Tuning and minimizing lipidome composition in *Mycoplasma mycoides* and the Minimal Cell JCVI-Syn3A

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Abstract

All cells are encapsulated by a lipid membrane which facilitates the interaction between life and its environment. The property and function of the cell membrane is critically dependent on its lipidome composition which can range from 10s to 100s of unique lipid structures. How life has evolved to exploit such diverse mixtures of lipids has been experimentally challenging to address. In this study, we introduce a simple approach to minimize and tune lipidome composition in *Mycoplasma mycoides* and the Minimal Cell (JCVI-Syn3A). By varying the lipid composition of the growth medium, and introducing unmodifiable diether phospholipids, we demonstrate that a lipidome composed of two lipids can support life. By systematically reintroducing phospholipid features, we show that acyl chain diversity is more critical for supporting growth than head group diversity. We also reveal significant effects of altering phospholipid chirality on membrane permeability, cellular robustness, and growth, demonstrating how subtle changes in lipid stereochemistry can have profound effects on cell fitness. Our approach to tune mycoplasma lipidome composition introduces a minimal living model membrane system to understand the design principles and the role of lipid complexity in bioengineering of cell membranes.

Introduction

Cell membranes are complex and responsive systems that serve to protect and mediate interactions of life with its environment. A large part of this molecular complexity is due to the diverse panel of lipids that make up the lipidome and which ultimately determine the form and function of the membrane. The complexity of cellular lipidomes can be staggering, from tens of unique structures in bacteria[1], to hundreds in eukaryotic organisms[2]. How life has evolved to utilize such complex mixtures of lipids to build cellular membranes remains an active area of exploration[3,4]. One approach to studying the role of lipidome complexity is to experimentally

tune lipidome composition and observe effects on cell fitness (e.g. growth). A recent study in bacteria, for example, revealed the importance of homeoviscous adaptation for electron transport by tuning acyl chain unsaturation in *Escherichia coli*[5]. However, for interpreting lipidome flexibility, *E. coli*, like many bacteria, is complicated by the fact that it has multiple membranes (e.g. inner and outer). Changes in a single membrane are obfuscated by whole cell lipid extracts, and purifying specific membrane types is laborious, and can lead to substantial experimental error due to varying purity. Gram positive organisms such as *Bacillus subtilis*, that have only a single membrane are better models in this regard[6, 7]. However, traditional genetic approaches to tuning lipidome composition have so far not afforded complete control over both lipid class composition, and phospholipid acyl chain composition. Thus, a cellular model system in which lipidome complexity can be reduced in a systematic fashion has not yet been established.

Mycoplasma are a class of genomically simple bacterial pathogens possessing several features that are promising as tunable living model membrane systems. Mycoplasmas have a single plasma membrane, and lack a cell wall[8]. Therefore, their membrane composition and biophysical properties can be examined in situ without the need for laborious membrane isolation. Having evolved to a parasitic lifestyle, mycoplasmas have lost many pathways for biomolecular synthesis, including most of their lipid synthesis pathways, which they take up from their hosts, or the growth media[8, 9, 10, 11, 12]. This affords the possibility to control their lipidome composition by controlling what lipids are provided in the media. Further, because of their relatively small genomes, the number of components involved in managing lipidome composition and membrane adaptation is within reach of being completely characterized and modeled[13]. Recently, a minimal cell, JCVI-Syn3.0, was engineered from *Mycoplasma mycoides subsp capri* strain *GM12* by systemically removing genes from *M. mycoides* to achieve an organism where every remaining gene is essential or quasi-essential[14]. To restore normal division and remove pleomorphism, 19 genes were added back to JCVI-Syn3.0 to create JCVI-Syn3A*;* a regularly dividing quasi minimal cell which offers a platform to study the role of lipids for the most fundamental requirements of life in a minimal genetic system[15].

In this study we establish an approach to modulate lipidome composition and reduce its complexity in *M. mycoides* and JCVI-Syn3A. By tuning the lipid composition of the growth medium and introducing diether phospholipids we introduce an approach to bypass cellular lipid remodeling, achieving a lipidome with only two lipids. Using these lipidomically minimal living membranes, we compare the relative importance of phospholipid head group vs. acyl chain complexity. Additionally, we observed profound effects resulting from subtle changes in the lipid chirality that distinguishes Archaea from the rest of life. By developing approaches to tune and minimize mycoplasma lipidome composition, we hope to introduce a new tool for deciphering the principles of living membranes and to introduce a new paradigm for understanding why has evolved to utilize so many lipids.

Results

Mycoplasmas as minimal model membrane systems

Mycoplasma mycoides is a pathogen of mammals that has historically been used as a simple model membrane system. There are a number of reasons why *M. mycoides* makes a good model membrane system. First, its small genome (~1,100,000 bp) limits the complexity of its genetic regulation, and has allowed researchers to fully sequence and annotate the genome (although a number of genes still have unknown or only putative functional assignments)[8,11]. Second, as a pathogen, the primary source of lipids for *M. mycoides* is through environmental uptake from the host rather than synthesis[8, 9, 10, 11, 16]. In a laboratory setting using growth medium as the lipid source, this feature provides the experimenter with direct control over the lipid components available for synthesizing and maintaining the membrane; by adding or removing specific lipids from the growth medium of *M. mycoides*, the composition of the membrane can be altered. Third, *M. mycoides* has a single plasma membrane and no cell wall or organelles, making it easy to interrogate the membrane and ensuring that membrane targeting probes in *M. mycoides* are acting on the membrane and not another structure[8]. Fourth, and perhaps most importantly for this research, *M. mycoides* is unable to synthesize or alter fatty acid composition[8, 9, 10, 11] This means that, while it can alter the acyl chain composition of its membrane lipids, it is limited in its ability to do so by the pool of fatty acids it has access to. These factors combine to make *M.*

mycoides one of the model systems in which membrane remodeling is both the simplest and most controllable, and as such it is an excellent model system to study membrane remodeling in living organisms[17].

In *M. mycoides* membranes phospholipids, sterols, and free fatty acids are taken up from the environment and either incorporated into the membrane or taken up into the cytoplasm (Fig. 1a; 1)[8, 18, 19, 20]. *M. mycoides* can cleave acyl chains from exogenous phospholipids (Fig. 1a; 2)[21, 22, 23] When a pool of free fatty acids is present, *M. mycoides* can use those fatty acids to modify the acyl chain composition of phospholipids taken up from the media, or can synthesize the phosphotidylglycerol (PG) class of lipids which can in turn be modified and used to synthesize cardiolipins (Fig. 1a; 3, 4, 5)[8, 20, 21, 24, 25, 26, 27]. These newly synthesized or modified lipids can be broken down to replenish the fatty acid pool, or reinserted into the membrane (Fig. 1a; 6)[25]. With the exception of the modification of PG headgroups to make cardiolipins, all lipid remodeling in *M. mycoides* is acyl chain remodeling–that is, it relies on having access to a pool of free fatty acids to modify the existing acyl chain composition of phospholipids or synthesize *de novo* PGs[8, 9]. Cholesterol is essential for growth of *M. mycoides* (Fig. 1a; 8)[28, 29, 30]. For the first time, we report the feeding of *M. mycoides* on a defined lipid diet which contains no exogenous source of free fatty acids. When fed such a diet, *M. mycoides* is forced to rely completely on acyl chain scavenging from exogenous phospholipids as its only source of acyl chains for which to remodel its membrane lipidome (Fig. 1b).

