endosomes and perhaps the Golgi. Using filipin, genome-wide screening for genes regulating sterol localization was conducted in both yeast and mammalian cells. These studies identified many genes, suggesting the complexity of intracellular sterol localization. For example, in yeast mutants deleted for phospholipase C (*PLC1*) or protein phosphatase type 2C (*PTC1*) gene, intense filipin staining was observed in intracellular compartments, probably late endosomes (Fei et al. 2008). Thus, signal transduction pathways involving these proteins may sense and/or control sterol homeostasis. The screening of mammalian cells identified a novel endolysosomal protein TMEM97 whose knockdown reduced the intensity of filipin staining as well as uptake of fluorescent LDL, and this protein was co-immunoprecipitated with NPC1 (Bartz et al. 2009). Independent of such screening, abnormal filipin staining was observed in yeast mutants defective in GPI biosynthesis (Schonbachler et al. 1995; Kajiwara et al. 2008). Some *gpi* mutants are also defective in sphingolipid biosynthesis, possibly affecting the step of ceramide transport, suggesting that GPI-anchored proteins may facilitate sterol and sphingolipid transport to the cell surface. The existence of the GPI moiety, whose structure is remodeled from a diacylglycerol to ceramide in yeast may stabilize or participate in sterol-sphingolipid interactions affecting transport.

Ergosterol biosynthesis is a common target of antifungal drugs, such as polyene antibiotics amphotericin B, natamycin, and nystatin. Recently, a natural antifungal compound theonellamide (TNM) was shown to target  $\beta$ -hydroxysterol (Ho et al. 2009; Nishimura et al. 2010). TNM is an unusual bicyclic peptide and represents a novel class of sterol-binding compounds. As described below, yeast cells can be stained using a fluorescent version of TNM.

The initial steps of sphingolipid biosynthesis, including sphingoid base and ceramide formation, take place in the endoplasmic reticulum

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(Futerman and Riezman 2005). Higher sphingolipids are produced in the Golgi apparatus where they are transported by different mechanisms. In mammalian cells, ceramides for glycosphingolipid production seem to be transported via a different pathway than SM, which is produced from a specific ceramide pool transported via a nonvesicular pathway mediated by the CERT protein (Hanada et al. 2003). In yeast cells both vesicular and nonvesicular ER to Golgi ceramide transport exists (Funato and Riezman 2001). Most higher sphingolipids are transported to the plasma membrane.

### METHODS OF SEPARATION AND DETECTION

Mass spectrometric shotgun lipidomics as described by Schwudke et al. (2011) is a powerful analytical technique with many advantages but also limitations with respect to sterol and sphingolipid analysis even though careful sample preparation and high dilutions can reduce ion suppression by glycerophospholipids in the nonseparated extract. Utilization of a chip-based nano-spray setup provides high reproducibility, whereas removal of phosphoglycerolipids and triacylglycerides by base treatment makes it possible to detect a variety of sphingolipids (Jiang et al. 2007), although there are isomeric species that cannot be distinguished (like glucosyl and galactosyl ceramides) and many overlapping isotopic species that could be resolved by high mass accuracy fourrier transformation mass spectrometry often show signals that are too weak to gain structural information based on fragmentation. Different protocols based on high performance liquid chromatography coupled to ESI-MS have been tailored for specific lipid classes (Sullards et al. 2007). Although column separation increases sensitivity and structural information it also introduces problems to the analysis, including changing ionization conditions during the course of the analysis and column memory. For quantitative analysis it is important to ensure that internal standards elute under the same ionization conditions as the respective analytes, which is easier on normal-phase

columns in which lipids elute as lipid classes. Absolute quantification would require a standard curve for every analyzed molecule at the respective ionization condition or a corresponding stable isotope labeled standard for each molecule. Although both possibilities are not realistic in a vertebrate system the second one is actually feasible for the rather simple sphingolipidome of *S. cerevisiae* (Ejsing et al. 2009). In most systems a sufficient number of internal standards containing unnatural and stable isotope labeled lipids can ensure semi-quantitative lipid analysis.

