Tools for the analysis of metabolic flux through the sphingolipid pathway

Fernando Martínez-Montañés and Roger Schneider

University of Fribourg, Department of Biology, 1700 Fribourg, Switzerland

Running Title: Flux analysis through the sphingolipid pathway

Key Words: long-chain base, ceramide, sphingolipids, fluorescent sphingolipid, stable isotope, odd-chain tracer, click-chemistry, metabolic flux, fungal sphingolipids, mass spectrometry

Corresponding author: Roger Schneider, University of Fribourg, Department of Biology, Chemin du Musée 10, 1700 Fribourg, Switzerland; email: roger.schneider@unifr.ch

Abstract

Discerning the complex regulation of the enzymatic steps necessary for sphingolipid biosynthesis is facilitated by the utilization of tracers that allow a time-resolved analysis of the pathway dynamics without affecting the metabolic flux. Different strategies have been used and new tools are continuously being developed to probe the various enzymatic conversions that occur within this complex pathway. Here, we provide a short overview of the divergent fungal and mammalian sphingolipid biosynthetic routes, and of the tracers and methods that are frequently employed to follow the flux of intermediates throughout these pathways.

1. Introduction

Sphingolipids in conjunction with sterols and glycerophospholipids constitute the main lipids of the plasma membrane of eukaryotic cells. They are essential to maintain the permeability barrier of this limiting membrane, but also function as signalling molecules that coordinate surface expansion with cell growth and division. Sphingolipids are amphipathic lipids composed of a hydrophobic part, termed ceramide, which can be substituted by an array of hydrophilic head groups. Ceramide itself is composed of a sphingoid long-chain base (LCB) that is amide-bound to a fatty acid. Despite the fact that sphingolipid constituents such as LCBs and ceramides have emerged as potent signalling molecules, the number of publications on sphingolipids over the last 5-10 years has not increased as dramatically as those containing “lipids” or “fatty acids” as key words (Fig. 1).

Nevertheless, our understanding of sphingolipid synthesis and regulation, particularly in genetic model organisms such as *Saccharomyces cerevisiae*, has been greatly expanded over the last couple of years. This advancement is at least partially due to the development of new analytical tools, most notably mass spectrometry, which opened the door to lipidomics. The steps of sphingolipid biosynthesis and turnover and their homeostatic regulation in both yeast and mammalian cells have recently been reviewed and is not the subject of this more methodologically oriented review [1, 2]. Instead, we highlight significant differences in sphingolipid metabolism in less studied models and pathogenic yeast and discuss advantages and shortcomings of different strategies to chemically label different intermediates to follow their flux through the pathway.

2. Diversity of sphingolipid biosynthetic pathways in yeast species

The diversity among different yeast species is large and one of the reasons for their exploration as models to study particular biological processes. While *Schizosaccharomyces pombe* is a preferred subject to study cell division, *Pichia pastoris* is being used as a workhorse for the heterologous expression of eukaryotic proteins. *Saccharomyces cerevisiae*, on the other hand, has yielded many important insights into sphingolipid metabolism, particularly due to the pioneering studies of Lester and coworkers [3, 4]. Even with complete genome sequences of the fission yeast *S. pombe*, the methylotrophic yeast *P. pastoris* and several other pathogenic fungi at hand today, compared to *S. cerevisiae*, only relatively few studies on the sphingolipid pathway in these organisms have been reported [5-9].

The synthesis of the sphingoid backbone is conserved among eukaryotes and begins in the endoplasmic reticulum with the condensation of L-serine and palmitoyl-CoA, the preferred substrates of serine palmitoyltransferase (SPT), to form 3-ketosphinganine. This sphingoid base is then reduced to dihydrosphingosine (DHS). DHS is hydroxylated to yield phytosphingosine (PHS), and serves as precursor to desaturated ceramides typically produced by *Candida albicans*, *P. pastoris*, *S. pombe*, *Kluyveromyces lactis* and *Saccharomyces kluyveri* [5, 6, 10, 11] (Fig. 2, branches on brown/green backgrounds). The diversity of long-chain bases, however, is bewildering and covered in depth in more specialized reviews [12, 13].