<u>Minimizing the lipidome</u>

Our first goal was to determine the minimal lipidome that can support growth of *M. mycoides*. Sterols, preferably cholesterol, are required for growth, and must be included in any lipid diet. *M. mycoides* can synthesize several phospholipids (e.g. PG and CL) when provided free fatty acids[20, 29, 30]. Therefore, to minimize the phospholipid diversity, we removed free fatty acids from the lipid diet and instead provided a single phospholipid, POPC, along with cholesterol, yielding a minimal 2-component lipid diet. When transferred from growth medium containing fetal bovine serum (FBS, a complex undefined lipid source), to the two-component lipid diet, cells

initially grew poorly and tended to aggregate in clumps (not shown). Thin layer chromatography (TLC) of cell lipid extracts showed that, initially, PG and CL (both internally synthesized lipids) disappeared, and only traces of SM remained, presumably carry over from growth on FBS (Fig. 2a). After adaptation, sphingomyelin was no longer visible, however, PG and CL reappeared along with lyso-PC (a PC lipid with one of its acyl chains removed). Since *M. mycoides* cannot synthesize phospholipids in the absence of fatty acids, these observations indicate that acyl chains were being scavenged from POPC for the synthesis of PG and CL, presumably as a result of lipase activity (Fig. 2b). A systematic analysis of PG and CL production on minimal lipid diets composed of varying phospholipid species demonstrated that *M. mycoides* is capable of scavenging acyl chains from a broad range of phospholipid head groups (Fig. S1, TableS1), demonstrating a robust capacity for *M. mycoides* to procure acyl chains for internal PG and CL synthesis from nearly any phospholipid source.

The capacity for *M. mycoides* to scavenge acyl chains from exogenous phospholipids provided a hurdle to our goal of minimizing the lipidome. Shotgun mass spectrometry of cells grown on the minimal 2-component lipid diet revealed 28 lipid structures generated from the remodeling of POPC, and presence of phospholipids including PG, CL, PA, and DAG (Fig. 2c). Since the source of acyl chains for internal phospholipid synthesis presumably came from exogenous POPC, we reasoned that internal lipid synthesis could be eliminated by blocking acyl chain scavenging. To do this, we replaced POPC with an analogous lipid containing ether-linked 16:0 and 18:1 hydrocarbon chains, which are inert to lipase activity[31, 32]. Following the transfer of cells from FBS or minimal POPC-cholesterol diet to a minimal diether PC-diet, TLC analysis of lipid extracts over several passages showed the disappearance of CL and PG, and presence of only two bands corresponding to cholesterol and diether PC (Fig. 2d). Shotgun lipidomic analysis demonstrated that cholesterol and diether PC accounted for greater than 99 mol% of the detected lipids (the remaining very low abundance lipids are derived from impurities in the media, in particular from yeast extract). Thus, by introducing an enzymatically inert phospholipid, diether PC, to the minimal lipid diet, we could achieve a minimal lipidome composed predominantly of only two lipid structures, cholesterol and diether PC (Fig. 2e). When cells grown on the minimal lipid diet

are switched to a diet of POPC + cholesterol there is a period of adaptation similar to that seen in Fig. 2a. After the first passage, before adaptation, the cells do no yet undergo significant acyl chain scavenging, and the membrane composition remains predominantly composed of the two lipids POPC and cholesterol (Fig. 2f). This delayed adaptation to acyl chain scavenging allows us a brief window to study simplified membranes composed of lipids *M. mycoides* could normally scavenge acyl chains from. The simplified membranes only persist until *M. mycoides* is adapted to the new diet.

Tuning lipidome composition

Minimizing the lipidome of *M. mycoides* to two lipids resulted in a two-fold decrease in growth rates. We next asked which components of the lipidome are critical for growth. By feeding cells with diether PC, we had reduced lipidome composition in several ways. First, we eliminated the presence of internally synthesized phospholipids such as PG and CL. Therefore, minimizing the diversity of phospholipid head groups could have contributed to impaired growth. Second, we eliminated phospholipid hydrocarbon chain diversity by limiting the cell to one configuration (16:0/18:1). Thus, it is also possible that restricting the diversity of phospholipid hydrocarbon chain configurations could have impaired growth.

To test whether head group diversity was a factor in determining growth rate we provided cells with lipid diets containing diether PG and a mixture of diether PG with diether PC. When lipid extracts of cells grown on diether PG were analyzed by TLC, we observed bands corresponding to both PG and CL, indicating that CL synthesis can proceed from diether PG (Fig. 3a). When grown on diether PG and PC, TLC analysis showed three bands corresponding to PC, PG, and CL (Fig. 3b). Therefore, cells grown on diether PG generate lipidomes containing two phospholipid head groups and when grown on diether PG and PC generate lipidomes with three head phospholipid head groups. Surprisingly, growth rates for both diether PG and diether PG+PC diets were lower than for diether PC alone (Fig. 3e). Further, growth of cells on POPC prior to adaptation and ability to synthesize PG and CL, show only slightly higher growth rates than diether PC diets, indicating that reduced growth is not solely due to the introduction of diether

phospholipids. Phospholipid headgroup diversity alone, therefore, is not sufficient to rescue growth.

We next asked whether phospholipid acyl chain diversity could account for impaired growth. To this end, we grew cells on a diet of cholesterol and two fatty acids (palmitate and oleate) designated as '2FA'. On this diet, the phospholipidome is composed predominantly of PG and CL (Fig. 3c) and cells can synthesize phospholipids with a variety of acyl chain configurations (e.g. 16:0/18:1, 16:0/16:0, etc…). Thus, headgroup diversity is effectively two, comparable to growth on diether PG, however, diversity of acyl chain configurations is higher. Growth rates on the 2FA diet were more than twice growth on diether PG, and approached growth rates of cells adapted to growth on POPC (Fig. 3e). Therefore, for a lipidome with two phospholipid headgroups, increased diversity of acyl chain configurations rescues growth, demonstrating the importance of acyl chains for growth.

