Engineering the bilayer: emerging genetic toolkits for mechanistic lipid biology

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Abstract

The structural diversity of lipids underpins the biophysical properties of cellular membranes, which vary across all scales of biological organization. Since lipid composition results from complex metabolic and transport pathways, its experimental control has been a major goal of mechanistic membrane biology. Here, we argue that in the wake of synthetic biology similar metabolic engineering strategies can be applied to control the composition, physicochemical properties, and function of cell membranes. In one emerging area, titratable expression platforms allow for specific and genome-wide alterations in lipid biosynthetic genes, providing analogue control over lipidome stoichiometry in membranes. Simultaneously, heterologous expression of biosynthetic genes and pathways has allowed for gain-of-function experiments with diverse lipids in non-native systems. Finally, we highlight future directions for tool development, including recently discovered lipid transport pathways to intracellular lipid pools. Further tool development providing synthetic control of membrane properties can allow biologists to untangle membrane lipid structure-associated functions.

Introduction

A long-standing question in membrane biology is simply “why are there so many lipids?” [1]. Even simple cell compartments, like the *E. coli* inner membrane (IM), feature hundreds of distinct components resulting from distinct lipid classes and their combinatorial diversity of modifications. The heterogeneity of lipid composition across lipid bilayers is represented across multiple scales: between organisms, cell and tissue types, between organelles, and even individual bilayer leaflets. Advances in lipidomics, informatics, and imaging tools have begun to unravel the complexity of biological membrane composition. For example, the molecular asymmetry of the eukaryotic plasma membrane, although long recognized, has only recently been fully described in its molecular details [2]**. The composition of lipids in a bilayer dictates its structural (packing, thickness, surface charge, phase behavior) as well as dynamic (permeability, diffusivity, response to deformation) properties, which are utilized by nearly all membrane-associated cellular processes. Lipid composition thus represents the chemical lever by which these biophysical concepts can be tested in cells.

In addition to compartmentalizing biology, membranes also serve as molecular microenvironments for a range of processes in metabolism, protein biogenesis and transport, and signaling pathways and action potential generation [3]. Natural selection has likely tailored the biophysical properties of individual membranes for specific functions across biological systems. Indeed, membrane composition is dynamic in organisms, responsive to environmental cues [4] and developmental stages [5]. We are learning more about how lipids are altered in neurological

and metabolic diseases [6] and aging processes [7]. In one recent example, shotgun lipidomics of mouse brain tissue resolved cell-specific lipids and unique lipid class signatures across different regions of the brain in response to aging, including plasmenyl ether lipids (plasmalogens) [8]. Plasmalogens have been implicated in ferroptosis [9] and mitochondrial respirasome assembly [10], two major causes of reactive oxygen mediated cell death. However, like for many lipid classes, we still do not understand their fundamental roles in cells. Identifying functional changes in lipid composition and underlying biochemical pathways is thus directly relevant to human health for the development of therapeutics and diagnostic platforms [11].

Despite advancements in lipidomics and other technologies, mechanistic understanding of membrane structure and lipid function is still lacking. In our view, there are several key experimental challenges that contribute to this. Structural lipids act via their subtle effects on membrane packing and dynamics, in contrast to protein binding and enzyme kinetics that are highly nonlinear and saturate quickly. Thus, quantitative manipulation of lipid stoichiometry is key to understanding their function. Historically, this has been difficult because lipids are not easily amenable to manipulation by classical genetic methods. Lipid synthesis is often controlled by redundant and inter-dependent gene products and many pathways are essential for cell viability. Supplementation strategies for adding or depleting specific lipids to cells have long sought to have such quantitative control over composition. However, the unique properties of lipids, namely their insolubility and non-linear partitioning between different aggregate and bound states, pose a challenge for their controlled incorporation. Chemical inhibitors of specific lipid synthesis pathways, such as in early sphingolipid metabolism [12], can provide dosed control over some pathways, but fail to canvas the diversity of lipids found across organisms.

Here our focus is on genetic manipulation of lipid biosynthesis as a general strategy for untangling membrane function. Compared to functional studies of macromolecules (protein and nucleic acid), genetic control of lipids is challenging because they are not direct gene products, but produced through complex metabolic pathways. However, advances in precise genetic tools, often developed with totally different applications in mind, are allowing for increasingly fine-tuned control of lipid composition in living cells. We highlight recent advances made using this approach, which we trace back to experiments carried out in model microorganisms twenty years ago.