The long-chain bases, DHS and PHS are N-acylated to yield dihydroceramide and phytoceramide, the hydrophobic backbones of the more mature and complex sphingolipids. In *S. cerevisiae*, ceramide is then uniquely converted to phospho-inositol-containing sphingolipids. On the other hand, *P. pastoris*, *C. albicans* and other fungal

pathogens, are able to produce two types of mature sphingolipids, the acidic phospho-inositol containing ones and the neutral glucosylceramides, which contain glucose instead of the phospho-inositol, as their polar head group [7, 14, 15] (Fig. 2). Interestingly, the ceramide synthases of *C. albicans* possess distinct substrates specificities and physiological roles: CaLag1p predominantly synthesizes ceramides with C24:0/C26:0 fatty acid moieties for the generation of phospho-inositol-containing sphingolipids, whereas CaLac1p produces ceramides with C18:0 fatty acid moieties, which serve as precursors for glucosylsphingolipids [8]. It is noteworthy, that the fungal glucosylceramide structurally differs from mammalian and plant glucosylceramides [16]. The double bonds in position $\Delta 4$ and $\Delta 8$ and the fungal specific C9 methyl group are inserted sequentially into the LCB backbone of ceramide [7, 9, 17, 18] (Fig. 2).

Comparative analyses of the sphingolipid composition of *S. cerevisiae* and other yeast revealed additional differences [19, 20]. For example, while mannosyl-diinositolphosphorylceramide (M(IP)₂C) is the most abundant mature sphingolipid species in *S. cerevisiae*, the major sphingolipid in *S. pombe* is mannosyl-inositolphosphorylceramide (MIPC) [19-21]. In *S. cerevisiae*, the final step in the biosynthesis of complex sphingolipids requires the inositolphosphotransferase, Ipt1, for synthesis of M(IP)₂C, however, *S. pombe* does not have an orthologue of Ipt1 and thus cannot convert MIPC to M(IP)₂C. Indeed, only the budding yeasts *C. glabrata*, *C. albicans* and *K. lactis* contain Ipt1 homologues [22].

Despite the fact that *S. cerevisiae* could be considered as a somewhat exceptional yeast, with a particularly simple sphingolipid composition, the pioneering studies in this organism have provided the fundament to explore more complex pathways in other species. Particularly the combination of genetics with lipid tracer experiments has allowed to gain insight into the pathway and its regulation. Different strategies to follow

the activity of key enzymes within the pathway and the respective biosynthetic intermediates are summarized in the next section.

3. Isotope-assisted methods to follow sphingolipid metabolites

3.1. Radioactive tracers

Traditionally, sphingolipids have been studied using radioactive precursors, such as [^3H]myo-inositol, [^3H]DHS, [^3H]L-serine, [^{14}C]palmitate or [^{32}P]phosphate. Cells are labelled with the corresponding tracer for the desired time, usually 24 hours for steady-state measurements or several minutes to few hours for pulse-chase experiments. Incubation times and amount of radioactivity added depend on the type of precursor used, its specific activity, and the type of information one would like to retrieve by the experiment. After labelling, cells are placed on ice and/or treated with trichloroacetic acid (TCA) and lipids are extracted. For detection, lipids are typically separated by thin layer chromatography (TLC) and quantified by scrapping the band of interest and measuring the tracer by scintillation counting. Alternatively, TLC plates can be scanned by a radioscanner, exposed to traditional film or to a phosphorimager screen.

The 90's and the first years of the 2000's were prolific in terms of identification and characterization of many of the sphingolipid biosynthetic enzymes in *S. cerevisiae* [23, 24]. Since then, *in vitro* and *in vivo* assays using radiolabelled precursors and intermediates have been routinely used to study the regulation of the pathway, ceramide transport and LCB uptake and release [25-33].

Despite their invaluable help in deciphering sphingolipid metabolism, the toxicity of radiolabelled compounds has promoted the development of alternatives, such as stable

isotopes, fluorescently labelled compounds, click chemistry-based or odd-chain analogues of sphingolipid substrates.