Finally, we asked whether growth could also be rescued by providing the full suite of ~28 lipids in cells adapted to growth on POPC. It is possible, for example, that growth is impaired by the disruption of internal phospholipid synthesis or remodeling pathways due to coupling of lipid synthesis with cellular growth. To test this, we transferred cells grown on FBS to a lipid diet derived from lipid extracts of cells adapted to growth on POPC (Fig. 3d), and measured growth in the first passage, before cells adapted to scavenge acyl chains from the dietary phospholipids. Growth rates on this transplanted lipidome diet resulted in a nearly complete rescue to levels observed in POPC cells after adaptation (Fig. 3e). These results indicate that internal phospholipid synthesis and remodeling is not required for optimal growth, and demonstrates a proof-of-principle that functional lipidomes can be engineered and transplanted to living membranes to support growth.

JCVI-Syn3A is an Even Simpler Cell

JCVI-Syn3A is a synthetic cell created by the J. Craig Venter Institute (JCVI) by removing every non-essential gene from *M. mycoides*[14]. As such, JCVI-Syn3A is an even simpler (genetically)

model system than *M. mycoides*, while still possessing all of the previously expressed characteristics of *M. mycoides* that make it a valuable model membrane system (Fig. 4A)[33]. In this study we used a strain that expresses an additional gene for the fluorescent mCherry protein, to create JCVI-Syn3A-*mCherry*[34], which for simplicity we subsequently refer to as JCVI-Syn3A. JCVI-Syn3A provides a good comparison organism to *M. mycoides*, and an experimental platform to examine the role of lipidome composition in supporting the minimal requirements for life. We therefore asked whether the JCVI-Syn3A lipidome could also be minimized.

Since JCVI-Syn3A has a truncated set of genes compared to *M. mycoides*, we first tested whether they retained the capacity to scavenge acyl chains from exogenous phospholipids for the internal synthesis of PG and CL. Unexpectedly we observed that JCVI-Syn3A could still synthesize PC and CL when fed with a minimal 2-component diet of cholesterol and POPC, as well as with a range of other phospholipid head groups (Table S1). This suggests that an undiscovered lipase remains in the JCVI-Syn3A genome. As a more minimal system, JCVI-Syn3A is a more fragile organism, and not all cells for a given lipid diet survived the growth protocol. To not bias the growth rates, these cells were removed from the growth rate analysis. An interesting feature was noticed, however, that a much higher fraction of cells that could not undergo acyl chain scavenging were non-viable than cells that could (Fig. 4b) further illustrating the decreased fitness of cells unable to remodel acyl chain composition.

Next, we assayed growth of JCVI-Syn3A on a 2-component lipid diet of cholesterol + diether PC (16:0/18:1). Growth for JCVI-Syn3A and *M. mycoides* on the Diether POPC + Cholesterol diet are commensurate, but on the POPC + Cholesterol diet *M. mycoides* has significantly improved growth, which is consistent with the literature (16) (Fig. 4c). Shotgun lipidomic analysis confirmed that JCVI-Syn3A cells grown on a 2-component diether PC + Cholesterol diet yielded a lipidome with over 99 mol% composed of only two lipid species (Fig. 4d). As such, this work has developed the simplest known living membrane in one of the simplest known living organisms. As complexity is often a hindrance in understanding biological systems, the value of such a simple model system is readily apparent.

Enantiomeric Lipids Inhibit Cell Growth

Having explored the minimal requirements for lipidome complexity in *M. mycoides* and JCVI-Syn3A, we next sought to leverage these model systems to probe another fundamental aspect of lipid biology: chirality. Glycerolipids (including phospholipids) have a chiral center in the glycerol backbone leading to enantiomeric lipids that are mirror images of each other (Fig. 5a). In bacteria and eukaryotes, glycerolipids are synthesized with acyl chains at the sn-1 and sn-2 positions, and the phosphate head group at the sn-3 position (Fig. 5A: G3P enantiomer). Conversely, archaea synthesize glycerolipids with the phosphate at the sn-1 position and the acyl chains at the sn-2 and sn-3 positions (G1P enantiomer)[35, 36]. Known as the 'Lipid Divide', this difference in stereochemistry between lipids of archaea and the rest of life has long stood as an unexplained enigma[37, 38, 39, 40]. Did a last common ancestor have membranes with both enantiomers? What are the consequences of having a racemic mixture of phospholipids in a living membrane? To date, no naturally occurring organism has been found that has comparable amounts of both enantiomers in its membrane. Furthermore, experimentally modulating phospholipid chirality in the lipidome through genetic approaches presents significant challenges, as shown by the work of Caforio et al[41]. However, the ability of mycoplasma to uptake exogenous lipids makes them an exceptionally well-suited model for unraveling this elusive problem in membrane biology.

To establish whether *M. mycoides* and JCVI-Syn3A can grow on a G1P phospholipid enantiomer, we prepared a lipid diet consisting of cholesterol and enantiomeric POPC (entPOPC), as well as a racemic mixture of cholesterol and POPC:entPOPC (1:1 mol%). We compared the two diets containing entPOPC against growth on cholesterol + POPC (Fig. 5b). Growth rates of both organisms show that the introduction of entPOPC to the lipid diet results in impaired growth, with the racemic diet yielding the most pronounced decrease in growth rate. Thus, we demonstrate that cells are viable when fed enantiomeric phospholipids.

We next asked how the introduction of entPOPC affected the mechanical robustness and permeability of the membrane. To assay membrane robustness, we measured sensitivity of cells

to hypoosmotic shock, by determining what fraction of cells are lysed following a shock. Since *M. mycoides* and JCVI-Syn3A lack a cell wall or cytoskeleton, cell lysis during rapid hypoosmotic shock is indicative of membrane rupture strength. Hypoosmotic sensitivity increased significantly for cells grown on entPOPC diets, demonstrating that the enantiomeric lipids affect the mechanical robustness of the membrane and, consequently, the whole cell (Fig. 5c,d). To assay permeability, we measured the rate of permeation of fluorescein diacetate (FDA), a non-chiral molecule, across the cell membrane[42, 43]. Membrane permeability, as measured by the permeability coefficient of FDA increased significantly upon the introduction of enantiomeric lipids, but was the highest for both *M. mycoides* and JCVI-Syn3A for the racemic lipid diet (Fig. 5e, f). When plotted against growth rate, there is an apparent correlation of lower growth with higher permeability (Fig 5g,h), possibly indicating membrane leakiness one of the factors underlying impaired growth. The found permeability coefficients were similar to those previously reported in mammalian cells[43].

Increased permeability and reduced membrane robustness could be due either to changes in the property of the lipid bilayer, or through changes in lipid-protein interactions. Previous work on phosphatidylcholine enantiomers in model membranes revealed that modest changes in permeability to calcein occur in membranes composed of both enantiomers, in particular scalemic (not 1:1) mixtures. However, such small changes would not account for the large change in permeability we observe. To determine if introducing enantiomeric POPC affected membrane bilayer robustness or permeability we reconstituted cellular lipid extracts from *M. mycoides* into liposomes and evaluated C-Laurdan fluorescence (Fig. 6). The C-Laurdan General Polarization index (GP) reports bilayer hydration, which is closely coupled with permeability and mechanical robustness of the membrane[44, 45, 46]. Surprisingly, GP values did not vary significantly across all three POPC diets, indicating that the lipid bilayer itself was not disrupted by changes in chirality, and implicating an effect on lipid-protein interactions. Indeed, several studies have demonstrated an effect of varying lipid chirality on lipid-peptide interactions, and the permeability of membranes to chiral amino acids[47, 48]. Thus, disrupted lipid-protein interactions are the most likely basis for the observed phenotypes, setting the stage for future work

employing *M. mycoides* and JCVI-Syn3A as model systems to explore the significance of lipid chirality on lipid-protein interactions in a living membrane.