Modulation of membrane composition through metabolic engineering

Through broad advancements in genetics, genome sequencing, and recombinant DNA technologies, the characterization and heterologous recombination of central dogma components has enabled the emergence of metabolic engineering as a source for building key tools to manipulate and study metabolic pathways. Since the 1960s, lipid biologists have used genetic manipulation, e.g. knockouts, disruptions, and temperature sensitive alleles, to deduce the diversity of biosynthetic pathways underlying lipid metabolism. Initially, loss-of-function experiments in lipid pathways used gene disruptions or temperature sensitive alleles [13] in bacteria to map out lipid pathways, such as phospholipid biosynthesis by Eugene Kennedy and his lab members [14] and fatty acid synthesis by John Cronan and colleagues *[15]*. The essential work of identifying lipid biosynthetic pathways still continues to this day and is the basis for all subsequent efforts at lipid manipulation. For example, only within the past year, have key enzymes for plasmalogen biosynthesis been identified in mammals [16] and bacteria [17].

In efforts led by Bill Dowhan's lab 20 years ago, lipid biologists started applying the genetic manipulation of biosynthetic pathways not just for their discovery, but to study lipid function itself (Figure 1). This conceptual advancement is best demonstrated by the elegant work the Dowhan

lab carried out on the role of phospholipid headgroup composition in membrane protein topology using *E. coli* mutants deficient in phosphatidylethanolamine (PE). Active transport by LacY was used as a read out of properly oriented transmembrane (TM) protein, which became inverted upon PE loss [18]*. Importantly, physiological results were coupled to reconstitution experiments showing LacY topology was altered by PE content in vesicles. These efforts to interrogate the role of phospholipid composition in TM protein folding are still ongoing, but have led to significant revisions of the classic "positive inside" rule of TM protein topology [19]

While gene disruptions can provide functional information in specific cases, fine-tuned control of lipid stoichiometry is needed to model the cellular and biophysical roles for most bulk lipid components. One of the cornerstone tools of metabolic engineering has been the development and application of titratable promoters that can fine tune gene expression levels in response to concentrations of an inducer or repressor molecule. When the synthesis of enzymes controlling rate-limiting steps in a pathway are placed under such promoters, control over the stoichiometry of the resulting lipid species can be achieved. Commonly used engineered promoters include mammalian [20] and yeast [21] systems based on bacterial Tet Repressor proteins and bacterial systems based on pBAD promoters [22]. An early example of applying these to lipid biology was the Tet-based repression of yeast cardiolipin synthase by the Dowhan lab, first demonstrating that this mitochondrial lipid was involved in the stability of respiratory supercomplexes [23].

In our experience, the more precise a lipid component can be manipulated experimentally, the more likely a researcher has of uncovering specific mechanisms underlying their function. There are two reasons for this: 1) For lipids that have pleiotropic effects, careful titration of their stoichiometry allows identification of processes most sensitive to their depletion i.e., what breaks first? 2) With sufficient resolution, titration of lipid composition allows for the building of mechanistic models based on specific biophysical measurements. Genetic tools, such as titratable promoters, that allow for analogue control of lipid synthesis are thus generally preferable over those, such as knockouts, that allow only binary or on/off control. An important consideration for these systems is the homogeneity of their repression, since endogenous promoter systems feature feedback loops to generate "on or off" transcriptional responses. Without validation, titration across a population can be deceiving, and in fact reflect the proportion of cells exhibiting strictly "on" or "off" state [24]. Synthetic titratable systems or engineered cell backgrounds have been developed to overcome these limitations, but evaluation of their performance with single cell analytical techniques (e.g. flow cytometry) is still important.

The power of highly titratable lipid synthesis platforms is highlighted by our recent efforts to understand cellular roles for unsaturated fatty acids (UFA), essential lipid components whose exact stoichiometry regulates the viscosity or fluidity of membranes. In *E. coli*, we focused our efforts on titration of *fabB*, whose gene product had been found by the Cronan lab to be rate-limiting for UFA biosynthesis almost 40 years ago [25]. Using a highly engineered K12 background that allows for homogenous and titratable expression off the pBAD promoter, we demonstrated that unsaturated lipid levels in the inner membrane (IM) could be manipulated across the entire viable range [26]**. While very low levels of UFA led to pleiotropic defects in the cell envelope, more gentle modulation revealed a specific phenotype in respiratory metabolism. Because of the high performance of our genetic system, we were able to measure the exact dependence of respiratory function, as well as related biochemical parameters - membrane viscosity, diffusion rates of ubiquinone, ETC enzyme activities - as a function of UFA levels. These data were integrated into a quantitative model of the respiratory chain that highlighted a role for ubiquinone diffusion, which in turn is dependent on IM lipid composition.