3.2. Stable isotopes

Some of the most commonly used stable isotopes are deuterium [^2H], [^{13}C] and [^{15}N]. These are commercially available as sphingolipid precursors such as [$\text{U-}^{13}\text{C},^{15}\text{N}$]serine, D_9 -choline, [$\text{U-}^{13}\text{C}$]palmitate and can be employed to analyse sphingolipid synthesis and metabolism in eukaryotic cells [34].

The analysis of metabolic fluxes following labelling with such stable isotopes is an important tool for elucidating the fate of substrates through lipid metabolic pathways [34]. However, the utilization of stable isotopes also faces its limitations. First, unambiguous detection of putative isobaric or isomeric metabolites requires high resolution LC-MS/MS, an expensive technology that many smaller research laboratories cannot easily afford. In addition, the interpretation of data may require a time-consuming analysis of isotopologues (molecules with the same chemical structure but different isotopes at specific structural positions) and to calculate isotopomers (molecules with the same number of isotopic atoms but incorporated into different structural positions) [35, 36]. Moreover, compared to radioisotopes, some stable isotopes, particularly [^{13}C] has a reduced signal to noise ratio because of its relatively high natural abundance [37].

4. Fluorescent-based methods

Sphingolipid precursors labelled with a 7-nitro-2-1,3-benzoxadiazole (NBD) fluorescent group have been used for decades to assay the activity of sphingolipid biosynthetic enzymes and monitor trafficking in living cells circumventing radioactive-associated issues [38]. For instance, NBD C₆-ceramide has been used in living cells or in *in vitro* assays as a substrate to study IPC synthase activity in *S. cerevisiae* [39]. The resulting fluorescent product C₆-NBD-IPC can then be detected by HPLC-MS or by TLC [40, 41]. In mammalian cells, sphingomyelin synthase activity can be measured by monitoring the transfer of phosphocholine from phosphatidylcholine to NBD C₆-ceramide [42].

NBD-sphingosine and NBD-sphingosine-1-phosphate (NBD-S1P) were used to assay S1P lyase activity in extracts from mammalian cells or *S. cerevisiae* [43]. NBD-DHS, for example, was employed to monitor LCB export from *C. albicans* or ceramide synthase (CerS) activity in cultured cells or tissue homogenates [44, 45]. The resulting NBD-dihydroceramide can be detected either on TLC plates, following separation by reverse phase chromatography, or after solid phase extraction on a C18-substituted matrix [45-47].

As an alternative to NBD, the borondipyrromethene (BODIPY) can be used as a fluorophore to investigate sphingolipid enzymatic activities and dynamics in living cells [48, 49]. BODIPY is more photostable than NBD and allows visualization of sphingolipids concomitantly with GFP-tagged proteins. However, a general drawback of using these and other fluorescently-labelled substrates is that they generally contain voluminous tags that can affect the hydrophobicity of the lipid and its binding to enzymes, thereby affecting their kinetic parameters [48]. Consequently, smaller probes

would be of great value. For example, polyene lipids, containing multiple double bonds in their hydrocarbon chains, are intrinsically fluorescent and would be excellent probes to study sphingolipid dynamics and metabolism. However, their photostability is limited [50].

5. Click chemistry-based methods

Click chemistry allows the unambiguous detection of compounds containing azido groups or terminal alkynes, which can be integrated into the sphingoid- or fatty acid-backbone [50, 51]. Alkyne lipids, containing a triple bond, are not intrinsically fluorescent but can be visualized by microscopy after a chemical reaction. Such click chemistry-based probes have recently been used for tracing fatty acid, cholesterol and sphingolipid flux in mammalian cells [51-54].

As is the case with fluorescently-labelled sphingolipids, click-labelled lipids are not identical to their natural counterparts, and their use is also not free of limitations. For instance, the rapid degradation and incorporation of a photoactivatable sphingosine analogue with a clickable alkyne group into glycerolipids limits its use to studies in S1P lyase knock-out cell lines only, because the labelled sphingoid substrate cannot escape into the anabolic pathway [55].