Discussion

In this study, we introduce *M. mycoides* and the Minimal Cell JCVI-Syn3A as minimal model organisms with tunable lipidomes for studying the role of lipid complexity. By choosing a model membrane system incapable of synthesizing or modifying fatty acids, and developing a set of defined lipid diets for that system, we have demonstrated the creation of a platform in which the lipidome can be tuned in terms of phospholipid head group, acyl chain composition or lipid chirality. Using this platform, we created the simplest living membrane, and one incapable of undergoing acyl chain remodeling. These tunable living membranes allowed us to quantitatively examine the contribution of lipidomic features to the fitness of a minimal living system.

Lipid scavenging

Our observations broadly demonstrate how lipidome composition is crucial even for relatively simple microorganisms. This is anecdotally illustrated by the capacity we observe for mycoplasma to produce a complex lipidome from a single exogenous phospholipid, which is an impressive evolutionary adaptation to their pathogenic lifestyle. Indeed, this acyl chain scavenging activity, which confounded our initial attempt to simplify the lipidome, suggests a potential target for treating mycoplasma infection. Although mycoplasma can survive in pure culture with a reduced lipidome, they may fare much worse in the context of a host immune system. Furthermore, the ability for the Minimal Cell to grow with a lipidome of two lipids implies the possibility for further minimization of the genome, through the deletion of pathways involved in the scavenging of phospholipid acyl chains and the internal synthesis of phospholipids.

Minimal lipidomes

A long-standing challenge in membrane biology has been to understand why life has evolved such complex lipidomes, and to identify the essential features of lipidomes required for optimal membrane function and cellular fitness. The simplest lipidome so far reported was from a Gram-

negative bacterium and was composed of 27 lipid species, excluding outer membrane lipopolysaccharides that were not analyzed. By reducing the lipidome of *M. mycoides* and JCVI-Syn3A down to 2 lipids, we show that a complex lipidome is not essential for life, but that two lipids are far from optimal. By systematically reintroducing lipidomic features to cells with a minimal lipidome, we were able to show that diversity of acyl chain configurations is more crucial than diversity of phospholipid head groups. This observation is consistent with previous work showing that reducing the head group diversity of *B. subtilis* had relatively little effect on growth[49]. This is not to say that having a diversity of head groups is not important. There are well-characterized examples of head group chemistry influencing bioactivity, for example the dependence of bacterial peptide secretion machinery on phophatidylethanolamine[50]. We previously showed how headgroup structure influences the biophysical effect of acyl chain composition[4]. Thus, having the right phospholipid head group may be as important as having diverse headgroups. However, acyl chain structure directly influences the properties of the bilayer and the activity of integral membrane proteins. Indeed, the critical role of phospholipid acyl chain unsaturation was recently demonstrated for the assembly and function of the nuclear pore complex in Eukaryotes[51]. Our results demonstrate that the diversity of acyl chain configurations is one of the minimal parameters required for a functional lipidome.

One limitation of this work is that the growth medium is not defined, and there is a very small contribution of lipids from components of the growth medium, such as the yeast extract. Thus, while the majority (>99%) of the minimal lipidome is composed of 2 lipids, there is a fraction of a percent of very low abundance lipids derived from the media and it is possible, but unlikely, that these trace lipids play a significant role. In this regard, development of a defined growth medium will be essential in the continued development of mycoplasmas as minimal model membrane systems[26]. Another factor limiting the minimization of the lipidome to one lipid is that cholesterol (or an analogue) is required for growth, but also cannot form a bilayer alone. So, it is possible that even one bilayer forming lipid could support life in a cell that has not evolved to require sterols. It is also, however, possible that having a membrane reinforcing sterol or sterol analogue is critical for building a stable cell membrane with only one phospholipid. A

mycoplasma-like organism such as Mesoplasma that does not require sterols for growth would provide a means to test this hypothesis[52]. Nonetheless, a JCVI-Syn3A membrane with two lipids comprising over 99% of the lipidome is currently the simplest living membrane that has been reported.

Lipid chirality and the lipid divide

The lipid divide, represents another major problem in membrane biology. Homochirality is a fundamental feature of biomolecular chemistry. Biomolecules exhibit enantioselectivity for chirally compatible interaction partners. Consequently, life has evolved to rely on homochiral molecules (e.g. L-amino acids and nucleic acids). An interesting twist is in the divide between the chirality of phospholipids made by Archaea and the rest of life[35]. The divide raises questions about whether the last universal common ancestor (LUCA) possessed a heterochiral lipidome, or whether the divide happened after the divergence of Bacteria and Archaea[53, 54]. Further, current theories that eukaryotes emerged from an Archaeal ancestor[55], create a conundrum in explaining why Eukaryotes don't have Archaeal lipid enantiomers, and whether a gradual transition involving heterochiral lipidomes could have occurred. At the crux of these conundrums is whether heterochiral lipidomes can support stable membranes and optimal cellular fitness.

We took advantage of the tuneability of *M. mycoides* and JCVI-Syn3A lipidomes to observe how heterochiral lipidomes influence the membrane and cellular growth. Previous work in living systems has achieved lipidomes with a mixture of structurally diverse bacterial and archaeal lipids that differed not only by chirality, but also in a variety of other ways[56]. A unique feature of this study is that we were able to introduce two enantiomers of a single lipid structure (POPC) into a living membrane. Our results demonstrate that a heterochiral lipidome results in a leakier membrane and impaired cellular robustness and growth. In contrast, previous work in E. coli indicated that heterochiral lipidomes did not affect growth[56]. E. coli has an outer membrane and cell wall that might compensate for lack of stability caused by heterochirality. The lipids in the E. coli study were not stereoisomers, but rather entirely different lipid structures (ether vs ester acyl chain linkages, isoprenoid tails vs. fatty acid tails). It is possible that these structural

differences somehow obscured the destabilizing effects of heterochirality. Ultimately, the basis for these diverging observations will provide insight into the biological significance of lipid chirality. Nonetheless, our work shows that in a minimal organism with a single membrane, heterochiral lipidomes can support growth, but lead to impaired robustness and fitness. This demonstrates that ancestral heterochiral lipidomes could have supported life.