In addition, the precise identification of most sterols cannot be determined based solely on mass measurements. Different chromatographic methods have been developed to increase sensitivity by removing ion-suppressing background and to differentiate molecular structures based not only on mass and fragmentation but also on retention time, which is determined by structural differences even between isomeric species. In the case of sterols, gas chromatography coupled to an EI-source has proven to give reliable results (Guan et al. 2009).

### GENETIC APPROACHES TO STEROL AND SPHINGOLIPID FUNCTION

Both sterols and sphingolipids play important structural roles in many cellular membranes. Sterols have been shown to influence fluidity and permeability of membranes, both in vitro and in vivo (Haines 2001; Emter et al. 2002). Still, at least some eukaryotic cells, like cultured insect cells, can function without almost any sterol in their membranes (Silberkang et al. 1983) and it has been proposed that sphingolipids with dien-sphingoid bases can replace sterols in the membrane (Carvalho et al. 2010). Certain glycosphingolipids have also been proposed to serve a structural role, for example in the chilling response of plants (Pata et al. 2010). Another very important role of sterols that has to be kept in mind when studying their cellular functions is the production of different signaling molecules. Multicellular organisms rely on sterol-derived hormones like the steroids of vertebrates, brassinosteroids of plants and

ecdysteroids, or other molting hormones of invertebrates. Other sterol-derived signaling molecules include Vitamin D, bile acids, and oxysterols (Kurzchalia and Ward 2003).

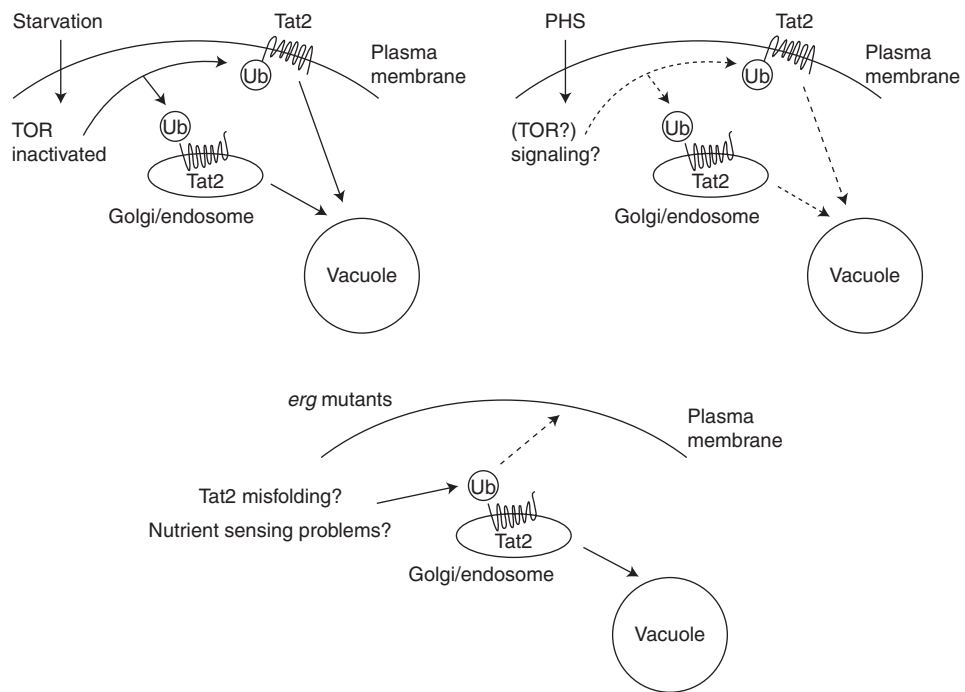
Yeast genetics has been a powerful tool to elucidate the biosynthesis and functions of sterols and sphingolipids. Mutants defective in various steps of these metabolic pathways were isolated. Many efforts have focused on mutants (*erg2*, *erg3*, *erg4*, *erg5*, and *erg6*) defective in the last five steps of the biosynthesis, because each is a nonessential step. In these mutants, ergosterol is absent and specific sterol intermediates are accumulated. The *erg* mutants are defective in many cellular processes, indicating that ergosterol cannot be replaced by the sterol intermediates. The differences between ergosterol and the intermediates are the desaturation status of the A and B rings and the side chain modifications. Despite the slight differences from one another, each *erg* mutant has distinct defects. For example, endocytic internalization from the plasma membrane is strongly affected in the *erg2* mutant, defective in the C-8 sterol isomerase activity, but not drastically altered in the *erg6* mutant, defective in the C-24 sterol methyltransferase activity (Munn et al. 1999; Heese-Peck et al. 2002). This highlights the structural importance of sterol. Because yeast cells do not take up sterol from the medium under aerobic conditions, the sterol composition of each mutant is clearly defined and can be quantitatively determined by GC-MS. Notably, changes in ergosterol structure in the different *erg* mutants confer different sphingolipid compositions, without affecting glycerophospholipids, as determined by mass spectrometry (Guan et al. 2009). Thus, cells adjust their sphingolipid profile in response to changes in sterol structures, strongly suggesting that sterols and sphingolipids interact, perhaps physically. Using combinations of mutations in sterol and sphingolipid biosynthesis a number of synthetic and suppression phenotypes were found. These findings led to the conclusion that sterols and sphingolipids interact functionally in biological membranes.