6. Odd-chain length long-chain bases

Last, but not least, lipids with a simple modification in their chain length are exceptionally useful probes to assay specific enzymatic activities or even to analyse the

flux of substrates through the multiple steps of the sphingolipid pathway. *In vitro* assays to monitor sphingosine kinase or ceramide synthase activities by using non-naturally occurring C17-sphingosine or C17-DHS as substrates followed by product detection by mass spectrometry have been described previously [56]. More recently, administration of C17-PHS to living yeast cells was employed to monitor ceramide synthase and IPC production *in vivo* and yielded insights into the complex regulation of the pathway [57].

One important principle of using tracers to study biological processes is that they should be provided in low concentrations in order not to affect the steady-state concentrations of the endogenous metabolites. This is particularly crucial when studying mutants of a given pathway as they may already possess elevated levels of metabolic precursors and thus may be particularly sensitive to further increases in their levels. This, for example, is the case with Orm mutant cells. The conserved Orm proteins are negative regulators of the rate-limiting enzyme of sphingolipid synthesis, serine palmitoyltransferase [58, 59]. Cells lacking the Orm proteins thus have high levels of long-chain bases [58, 59]. Whereas wild-type cells respond in a dose-dependent manner to the addition of C17-PHS, Orm mutant cells display a reduction in ceramide and IPC synthesis if higher concentrations of C17-PHS are supplied [57].

7. Conclusions

Our understanding of the sphingolipid pathway has progressed tremendously over the last few decades. Advances in the detection and analysis of lipids, particularly by mass spectrometry, and its integration with other approaches such as synthetic genetic arrays or chemogenomics will likely help to further decipher the mechanism that regulate this pathway. The use and limitations of different chemical probes and tracers to analyse

particular steps in the pathway, however, must be considered carefully before embarking into new territory.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank members of the lab for their support and helpful discussions, particularly Stéphanie Cottier and Vendula Stradalova for their helpful comments on the manuscript. This work was supported by the canton of Fribourg, the Novartis Foundation and by the Swiss National Science Foundation.

Figure legends

Figure 1. Lipid-related publications from 1990 to 2015.

The number of publications containing the indicated key word in the title were calculated using PubMed Single Citation Matcher (<http://www.ncbi.nlm.nih.gov/pubmed/citmatch>).

Datasets were generated using the following key words: black (lipid/s); green (fatty acid/s); orange (sterol/s, cholesterol/s, ergosterol/s); blue (phospholipid/s, glycerophospholipid/s, phosphoinositide/s, phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, phosphatidylserine, cardiolipin); red (sphingolipid/s, phosphosphingolipid/s, sphingosine/s, ceramide/s, ceramidase/s, sphingomyelin/s, sphingomyelinase/s).

Figure 2. Overview of the *de novo* sphingolipid biosynthetic pathways in fungi and mammals.

For clarity, only anabolic processes are depicted, with the exception of sphingosine and sphingadienine formed by ceramide hydrolysis. Early steps in the pathway until the formation of dihydroceramide are common between fungi and mammals. *S. cerevisiae* produces inositol-containing sphingolipids (left, on blue background), whereas other yeast species can also synthesize glucosylceramides (center, on brown background). On the other hand, mammals (right, on green background) produce glycosphingolipids by

addition of one (glucosylceramides, galactosylceramides) or more (gangliosides) sugar residues to the 1-hydroxy group of ceramide.