The changes in membrane robustness and permeability that we observed do not seem to be explained by changes in lipid order of pure lipid vesicles reconstituted from cell lipid extracts of homo- and heterochiral lipidomes. This implies that lipid-protein interactions may be affected by lipid chirality in ways the impair membrane robustness and function. Numerous in vitro studies have demonstrated the effect of lipid chirality on lipid-protein interactions, consistent with the possibility that the phenotypes we observed are rooted in perturbed lipid-protein interactions. Looking forward, *M. mycoides* and JCVI-Syn3A will be excellent model system to explore the functional consequences of lipid chirality on lipid-protein interactions.

Outlook

Our approach to employ *M. mycoides* and JCVI-Syn3A as minimal living model membrane systems paves a new path towards unraveling the role of lipidome diversity and complexity. Our observations reveal that life does not require complex lipidomes. However, minimization comes with clear trade-offs in cellular fitness. We further demonstrate the capacity of these model systems to serve as chassis for exploring fundamental questions in membrane biology. The ability of this system as a testing platform for lipid diets with a variety of features (including class and acyl chain composition) makes its a useful and simple in vivo model for design-test-build applications of membrane composition experiments. Furthermore, we demonstrated the ability to force *M. mycoides* and JCVI-Syn3A to take up and incorporate enantiomeric lipids in their membranes, the first time this has been shown in eukaryotic or prokaryotic organisms, and an exciting first step to allow us to probe questions about the lipid divide and the role of chirality in membrane stability and cell fitness. Overall, we have demonstrated the creation of a simple,

tunable, living, model membrane system that can be used as a novel platform for probing the design principles of living membranes.

Acknowledgements

The authors wish to thank the Saenz lab; Nataliya Safronova, Lisa Junghans, Ha Ngoc Anh Nguyen, and Tomasz Czerniak for discussions and feedback through the process, and for help with experimental designs; the JCVI, and specifically John Glass and Kim Wise, for providing *M. mycoides* and JCVI-Syn3A and for further feedback, including manuscript comments; Jennifer Katz-Brandoli for assisting with robot runs and help with figure layout. We thank Telesis Bio, Inc. for allowing us to use JCVI-syn3A and Lipotype GmbH for lipidomic analyses. This work was supported by the B CUBE of the TU Dresden, a German Federal Ministry of Education and Research BMBF grant (to J.S., project 03Z22EN12), and a VW Foundation ''Life ''grant (to J.S., project 93090).

Materials and Methods

Cell Culture:

M. mycoides and JCVI-Syn3A were grown in liquid culture at 37ºC on a modified SP4 growth medium with Phenol Red[57]. The lipid source was provided either by Fetal Bovine Serum (FBS), complex natural source of mammalian lipids, or by complexing lipids with delipidated bovine serum albumin (BSA). Lipids were dissolved in ethanol and added to the medium at 37ºC immediately before the addition of cells, in the concentrations given in Supplementary Table 2. *M. mycoides* was passaged to new growth medium by adding 50 µL of cell culture at OD600 0.4-0.8 into 7 mL of freshly prepared media in a T25 Flask (Stand., Vent. Cap) or 10mL of media in a Duran 100mL glass flask. This corresponded to roughly one passage per day. JCVI-Syn3A was passaged to new medium by adding 200 µL of cell culture at OD600 0.2-0.6 into 7 mL of freshly prepared media. This corresponded to one passage every two to four days, depending on cell growth rates. Cells were grown at 37ºC and 40RPM in a KuhnerShakerX incubator.

Growth Rates:

Metabolic Growth Index:

Due to differences in cell size and behavior between *M. mycoides* and JCVI-Syn3A, measuring growth curves by relying on taking the absorbance at 600nm proved to be an unreliable method

to achieve growth estimates that were robustly comparable between the two organisms. Phenol red is a pH indicator that is commonly used as a readout for growth in mycoplasma cell culture[58] as it detects changes in pH as metabolic activity acidifies the culture media. To obtain the rate of pH change over time cells were grown on the liquid handler system Biomek i7 Automated Workstation, and the absorbance at 562 nm was recorded hourly to record changes in phenol red absorbance as cell growth acidifies the media[13, 58, 59]. All absorbances were then plotted with respect to time, and then fitted to the logistic function $Nt = K (1 + (K-No)/No) e^{-rt}$) where Nt is population at time t, K is the carrying capacity, N0 is the initial population size, and r is the growth rate; or, in this case, the rate of pH change. r was determined by calculating the slope of change at the steepest point of the logarithmic curve, corresponding to mid exponential growth. To validate the relevance of this method as a readout for cell growth, growth rates for a representative set of *M. mycoides* diets were calculated using the absorbance of the cells at 600nm and were compared to the rates calculated with the phenol red method. The result was a strong linear correlation between the two methods (Supplementary figure 5a). Representative curves of the entire period of growth for *M. mycoides* on one diet and generated by the two methods is also shown (Supplementary figure 5b).

Cellular growth data was obtained by growing cells on the liquid handler system Biomek i7 Automated Workstation with absorbance readings taken hourly at OD 562 nm to record changes in phenol red absorbance as cell growth acidifies the media[13, 58, 59]. Growth rates were determined by calculating the slope of change at the steepest point of the exponential growth curve, corresponding to mid exponential growth.

Cell growth data measured by optical density at 600nm was obtained through hourly manual measurements from cultures grown in 30mL in a Duran 100 mL glass flask at 37ºC using a DeNovix Spectrophotometer (Supplementary figure 6a,b).

<u>Lipid Extraction and Thin Layer Chromatography:</u>

Lipids were extracted using a Bligh-Dyer lipid extraction protocol[60]. When appropriate, lipid concentration was determined with a phosphate assay. TLC plates with Silica Gel and a 10x concentrating zone were pre-washed with chloroform to remove debris and other artifacts, and were then dried at 60ºC for > 30 minutes to ensure complete solvent evaporation. Lipid samples were then loaded on the plate and were dried at 60ºC for > 10 minutes to again ensure complete solvent evaporation. The plate was then run in a closed glass chamber using a running solution of Chloroform:Methanol:Acetic Acid:Water (85:25:5:4), and were subsequently dried again at 60ºC for > 30 minutes. TLC plates were then rinsed with a 3% Copper acetate and 8% phosphoric acid solution and heated by a handheld heat gun at 280ºC to char lipid spots.

Liposome Preparation: Lipids of a known concentration (either in stock solutions or extracted from cells and validated with a phosphate assay) were prepared in a 2mL glass by having their solvents evaporated overnight under a 10-17mbar vacuum. Lipids were then resuspended in the appropriate volume of Liposome buffer (LiB) (10mM HEPES, 100 mM NaCl) to achieve 100mM liposome solution and incubated for 30 minutes at 37ºC. Subsequently, liposomes were homogenized with ten freeze thaw cycles (1 minute in liquid nitrogen followed by 5 minutes at 60C) and 7 extrusion cycles in a Hamilton syringe setup through a 100nm filter.