Current limitations in precise control of lipid stoichiometry

There are two major limitations in current lipid engineering strategies based on titratable promoters or libraries of constitutive promoters [27]. First, there are a very limited number of high-performing promoters systems, which both limits their application to specific organisms and/or cell backgrounds and current systems do not allow for simultaneous manipulation of multiple lipid components. The latter could be especially important for probing lipid interactions underlying models of cell organization [28] and transport pathways [29]. Second, the ability of a promoter systems suitability to alter pathway production must be empirically characterized in each application, which is laborious and not guaranteed to work. Generally, expression levels must be titrated in a specific and highly gene-dependent range in order to affect the final abundance of a lipid product at steady state. Promoters that are too strong or weak can be modified by point mutations or through changes to ribosomal binding sites [30] or elements affecting transcript stability, but such tools are not yet commonplace outside of core systems like *E. coli*.

An attractive path forward to overcome these limitations is through programmable genetic systems, such as CRISPR-Cas, which are now being applied to modulate gene expression in numerous biological systems. CRISPR-Cas9 inhibition (CRISPRi) has emerged as an especially versatile tool for the knockout or knockdown of genes with targeted single guide RNAs (sgRNA) [31]. While titratable CRISPRi repression has so far had limited applications in lipid pathways, the recent knockout of the mammalian fatty acid desaturase gene, *SCD1*, in goat mammary epithelial cells demonstrates the potential for using CRISPRi to alter cellular lipid composition [32]. Bidirectional titratable CRISPR transcription has been recently demonstrated with the implementation of sgRNA libraries varying in target locations for the controlled tuning of metabolic pathway genes [33–35]**. In addition, design of biological circuits to quantitatively control repression levels has been shown through mediating the expression of CRISPRi sgRNA with titratable promoters [36,37]. These early studies demonstrate the opportunity to overcome the traditional confines of binary gene deletion or overexpression in genetic approaches and study gene function at intermediate expression levels. In application to lipid biology, these metabolic engineering methods can be used to stoichiometrically vary specific lipid content in membranes, modulate chemical and biophysical membrane properties, and study lipid membrane mechanics when combined with biochemical, microscopic, and -omics analysis.

Plug n' play: porting lipid components between organism to test their function

Heterologous protein expression in non-native hosts is a well-established tool in elucidating enzyme function. In principle, this methodology can be further extended to investigate the function of enzyme products (e.g. lipids) by engineering their synthesis in non-native cell systems. Since the behavior of structural lipids within membranes is governed by physical properties, heterologous expression between organisms can allow deep inference into membrane structure-function that is removed from specific lipid chemistries (Figure 2). The first example of applying heterologous expression to lipid function was again carried out by the Dowhan lab to address the determinant of TM protein topology. In PE mutants that were earlier shown to have defective LacY topology, heterologous synthesis of the non-native phosphatidylcholine (PC) was used to demonstrate the general role for zwitterionic lipids. In separate experiments, glycosylated diacylglycerolipids, neutral lipids common in cyanobacteria and plants, engineered in *E. coli* through expression of glycosyltransferases could also rescue PE mutants in restoring *LacY* function and topology [38]*. This finding supported a model in which the reduction of membrane charge density by either zwitterionic or neutral lipids was sufficient for proper TM topology.

Interestingly, longer diglycosylated headgroups were unable to restore this function, revealing a likely role for lipid spontaneous curvature [38]*.