References

1. D. K. Olson, F. Fröhlich, R. V. Farese, and T. C. Walther, Taming the sphinx: Mechanisms of cellular sphingolipid homeostasis, *Biochim Biophys Acta* (2015). 10.1016/j.bbalip.2015.12.021
2. A. Aguilera-Romero, C. Gehin, and H. Riezman, Sphingolipid homeostasis in the web of metabolic routes, *Biochim Biophys Acta* 1841 (2014) 647-656. 10.1016/j.bbalip.2013.10.014
3. S. Steiner, S. Smith, C. J. Waechter, and R. L. Lester, Isolation and partial characterization of a major inositol-containing lipid in baker's yeast, mannosyl-diinositol, diphosphoryl-ceramide, *Proc Natl Acad Sci U S A* 64 (1969) 1042-1048.
4. R. L. Lester, B. R. Withers, M. A. Schultz, and R. C. Dickson, Iron, glucose and intrinsic factors alter sphingolipid composition as yeast cells enter stationary phase, *Biochim Biophys Acta* 1831 (2013) 726-736. 10.1016/j.bbalip.2012.12.012
5. P. Ternes, T. Wobbe, M. Schwarz, S. Albrecht, K. Feussner, I. Riezman, J. M. Cregg, E. Heinz, H. Riezman, I. Feussner, and D. Warnecke, Two pathways of sphingolipid biosynthesis are separated in the yeast *Pichia pastoris*, *J Biol Chem* 286 (2011) 11401-11414. 10.1074/jbc.M110.193094
6. P. Ternes, S. Franke, U. Zähringer, P. Sperling, and E. Heinz, Identification and characterization of a sphingolipid delta 4-desaturase family, *J Biol Chem* 277 (2002) 25512-25518. 10.1074/jbc.M202947200
7. L. L. Guimarães, M. S. Toledo, F. A. Ferreira, A. H. Straus, and H. K. Takahashi, Structural diversity and biological significance of glycosphingolipids in pathogenic and opportunistic fungi, *Front Cell Infect Microbiol* 4 (2014) 138. 10.3389/fcimb.2014.00138
8. S. A. Cheon, J. Bal, Y. Song, H. M. Hwang, A. R. Kim, W. K. Kang, H. A. Kang, H. K. Hannibal-Bach, J. Knudsen, C. S. Ejlsing, and J. Y. Kim, Distinct roles of two ceramide synthases, CaLag1p and CaLac1p, in the morphogenesis of *Candida albicans*, *Mol Microbiol* 83 (2012) 728-745. 10.1111/j.1365-2958.2011.07961.x
9. M. Del Poeta, L. Nimrichter, M. L. Rodrigues, and C. Luberto, Synthesis and biological properties of fungal glucosylceramide, *PLoS Pathog* 10 (2014) e1003832. 10.1371/journal.ppat.1003832
10. S. Garton, L. V. Michaelson, F. Beaudoin, M. H. Beale, and J. A. Napier, The dihydroceramide desaturase is not essential for cell viability in *Schizosaccharomyces pombe*, *FEBS Lett* 538 (2003) 192-196.
11. N. Takakuwa, M. Kinoshita, Y. Oda, and M. Ohnishi, Isolation and characterization of the genes encoding delta(8)-sphingolipid desaturase from *Saccharomyces kluyveri* and *Kluyveromyces lactis*, *Curr Microbiol* 45 (2002) 459-461. 10.1007/s00284-002-3860-0
12. S. T. Pruett, A. Bushnev, K. Hagedorn, M. Adiga, C. A. Haynes, M. C. Sullards, D. C. Liotta, and A. H. J. Merrill, Biodiversity of sphingoid bases