Propidium Iodide Osmotic Shock Assay: Harvest 3X 0.4 ODU of cells with OD 0.2-0.4 (1 ODU = 1 mL of cells at OD 1). Spin down in pre-warmed centrifuge at 5000g, 7minutes, 37ºC, slow acceleration slow deceleration. Add 1 µL of 1mM Propidium Iodide dye to appropriate wells on a black or clear bottom 96well plate. Aspirate medium and resuspend each cell pellet in 400 µL of either H2O or 5X diluted mycoplasma wash buffer (MWB) (original MWB: 20mM HEPES, 200 mM NaCl, 1% W/V Glucose). Set up an extra tube with cells resuspended in 400 µL H2O to be boiled as a positive control. Boil control tube in 95ºC thermoshaker at 1000 rpm for 10 minutes. During that time, incubate all other tubes on the 37ºC thermoshaker at 600rpm. Add 100 µL of the cells in the appropriate buffer to each of their three analytical wells on the 96 well plate. Incubate 30 minutes with shaking at 37ºC in a TECAN platereader and subsequently measure the fluorescent signal at excitation 539nm emission 619nm.

Fluorescein Diacetate Permeability Assay: Harvest 3X 0.4 ODU of cells with OD 0.2-0.4 (1 ODU = 1mL of cells at OD 1). Spin down in pre-warmed centrifuge at 5000g, 7 minutes, 37ºC, slow acceleration slow deceleration. Wash 1x in MWB, resuspend in 400 µL MWB. Prepare a Fluorescein Standard curve in plate: 0, 0.25 ,0.5, 1.2, 2 µM Fluorescein Add 100 µL resuspended cells to proper wells (in triplicate), and 100 µL MWB to Fluorescein wells. Add FDA to cell wells to achieve final FDA concentration of 5 µM. Incubate and measure on TECAN (37ºC, 150 minutes, excitation 485 emission 525, measurements every 30 minutes). The permeability coefficient (P) of FDA was calculated using Fick's Law ($Q = P*A*(C_{out} - Ci_n)$) where Q is the flux across the membrane, and is a constant as the slope of fluorescein increase over time was linear; A is the area of the membrane, calculated using the assumption that the cross sectional area of POPC + Cholesterol in a bilayer is 45.1 $Å^2$ [61], the ratio of POPC : Cholesterol is roughly 1:1, the area of POPC + cholesterol is equivalent to the area of Enantiomeric POPC + Cholesterol, and the number of POPC molecules can be calculated from the phosphate assay described below; C_{out} and C_{in} are the concentrations of FDA outside and inside the cell (respectively); and Ci_n is 0 as FDA is immediately converted to fluorescein upon entering the cell.

C-Laurdan General Polarization Assay: Incubate liposomes at 37ºc and 1000rpm for 10 minutes on a tabletop thermoshaker. While incubating, remove C-Laurdan from freezer and let warm to temperature on bench. Add 1 µL C-Laurdan (Dilute 1mM stock 4x in EtOH. Add 1uL of diluted stock for final molarity 0.5 µM C-Laurdan) to 500 µL of liposomes. Incubate samples at 37ºc and 1000rpm for 10 minutes on a tabletop thermoshaker.Add 100 µL of each sample to a flat bottom 96 well plate well, repeating three times in order to achieve analytical triplicates. Measure on TECAN (2 channel fluorescence reading with excitation at 385nm and emission at 440 and 490 nm respectively). General polarization (GP) was calculated using the formula: GP = (I440 - I490) / (I440 + I490) where I is the fluorescence emission intensity at the respective wavelength.

Phosphate Assay: To estimate the concentration of phospholipids in a certain amount of cells, the amount of phosphate in the lipid extraction of 1ODU of cells was measured. To measure phosphate amount a modified version of the method of Chen et al. was used[62]. The lipid extraction was added to glass pyrex tubes with both biological and analytical triplicates (3 vials per biological replicate), and the solvent was evaporated by a brief (<5 minute) incubation at 200ºC. 50 µL of water were added to each sample. To prepare a standard curve of phosphate amounts, an ICP Phosphorus standard was diluted in water to give 5, 10, 20, 50 and 100, and 200 nmols of phosphate. 500 µL of 70% perchloric acid was added to each vial and, after brief vortexing, samples were incubated at 200ºC for 120 minutes. Tubes were cooled down in ice water, and 1mL of 10% w/v ascorbic acid followed immediately by 1 mL of 2.5% w/v ammonium heptamolybdate were added (with a brief vortex after adding each reagent). Samples were incubated at 37ºC for 30 minutes, and then absorbance at 820nm was measured on a TECAN plate reader by adding 200 µL of each tube to a clear bottom 96 well plate . A standard curve of absorbance at 820 vs phosphate amount was calculated using the phosphate standard, and lipid amount of each sample was calculated based on the absorbance value, standard curve, and assumption that one phosphate molecule equals one phospholipid.

Lipidomic Analysis: Lipids extracted from cells using the aforementioned Bligh-Dyer protocol were submitted to Lipotype for mass spectrometry based analysis[63]. The general procedure is described in Sampaio et.al, 2011[64]. For the analysis, samples were spiked with internal lipid standard mixture containing: cardiolipin 14:0/14:0/14:0/14:0, ceramide 18:1;2/17:0, diacylglycerol 17:0/17:0, hexosylceramide 18:1;2/12:0, lyso-phosphatidate 17:0, lyso-phosphatidylcholine 12:0, lyso-phosphatidylethanolamine 17:1, lyso-phosphatidylglycerol 17:1, lyso-phosphatidylinositol 17:1, lyso-phosphatidylserine 17:1, phosphatidate 17:0/17:0, phosphatidylcholine 17:0/17:0, diether phosphatidylcholine 16:0/18:1, phosphatidylethanolamine 17:0/17:0, phosphatidylglycerol 17:0/17:0, phosphatidylinositol 16:0/16:0, phosphatidylserine 17:0/17:0, cholesterol ester 20:0, sphingomyelin 18:1;2/12:0;0, triacylglycerol 17:0/17:0/17:0, and cholesterol D6. After

extraction, the organic phase was transferred to an infusion plate and dried in a speed vacuum concentrator. It was then resuspended in 7.5 mM ammonium acetate in chloroform/methanol/propanol (1:2:4, V:V:V) and a 33% ethanol solution of methylamine in chloroform/methanol (0.003:5:1; V:V:V). All liquid handling steps were performed using Hamilton Robotics STARlet robotic platform with the Anti Droplet Control feature for organic solvents pipetting. Samples were analyzed by direct infusion on a QExactive mass spectrometer (Thermo Scientific) equipped with a TriVersa NanoMate ion source (Advion Biosciences). Samples were analyzed in both positive and negative ion modes with a resolution of Rm/z=200=280000 for MS and Rm/z=200=17500 for MSMS experiments, in a single acquisition. MSMS was triggered by an inclusion list encompassing corresponding MS mass ranges scanned in 1 Da increments. Both MS and MSMS data were combined to monitor CE, DAG and TAG ions as ammonium adducts; PC, PC O-, as acetate adducts; and CL, PA, PE, PE O-, PG, PI and PS as deprotonated anions. MS only was used to monitor LPA, LPE, LPE O-, LPI and LPS as deprotonated anions; ceramide, hexosylceramide, sphingomyelin, LPC and LPC O- as acetate adducts and cholesterol as an ammonium adduct of an acetylated derivative.