It is theoretically possible that changes in membrane fluidity could account for many of

the defects in *erg* mutants. However, results measuring plasma membrane fluidity by fluorescence anisotropy of TMA-DPH did not correlate with the severity of the mutant phenotypes (Guan et al. 2009). It was postulated that defective protein-lipid interactions in the *erg* mutants caused the defects. One hypothesis is that some membrane proteins cannot fit into the altered membranes and their folding or activities are impaired. One of the noticeable phenotypes of the *erg6* mutant is reduced uptake of tryptophan from the medium (Gaber et al. 1989). This is because the high-affinity tryptophan permease Tat2 is inappropriately ubiquitinated and missorted to the vacuole (Fig. 1) (Umebayashi and Nakano 2003). The missorting of Tat2 is common to other *erg* mutants (Daicho et al. 2009; Guan et al. 2009), indicating that the sorting of Tat2 is very sensitive to sterol structures, strictly requiring ergosterol. One possibility is that Tat2 interacts with sterol-sphingolipid complexes and, in the *erg* mutants, Tat2 is misfolded and placed under ubiquitin surveillance. Identification of other membrane proteins that undergo ubiquitination in the *erg* mutants would help us to explain their diverse phenotypes. Alternatively, the *erg* mutants may have a defect in signaling, such as amino acid sensing, so that they always sort Tat2 to the vacuole. There have been two examples of signaling involved in Tat2 sorting. First, plasma membrane localization of Tat2 is regulated by the nutrient availability, which is sensed by the target of rapamycin (TOR) kinase (Fig. 1). Following starvation, TOR is inactivated and Tat2 is sorted to the vacuole in a ubiquitin-dependent manner (Beck et al. 1999). Downstream from TOR, the Npr1 kinase and the type 2A phosphatase-associated protein Tap42 are involved in this regulation (Schmidt et al. 1998). Second, addition of the exogenous LCB phytosphingosine (PHS) inhibits tryptophan uptake, most likely by transducing a signal that down-regulates Tat2 (Fig. 1) (Skrzypek et al. 1998). In addition to being substrates of ceramide synthases, LCBs are known as signaling molecules that activate yeast PDK1 kinase homologs Pkh1 and Pkh2 as well as AGC kinase homologs Ypk1 and Ypk2 (Sun



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**Figure 1.** Regulation of Tat2 sorting to the vacuole. (*Upper left*) Following starvation, the TOR signaling pathway is inactivated. This leads to ubiquitin-dependent sorting of both newly synthesized and plasma membrane-localized Tat2 to the vacuole. (*Upper right*) Exogenous PHS down-regulates Tat2, possibly by ubiquitination and vacuolar sorting. Signals activated by PHS remain to be elucidated. (*Lower*) In *erg* mutants, Tat2 is inappropriately ubiquitinated and sorted to the vacuole, not the plasma membrane. One possible reason is that Tat2 is misfolded by the altered sterol and sphingolipid composition. Alternatively, the sterol mutants may be defective in nutrient sensing.