- ("sphingosines") and related amino alcohols, *J Lipid Res* 49 (2008) 1621-1639. 10.1194/jlr.R800012-JLR200
13. A. H. Merrill, Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics, *Chem Rev* 111 (2011) 6387-6422. 10.1021/cr2002917
 14. L. Nimrichter, and M. L. Rodrigues, Fungal glucosylceramides: from structural components to biologically active targets of new antimicrobials, *Front Microbiol* 2 (2011) 212. 10.3389/fmicb.2011.00212
 15. E. Barreto-Bergter, G. L. Sassaki, and L. M. de Souza, Structural analysis of fungal cerebrosides, *Front Microbiol* 2 (2011) 239. 10.3389/fmicb.2011.00239
 16. J. E. Markham, D. V. Lynch, J. A. Napier, and T. M. Dunn..., Plant sphingolipids: function follows form, *Current opinion in plant ...* (2013).
 17. J. E. Markham, D. V. Lynch, J. A. Napier, T. M. Dunn, and E. B. Cahoon, Plant sphingolipids: function follows form, *Curr Opin Plant Biol* 16 (2013) 350-357. 10.1016/j.pbi.2013.02.009
 18. P. Ternes, P. Sperling, S. Albrecht, S. Franke, J. M. Cregg, D. Warnecke, and E. Heinz, Identification of fungal sphingolipid C9-methyltransferases by phylogenetic profiling, *J Biol Chem* 281 (2006) 5582-5592. 10.1074/jbc.M512864200
 19. C. S. Ejsing, T. Moehring, U. Bahr, E. Duchoslav, M. Karas, K. Simons, and A. Shevchenko, Collision-induced dissociation pathways of yeast sphingolipids and their molecular profiling in total lipid extracts: a study by quadrupole TOF and linear ion trap-orbitrap mass spectrometry, *J Mass Spectrom* 41 (2006) 372-389. 10.1002/jms.997
 20. G. Shui, X. L. Guan, C. P. Low, G. H. Chua, J. S. Goh, H. Yang, and M. R. Wenk, Toward one step analysis of cellular lipidomes using liquid chromatography coupled with mass spectrometry: application to *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* lipidomics, *Mol Biosyst* 6 (2010) 1008-1017. 10.1039/b913353d
 21. M. Nakase, M. Tani, T. Morita, H. K. Kitamoto, J. Kashiwazaki, T. Nakamura, A. Hosomi, N. Tanaka, and K. Takegawa, Mannosylinositol phosphorylceramide is a major sphingolipid component and is required for proper localization of plasma-membrane proteins in *Schizosaccharomyces pombe*, *J Cell Sci* 123 (2010) 1578-1587. 10.1242/jcs.059139
 22. M. Nakase, M. Tani, and K. Takegawa, Expression of budding yeast IPT1 produces mannosyldiinositol phosphorylceramide in fission yeast and inhibits cell growth, *Microbiology* 158 (2012) 1219-1228. 10.1099/mic.0.056184-0
 23. K. Funato, B. Vallee, and H. Riezman, Biosynthesis and trafficking of sphingolipids in the yeast *Saccharomyces cerevisiae*, *Biochemistry* 41 (2002) 15105-15114.
 24. R. C. Dickson, C. Sumanasekera, and R. L. Lester, Functions and metabolism of sphingolipids in *Saccharomyces cerevisiae*, *Prog Lipid Res* 45 (2006) 447-465. 10.1016/j.plipres.2006.03.004
 25. S. E. Brice, C. W. Alford, and L. A. Cowart, Modulation of sphingolipid metabolism by the phosphatidylinositol-4-phosphate phosphatase Sac1p through regulation of phosphatidylinositol in *Saccharomyces cerevisiae*, *J Biol Chem* 284