Materials:

SP4 (For 1L):

PPLO (3.5g) (Becton, Dickinson, and Company product no. 255420)

Tryptone (10g) (Sigma product no 70169)

Peptone (5g) (Sigma product no 70176)

20% Glucose (25mL) (Ross product no. X997.2)

20% Yeastolate (10mL) (Becton, Dickinson, and Company product no. 255772)

15% Yeast Extract (35mL) (Roth product no. 2904.3)

70g/L BSA (85mL) (Sigma product no. A7030) or FBS (170mL) (Biowest Product no. 5181H-500)

400,000U/mL Penicillin G-sodium salt (2.5mL) (Roth product no. HP48.1)

10mg/mL L-Glutamate (5mL) (Roth product no. HN08.2)

Sodium Bicarbonate (1.04g) (Honeywell product no. 71630)

CMRL (4.9g) (US Biological Lifesciences product no. C5900)

Phenol Red (11mg) (Sigma product no. P3532)

Lipids:

Cholesterol (Avanti product no. 700100)

Diether POPC (Diether 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no. 999983)

Diether DPPC (Diether 1-palmitoyl-2-palmitoyl-glycero-3-phosphocholine) (Avanti product no. 999992)

Diether DOPC (Diether 1-oleoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no. 999991)

Diether POPG (Diether 1-palmitoyl-2-oleoyl-glycero-3-phosphotdylglycerol) (Avanti product no. 999973)

POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no. 850457)

DPPC (1-palmitoyl-2-palmitoyl-glycero-3-phosphocholine) (Avanti product no. 850355)

DOPC (1-oleoyl-2-oleoyl-glycero-3-phosphocholine) (Avanti product no. 850375)

POPG (1-palmitoyl-2-oleoyl-glycero-3-phosphotdylglycerol) (Avanti product no. 840457)

DPPG (1-palmitoyl-2-palmitoyl-glycero-3-phosphoglycerol) (Avanti product no. 840455)

DOPG (1-oleoyl-2-oleoyl-glycero-3-phosphoglycerol) (Avanti product no. 850475)

POPE (1-palmitoyl-2-oleoyl-glycero-3-phosphoethanolamine) (Avanti product no. 850757)

DPPE (1-palmitoyl-2-palmitoyl-glycero-3-phosphoethanolamine) (Avanti product no. 850705)

DOPE (1-oleoyl-2-oleoyl-glycero-3-phosphoethanolamine) (Avanti product no. 850725)

Oleic Acid (c*is*-9-Octadecenoic acid) (Sigma product no. 01383)

Palmitic Acid (1-Pentadecanecarboxylic acid) (Sigma P0500)

Enantiomeric POPC (3-palmitoyl-2-oleoyl-glycero-1-phosphocholine) (Avanti product no. 850855)

16-0 Cardiolipin (1',3'-bis[1,2-dipalmitoyl-sn-glycero-3-phospho]-glycerol) (Avanti product no. 710333)

Assays:

FDA (Sigma product no. F7378)

Fluorescein (Fluka product no. 28803)

Propidium Iodide (Sigma product no. 537059)

C-Laurdan (Stratech Scientific Ltd. product no. T0001-SFC-1)

Cells:

JCVI-Syn3A-*mCherry (*From Telesis Bio)

*Mycoplasma mycoides subspecies capri strain GM12 (*From Telesis Bio)

Machines:

TECAN

Biomek i7 Automated Workstation

DeNovix DS-11 FX +

PicoQuant FluoTime 300 High Performance Fluorescence Lifetime and Steady State Spectrometer

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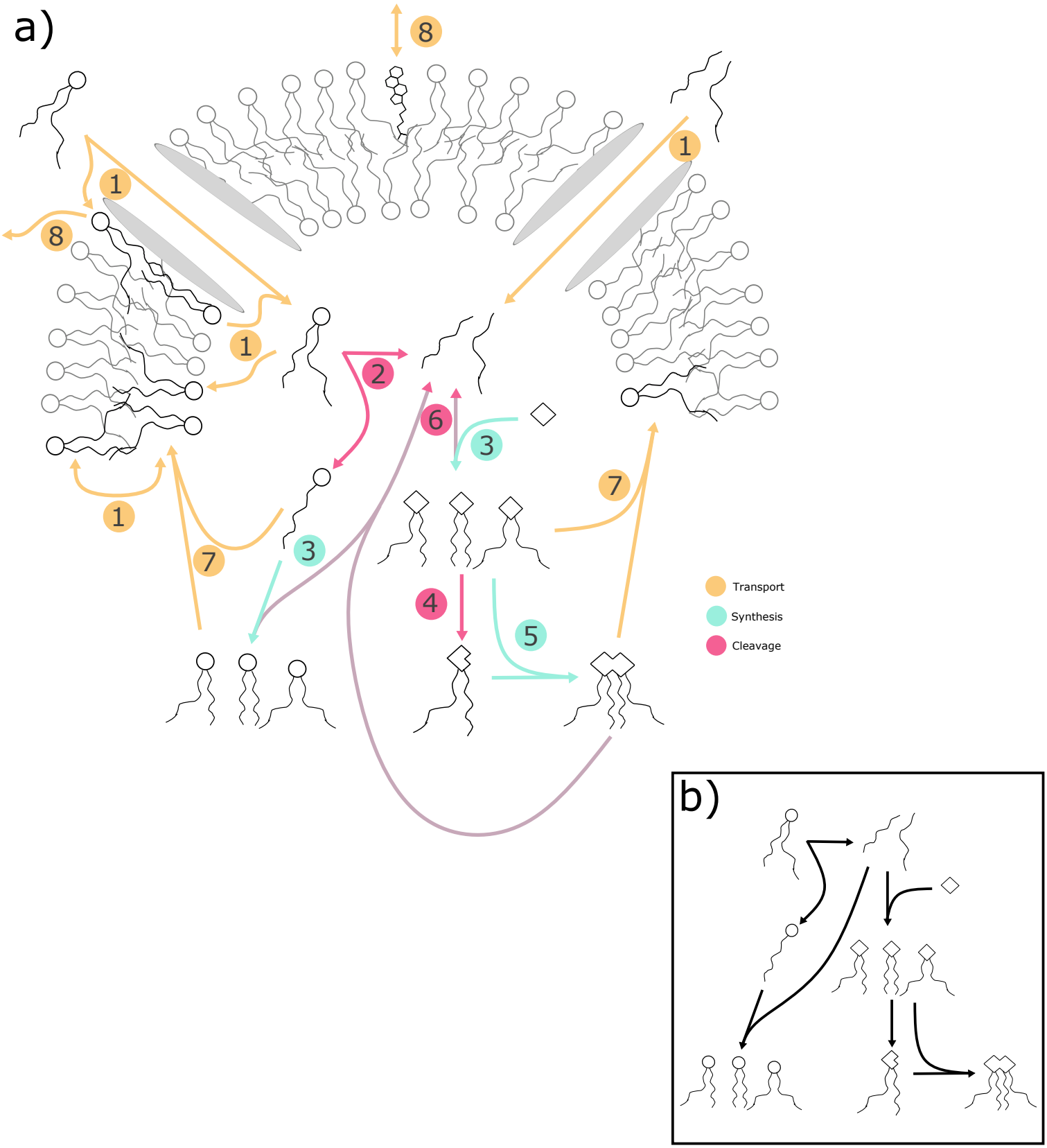