et al. 2000; Friant et al. 2001; Liu et al. 2005). Although the putative signaling pathway between the exogenous PHS and Tat2 down-regulation has not been characterized yet, this may be linked to the TOR pathway. Aronova et al. (2008) showed that TOR complex 2 (TORC2) regulates ceramide synthesis by activating the Ypk2 kinase. Considering that Tat2 localization is influenced by TOR signaling, it is interesting that sterols and sphingolipids have recently been implicated in regulation of TORC1 and TORC2. In yeast, it was shown that TORC2 activity is synthetically impaired in a sterol and sphingolipid double mutant (Guan et al. 2009). In mammalian cells, cholesterol trafficking defects such as NPC deficiency reduce the activities of the two TORCs (Xu et al. 2010). Since TORC1 couples amino acid availability to cell growth, sterols and

sphingolipids may influence amino acid sensing through TORC1. Notably, TORC1 is associated with the vacuole/lysosome membrane (Sturgill et al. 2008; Sancak et al. 2010). Its activity and/or localization could be sensitive to the lipid content of this organelle.

Lipid rafts have also been proposed to act as signaling platforms, clustering signaling molecules for efficient transmission. During mating, haploid yeast cells are polarized into pear-shaped “shmoo,” and MAPK signaling is activated to direct signaling required for mating. At the shmoo tip, both filipin staining and the MAPK scaffold protein Ste5 are concentrated (Pryciak and Huntress 1998; Bagnat and Simons 2002). One of the common phenotypes among the *erg2–6* mutants is reduced mating efficiency, including the MAPK signaling and subsequent plasma membrane fusion.



Strikingly, the *erg3* mutant is defective in the polarized plasma membrane localization of Ste5 (Jin et al. 2008). Thus, correct sterol composition is required for the polarized recruitment of a signaling molecule. Ste5p binds to PI(4,5)P<sub>2</sub> through its PH domain, and this is important for its correct localization (Garrenton et al. 2006). It seems that PI(4,5)P<sub>2</sub> is concentrated at the shmoo tip in wild-type cells, but not in the *erg6* mutant (Jin et al. 2008). Further work is needed to understand how sterol influences the PI(4,5)P<sub>2</sub> distribution.

Although ergosterol is enriched in the plasma membrane, the localization of sterol intermediates seems to be somewhat different. When fission yeast cells are stained with the fluorescent TNM, the cell periphery is clearly labeled in wild-type but the labeling is very weak in the *erg2* or *erg3* mutant (Nishimura et al. 2010). The binding of TNM to the sterol fractions prepared from these mutants was normal, strongly suggesting that the sterol intermediates are not preferentially localized to the plasma membrane. Consistent with this, the *erg3* mutant of the budding yeast shows abnormal filipin staining with significant labeling of cytoplasmic spots (Fei et al. 2008). The reason for the altered localization is unclear, but the structural changes in sterol-sphingolipid complexes may facilitate clearance from the plasma membrane or provide limited access to sterol transport proteins. In any case, reduced sterol levels in the plasma membrane could affect the sorting and function of plasma membrane proteins such as Tat2.

The above results clearly indicate that subtle differences in sterol structure drastically affect various membrane-related cellular events in yeast. Also in mammals, it has been reported that defects in late cholesterol biosynthetic steps cause severe diseases. Mutations in a  $\Delta^8$ - $\Delta^7$  sterol isomerase were identified in human X-linked dominant chondrodysplasia punctata as well as its relevant disease in mice (Derry et al. 1999). This enzyme isomerizes a double bond in the B ring, the same reaction as catalyzed by yeast Erg2p. Indeed, both the murine and human enzymes complement the yeast *erg2* mutant to produce ergosterol (Silve et al.

1996). Although the C7-C8 double bond introduced by Erg2/ $\Delta^8$ - $\Delta^7$  sterol isomerase persists in ergosterol, it is removed by 7-dehydrocholesterol reductase (DHCR7) to form cholesterol. Mutations in the DHCR gene cause Smith-Lemli-Opitz syndrome with developmental abnormalities (Fitzky et al. 1999). Thus, unlike yeast, the C7-C8 double bond has to be removed in mammals. These diseases tell us that correct sterol biosynthesis is vital also in mammals. It is not clear whether the phenotypes seen in yeast and human mutants are due to the lack of cholesterol or the presence of sterol intermediates. If the former is the case the defects might be alleviated by feeding cholesterol. If the latter, this strategy would not work. As mentioned above, the yeast *erg* mutants are defective in mating. When the *erg4* mutant was forced to take up ergosterol under an anaerobic condition, it restored shmoo formation but not subsequent cell fusion (Aguilar et al. 2010). However, under these conditions, de novo ergosterol synthesis is shut down because of lack of oxygen and the only source of ergosterol is that taken up from the medium.