(2009) 7588-7596. 10.1074/jbc.M808325200

26. M. Shimobayashi, W. Oppliger, S. Moes, P. Jenö, and M. N. Hall, TORC1-regulated protein kinase Npr1 phosphorylates Orm to stimulate complex sphingolipid synthesis, *Mol Biol Cell* (2013). 10.1091/mbc.E12-10-0753
27. E. Swain, K. Baudry, J. Stuke, V. McDonough, M. Germann, and J. T. J. Nickels, Sterol-dependent regulation of sphingolipid metabolism in *Saccharomyces cerevisiae*, *J Biol Chem* 277 (2002) 26177-26184. 10.1074/jbc.M204115200
28. K. Funato, and H. Riezman, Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast, *J Cell Biol* 155 (2001) 949-959. 10.1083/jcb.200105033
29. K. Kajiwar, A. Ikeda, A. Aguilera-Romero, G. A. Castillon, S. Kagiwada, K. Hanada, H. Riezman, M. Muñiz, and K. Funato, Osh proteins regulate COPII-mediated vesicular transport of ceramide from the endoplasmic reticulum in budding yeast, *J Cell Sci* 127 (2014) 376-387. 10.1242/jcs.132001
30. K. Kajiwar, R. Watanabe, H. Pichler, K. Ihara, S. Murakami, H. Riezman, and K. Funato, Yeast ARV1 Is Required for Efficient Delivery of an Early GPI Intermediate to the First Mannosyltransferase during GPI Assembly and Controls Lipid Flow from the Endoplasmic Reticulum, *Mol Biol Cell* 19 (2008) 2069-2082.
31. A. Kihara, and Y. Igarashi, Identification and characterization of a *Saccharomyces cerevisiae* gene, RSB1, involved in sphingoid long-chain base release, *J Biol Chem* 277 (2002) 30048-30054. 10.1074/jbc.M203385200
32. T. Sano, A. Kihara, F. Kurossu, S. Iwaki, and Y. Igarashi, Regulation of the sphingoid long-chain base kinase Lcb4p by ergosterol and heme: studies in phytosphingosine-resistant mutants, *J Biol Chem* 280 (2005) 36674-36682. 10.1074/jbc.M503147200
33. A. Kihara, and Y. Igarashi, Cross talk between sphingolipids and glycerophospholipids in the establishment of plasma membrane asymmetry, *Mol Biol Cell* 15 (2004) 4949-4959. 10.1091/mbc.E04-06-0458
34. J. Ecker, and G. Liebisch, Application of stable isotopes to investigate the metabolism of fatty acids, glycerophospholipid and sphingolipid species, *Prog Lipid Res* 54 (2014) 14-31. 10.1016/j.plipres.2014.01.002
35. L. You, B. Zhang, and Y. J. Tang, Application of stable isotope-assisted metabolomics for cell metabolism studies, *Metabolites* 4 (2014) 142-165. 10.3390/metabo4020142
36. C. A. Haynes, J. C. Allegood, E. W. Wang, S. L. Kelly, M. C. Sullards, and A. H. Merrill, Factors to consider in using [U-C]palmitate for analysis of sphingolipid biosynthesis by tandem mass spectrometry, *J Lipid Res* 52 (2011) 1583-1594. 10.1194/jlr.D015586
37. J. K. Kelleher, and G. B. Nickol, Isotopomer Spectral Analysis: Utilizing Nonlinear Models in Isotopic Flux Studies, *Methods Enzymol* 561 (2015) 303-330. 10.1016/bs.mie.2015.06.039
38. N. G. Lipsky, and R. E. Pagano, Sphingolipid metabolism in cultured fibroblasts: microscopic and biochemical studies employing a fluorescent ceramide analogue, *Proceedings of the National ...* (1983).

39. T. P. Levine, C. A. Wiggins, and S. Munro, Inositol phosphorylceramide synthase is located in the Golgi apparatus of *Saccharomyces cerevisiae*, *Mol Biol Cell* 11 (2000) 2267-2281.
40. W. Zhong, D. J. Murphy, and N. H. Georgopapadakou, Inhibition of yeast inositol phosphorylceramide synthase by aureobasidin A measured by a fluorometric assay, *FEBS Lett* 463 (1999) 241-244.
41. K. Sato, Y. Noda, and K. Yoda, Keil: a novel subunit of inositolphosphorylceramide synthase, essential for its enzyme activity and Golgi localization, *Mol Biol Cell* 20 (2009) 4444-4457. 10.1091/mbc.E09-03-0235
42. Y. Chen, D. A. Yurek, L. Yu, H. Wang, M. E. Ehsani, Y. W. Qian, R. J. Konrad, X. C. Jiang, M. S. Kuo, G. Cao, and J. Wang, Development of a quantitative biochemical and cellular sphingomyelin synthase assay using mass spectrometry, *Anal Biochem* 438 (2013) 61-66. 10.1016/j.ab.2013.03.016
43. P. Bandhuvula, H. Fyrst, and J. D. Saba, A rapid fluorescence assay for sphingosine-1-phosphate lyase enzyme activity, *J Lipid Res* 48 (2007) 2769-2778. 10.1194/jlr.D700010-JLR200
44. X. M. Jia, Y. Wang, Y. Jia, P. H. Gao, Y. G. Xu, L. Wang, Y. Y. Cao, Y. B. Cao, L. X. Zhang, and Y. Y. Jiang, RTA2 is involved in calcineurin-mediated azole resistance and sphingoid long-chain base release in *Candida albicans*, *Cell Mol Life Sci* 66 (2009) 122-134. 10.1007/s00018-008-8409-3
45. H. J. Kim, Q. Qiao, H. D. Toop, J. C. Morris, and A. S. Don, A fluorescent assay for ceramide synthase activity, *J Lipid Res* 53 (2012) 1701-1707. 10.1194/jlr.D025627
46. R. Tidhar, K. Sims, E. Rosenfeld-Gur, W. Shaw, and A. H. Futerman, A rapid ceramide synthase activity using NBD-sphinganine and solid phase extraction, *J Lipid Res* 56 (2015) 193-199. 10.1194/jlr.D052001
47. T. A. Couttas, X. Y. Lim, and A. S. Don, A three-step assay for ceramide synthase activity using a fluorescent substrate and HPLC, *Lipids* 50 (2015) 101-109. 10.1007/s11745-014-3969-5
48. P. Bandhuvula, Z. Li, R. Bittman, and J. D. Saba, Sphingosine 1-phosphate lyase enzyme assay using a BODIPY-labeled substrate, *Biochem Biophys Res Commun* 380 (2009) 366-370. 10.1016/j.bbrc.2009.01.106
49. R. Kim, K. Lou, and M. L. Kraft, A new, long-wavelength borondipyrromethene sphingosine for studying sphingolipid dynamics in live cells, *J Lipid Res* 54 (2013) 265-275. 10.1194/jlr.D029207
50. L. Kuerschner, and C. Thiele, Multiple bonds for the lipid interest, *Biochim Biophys Acta* 1841 (2014) 1031-1037. 10.1016/j.bbalip.2013.12.018
51. C. Thiele, C. Papan, D. Hoelper, K. Kusserow, A. Gaebler, M. Schoene, K. Piotrowitz, D. Lohmann, J. Spandl, A. Stevanovic, A. Shevchenko, and L. Kuerschner, Tracing fatty acid metabolism by click chemistry, *ACS Chem Biol* 7 (2012) 2004-2011. 10.1021/cb300414v
52. K. Hofmann, C. Thiele, H. F. Schött, A. Gaebler, M. Schoene, Y. Kiver, S. Friedrichs, D. Lütjohann, and L. Kuerschner, A novel alkyne cholesterol to trace cellular cholesterol metabolism and localization, *J Lipid Res* 55 (2014) 583-591. 10.1194/jlr.D044727