In the age of synthetic biology, metabolic engineering has made efforts to carry out heterologous expression commonplace through genome mining, codon optimization, and rapid design-build-test-learn cycles. Because of the technology focus on biofuels and other lipid-derived bioproducts, many of these approaches have themselves been demonstrated for central lipid pathways. As an instructive example, introducing a set of five genes from *Bacillus subtilis* into *E. coli* has been used to synthesize low melting temperature biofuels containing branched chain fatty acids (BCFAs) [39]. In our own work, we adapted this same engineering strategy to produce fluidizing acyl chains in *E. coli* phospholipids, demonstrating that decreased membrane viscosity due to BCFA incorporation also increased respiration rates [26]**. Another example is the insertion of the phosphoinositide (PI) and PI-phosphate biosynthetic pathway in *E.coli* [40]. PI-phosphates are scarce but essential polyphosphate lipids in eukaryotic cells, especially for cell signaling. In this work, *E.coli* is an engineered platform to study the role of PI-phosphates in a minimal system that can be used to bridge *in vitro* and endogenous *in vivo* studies.

One particularly striking endeavor in lipid engineering has been the effort by multiple labs to remodel the *E. coli* lipidome through the synthesis of isoprenoid-based ether lipids characteristic of archaeal cells [41,42]**. It has been hypothesized that the last universal common ancestor had a mixed heterochiral membrane composition containing ether-linked isoprenoid lipids (archaeal) and ester-linked fatty acid lipids (bacterial, eukaryotic), so such systems represent attractive models for understanding this evolutionary branching point. Because these lipids diverge in the chirality of glycerol-phosphate backbone, they represent distinct pathways that must be ported over in full. Initial experiments expressing six archaeal genes in *E. coli* could only produce modest amounts of final product, but recent advances have led to *E. coli* strains containing up to 30 mol%. Key to this achievement was the simultaneous overexpression of the native *E. coli* DXP pathway for producing isoprenoid building blocks, a strategy first pioneered by metabolic engineers to produce bulk amounts of terpenes for biotechnology [43].

While major lipid classes are largely conserved across eukaryotes, there is an underappreciated level of structural diversity within them among animals, plants, and fungi [44]. As a powerhouse of metabolic engineering, budding yeast (*Saccharomyces cerevisiae*) has proven to be an excellent platform to test the functions of chemical differences among eukaryotic lipids, such as sterols and sphingolipids, *in vivo*. Recently, *S. cerevisiae* sterol metabolism was engineered with plant enzymes as a heterologous production platform for plant sterols (phytosterols) [45]*. Engineered strains were capable of producing high titers of campesterol, without growth penalty, when heavily esterified and sequestered. However, free phytosterols produced in *are1/are2* sterol esterase mutants significantly impaired growth [45]*. Adapted laboratory evolution by repeated culture partially restored the growth phenotype of these strains while increasing free phytosterol production [45]*. However, the function of foreign phytosterols within *S. cerevisiae* membranes, in relation to the growth phenotype, and its adapted complementation, remains puzzling. To some extent, foreign sterols are toxic in Fungi, potentially because they can inhibit sterol transporters such as Osh proteins [46,47]. Hence, the effect of phytosterol production in yeast on intracellular lipid distribution, and comparative lipid distributions between plants and yeast, is of great interest. Similarly, *S. cerevisiae* has been engineered to synthesize glucuronic acid-bearing glycan head groups of plant sphingolipids (phytosphingolipids) [48,49]. Besides supporting enzyme function, the impact of non-native sphingolipid head group composition and charge, pertaining to analogue function in biological membranes, has not been investigated. Notably, membrane phase

partitioning mediated by intramolecular interactions between sterols and sphingolipids is one model by which organization within a membrane can occur [28].

Much of the emerging questions in membrane biophysics center around tissue-specific lipid compositions in animals, especially in the central nervous system [50]. Animal brains are highly enriched in polyunsaturated fatty acid (PUFA) containing phospholipids, plasmalogens, and sugar-modified sphingolipids (gangliosides), all of which still have poorly defined cellular functions. To explore these questions, lipid engineering in whole animal model systems is needed. *Drosophila melanogaster* is one powerful model system with strong genetic tools that allow for tissue and cell-type specific gene expression [51]. Recently, neuron-specific ectopic expression of PUFA-producing 12 fatty acid desaturase from *Caenorhabditis elegans* altered the thermoregulatory behavior of *D. melanogaster,* resulting in decreased reproductive temperature preference [52]**. In a similar strategy, neuron-specific synthesis of foreign GM3 gangliosides in *D. melanogaster* increased amyloid protein aggregation [53]. Thus, heterologous synthesis of lipids in multicellular organisms has the potential to interconnect lipid function and membrane properties with animal behavior and disease pathology. Because *Drosophila* also lacks enzymes involved in the synthesis of sterols and PUFAs, dietary manipulation of these components has also been demonstrated [54,55], but this lacks the tissue specificity that genetic approaches can provide.