Sphingolipids, especially LCBs, ceramides and their phosphorylated forms are important signaling molecules (Fyrst and Saba 2010) whose numerous functions in vertebrates will not be described in detail here. Sphingosine 1 phosphate is an extracellular signaling molecule that binds to G-protein coupled receptors and controls cell migration and many other physiological parameters. Recently, intracellular functions of sphingosine 1 phosphate in vertebrates have been revealed in control of protein ubiquitination (Alvarez et al. 2010) and transcription (Hait et al. 2009). In yeast, recent studies integrating genomic, transcriptomic, and lipidomic data have revealed a role for phyto-sphinganine 1 phosphate in regulating genes involved in mitochondrial respiration (Coward et al. 2010). Another sphingolipid connection to mitochondrial function comes from the dual localization of the yeast sphingomyelinase homolog, Isc1p, which is found in both ER and mitochondria (Vaena de Avalos et al. 2004).

Genetic approaches have allowed the identification of most of the enzymes in sphingolipid



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metabolism in yeast (Dunn et al. 2000; Dickson et al. 2006) and many homologs have been found in other systems (Futerman and Riezman 2005). Sphingolipids play a large number of roles in yeast, many of which have been recently reviewed (Dickson 2008). A number of roles for LCBs in yeast have been identified by use of a temperature-sensitive mutant in serine palmitoyltransferase (*lcb1-100*) activity, which was originally isolated as an endocytosis mutant (*end8*) (Munn and Riezman 1994). LCBs were shown to activate the Pkh1 and 2 kinases to regulate the actin cytoskeleton and the internalization step of endocytosis (Friant et al. 2000, 2001; Zanolari et al. 2000). Interestingly, sphingolipid metabolism also affects the endocytic pathway in *D. melanogaster*, genetically interacting with components of the internalization machinery, such as dynamin (Rao and Acharya 2008). The *lcb1-100* also shows defects in membrane protein maturation and trafficking, including the plasma membrane ATPase (Lee et al. 2002; Gaigg et al. 2005, 2006) and GPI-anchored proteins (Horvath et al. 1994; Bagnat et al. 2000; Watanabe et al. 2002). In both cases, the evidence suggests that the synthesis of ceramides is important. For the GPI-anchored proteins this could be because of the fact that most GPI-anchored proteins are remodeled to contain ceramide (Reggiori et al. 1997) and that this step is required for their concentration into ER exit sites (Castillon et al. 2009). Sphingolipids also play roles in regulation of translation affecting formation of RNA processing bodies (Coward et al. 2010) and regulating translation initiation during the heat shock response (Meier et al. 2006).

*S. cerevisiae* mutants with a defect in synthesis of MIPC show a hypersensitivity to calcium ions (Beeler et al. 1997), but MIPC synthesis does not seem to be required for targeting of plasma membrane proteins (Lisman et al. 2004). However, in *S. pombe* MIPC does seem to play a role in plasma membrane protein localization (Nakase et al. 2010).

In mammalian systems a large variety of functions have been ascribed to sphingolipids and functional insights have also been derived from genetic approaches, including the

identification of CERT (Hanada et al. 2003) and insights derived from the analysis of knockout mice, including mutants in glycosylceramide synthase (Yamashita et al. 1999; Sprong et al. 2001) and ceramide synthase 2 (Imgrund et al. 2009; Pewzner-Jung et al. 2010a,b). Mutants in ceramide synthases in *D. melanogaster* (Bauer et al. 2009) and *C. elegans* (Deng et al. 2008) have also provided new functions for sphingolipids in these organisms, including regulation of the response to anoxia (Menuz et al. 2009), while mutants in other steps of sphingolipid synthesis have been linked to gut development (Marza et al. 2009). We are still early in the period of the use of genetics to study sphingolipid and sterol function in animals and together with the new tools of lipidomics we can expect an exciting period in the years to come.

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