53. A. Gaebler, R. Milan, L. Straub, D. Hoelper, L. Kuerschner, and C. Thiele, Alkyne lipids as substrates for click chemistry-based in vitro enzymatic assays, *J Lipid Res* 54 (2013) 2282-2290. 10.1194/jlr.D038653
54. M. Garrido, J. L. Abad, G. Fabriàs, J. Casas, and A. Delgado, Azide-tagged sphingolipids: new tools for metabolic flux analysis, *Chembiochem* 16 (2015) 641-650. 10.1002/cbic.201402649
55. P. Haberkant, F. Stein, D. Höglinger, M. J. Gerl, B. Brügger, P. P. Van Veldhoven, J. Krijgsveld, A. C. Gavin, and C. Schultz, Bifunctional Sphingosine for Cell-Based Analysis of Protein-Sphingolipid Interactions, *ACS Chem Biol* 11 (2016) 222-230. 10.1021/acscchembio.5b00810
56. S. Spassieva, J. Bielawski, V. Anelli, and L. M. Obeid, Combination of C(17) sphingoid base homologues and mass spectrometry analysis as a new approach to study sphingolipid metabolism, *Methods Enzymol* 434 (2007) 233-241. 10.1016/S0076-6879(07)34012-3
57. F. Martínez-Montañés, and R. Schneiter, Following the flux of long-chain bases through the sphingolipid pathway in vivo using mass spectrometry, *J Lipid Res* (2016). 10.1194/jlr.D066472
58. D. K. Breslow, S. R. Collins, B. Bodenmiller, R. Aebersold, K. Simons, A. Shevchenko, C. S. Ejsing, and J. S. Weissman, Orm family proteins mediate sphingolipid homeostasis, *Nature* 463 (2010) 1048-1053. 10.1038/nature08787
59. S. Han, M. A. Lone, R. Schneiter, and A. Chang, Orm1 and Orm2 are conserved endoplasmic reticulum membrane proteins regulating lipid homeostasis and protein quality control, *Proc Natl Acad Sci USA* 107 (2010) 5851-5856. 10.1073/pnas.0911617107