Future Directions

Linking the genes involved in metabolic pathways for the production of lipids provided a map for the exploration of lipid function. Alongside the push to understand lipid function through genetic manipulation, metabolic engineering has emerged as both a conceptual approach and discrete set of tools for dissecting and controlling desired metabolic pathways [56]. In its application to lipid biology, metabolic engineering can be used to control biosynthetic pathways to modify the physicochemical parameters of cell membranes. As synthetic biology expands into broader and more complex systems [57], we anticipate that this strategy will become more powerful for answering fundamental questions about membranes and lipids.

Looking forward, a limitation of the approaches discussed here is that they are focused on modifying global lipidomes – the total lipid composition of cells. In contrast, the composition of specific organellar and sub-organellar membranes results partly from biosynthesis, but also intracellular trafficking, lipid transport, and sorting across eukaryotic compartments. Advances in lipidomics and biochemical fractionation have allowed us to understand heterogeneity of lipid composition across compartments in greater details, but we still lack strong tools to experimentally manipulate it As with the discovery of major lipid biosynthetic pathways over the past 50 years, we are now beginning to understand the biochemical machinery underlying lipid transport pathways [58]. In the future, it is likely that engineering efforts based on our knowledge of these systems will allow for tailored compositions for studying unique organellar properties and functions.

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Figure 1, 2 column:

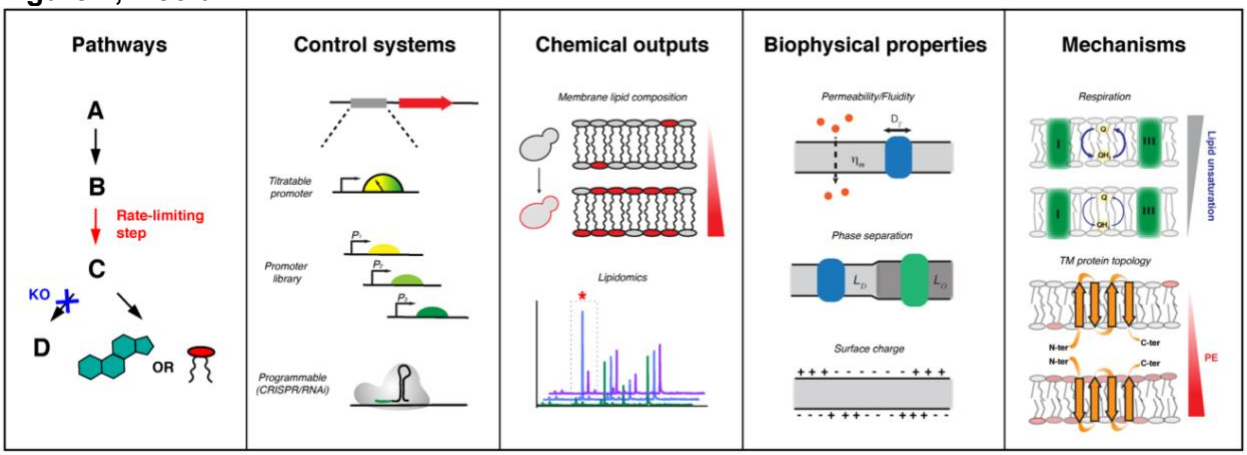

Figure 1. The metabolic engineering pipeline to investigate lipid function. Once well characterized, lipid biosynthetic **pathways** can be predictably modulated through the titration of enzyme levels carrying out rate-limiting steps or knockdown (KOs) of genes encoding non-essential enzymes. The former is done through engineered **control systems** which allow for controllable expression levels, primarily through the replacement or targeting of the endogenous promoter. The **chemical outputs** of engineered systems are first characterized through mass spectrometry-based lipidomics. Lipid composition defines the resulting **biophysical properties**, which can also be measured by spectroscopic and imaging approaches. These systems can then be used to investigate the **mechanisms** by which lipid composition acts in cells. Examples of this approach include the elucidation of how acyl chain unsaturation controls cellular respiration [26]** and how headgroup composition controls TM protein topology [38]*.

Figure 2, 1.5 column:

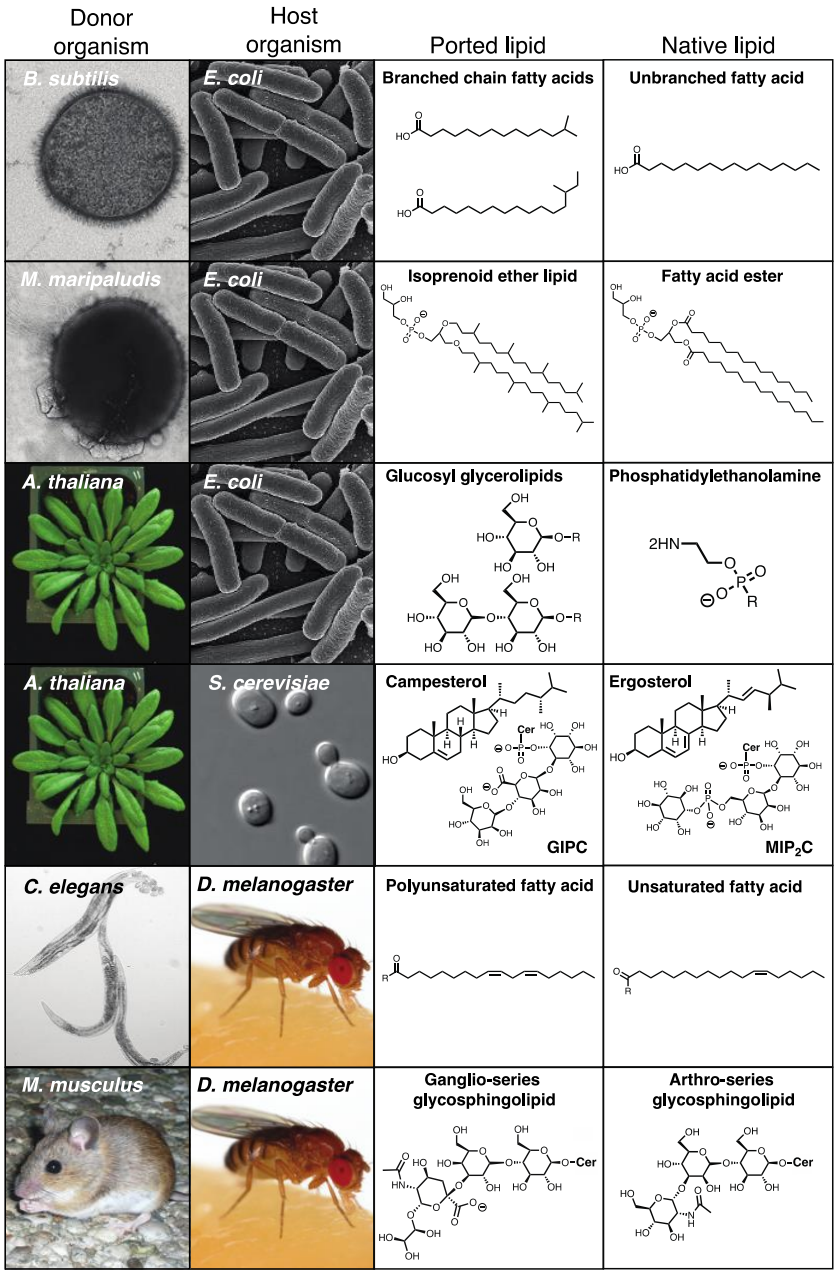

Figure 2. Examples of successful incorporation of heterologous lipid synthesis pathways in non-native hosts. Row 1: Branched chain fatty acids from *B. subtilis* produced in *E. coli* next to native unbranched fatty acid [26]**. **Row 2:** Isoprenoid linked ether lipids from Archaea produced in *E. coli* next to native fatty acid ester [42]**. **Row 3:** Mono- and di-glucosyl glycerolipids from *A. thaliana* produced in *E. coli* next to native phosphatidylethanolamine [38]**. Row 4:** Campesterol [45]* and mannosyl glycosyl inositol phosphorylceramide (GIPC) [49] from *A. thaliania* produced in *S. cerevisiae* next to native ergosterol and mannosyl di-inositol phosphorylceramide (MIP_2C). **Row 5:** Polyunsaturated fatty acid from *C. elegans* produced in *D. melanogaster* next to a native unsaturated fatty acid [52]**. **Row 6:** Ganglio-series

glycosphingolipid GM3 structure from *M. musculus* produced in *D. melanogaster* next to native arthro-series glycosphingolipid [53].

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