Figure 1: Membrane Remodeling in *M. Mycoides* is Dependent on Acyl Chain Scavenging

a) 1) *Mycoplasma mycoides* can take up phospholipids and free fatty acids from its environment[8, 18, 19, 20]. **2)** Cleaving ester bonds from glycerophospholipids results in a pool of free fatty acids[21, 22, 23]. **3)** With fatty acids, *M. mycoides* is able to synthesize Phosphatidylglycerols (PG) with various fatty acid compositions, as well as remodel other phospholipid classes[8, 20, 21, 24, 25]. **4)** Cardiolipins can be synthesized from two PGs, starting with cleavage of PG headgroups[26, 27]. **5)** *M. mycoides* can synthesize a diversity of Cardiolipins from PGs[8, 24, 26, 27]. **6)** Cleaving acyl chains from remodeled and synthesized phospholipids replenishes the pool of free fatty acids[25]. **7)** Glycerophospholipids are inserted into the membrane[23]. **8)** Cholesterol is essential for *M. mycoides* growth[28, 29, 30]. **b)** Acyl chain scavenging drives remodeling and membrane complexity in *M. mycoides*.

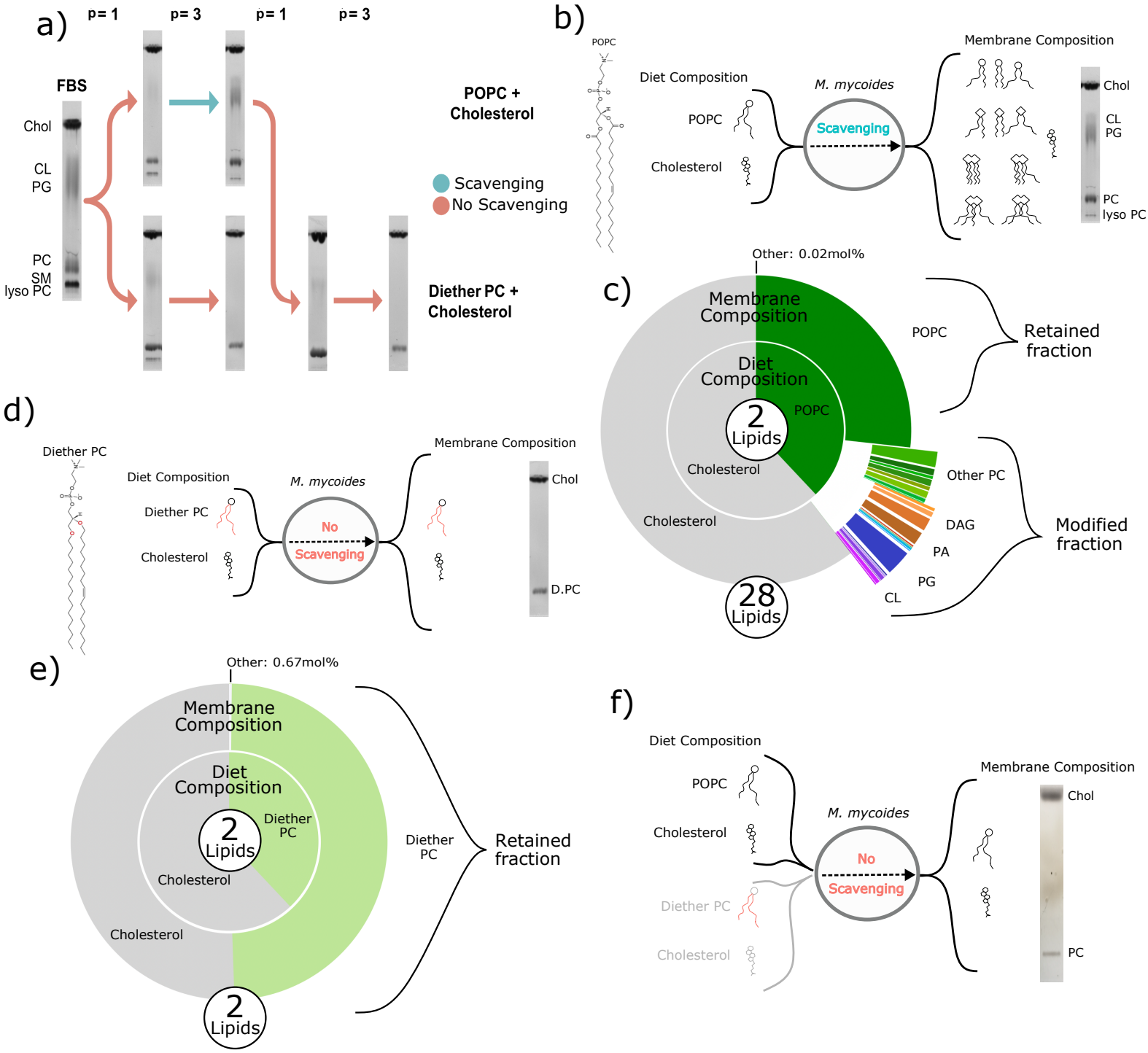

Figure 2: A Defined Lipid Diet Results in a Living Membrane with Two Lipids

a) *M. mycoides* can adapt to defined lipid "diets" with diester or diether phospholipids; resulting in simpler membranes than when grown on a complex diet (e.g. FBS). Adaptation to new diets occours after 3 passages ($p \geq 3$). **b)** *M. mycoides* can scavenge acyl chains from POPC, yielding a more complex membrane from a defined diet **c)** Lipidomic analysis shows acyl chain scavenging leads to the synthesis of a diversity of lipids, resulting in a membrane with 28 lipids from a diet of only two. **d)** 16:0-18:1 diether PC has ether-linked hydrocarbon chains that cannot be cleaved by *M. mycoides*, eliminating scavenging. **e)** Lipidomic analysis shows living cells with two lipids comprising 99.33 mol% of their lipidome. **f)** When switched from one diet to another, there is an adaptation period before acyl chain scavenging occours.

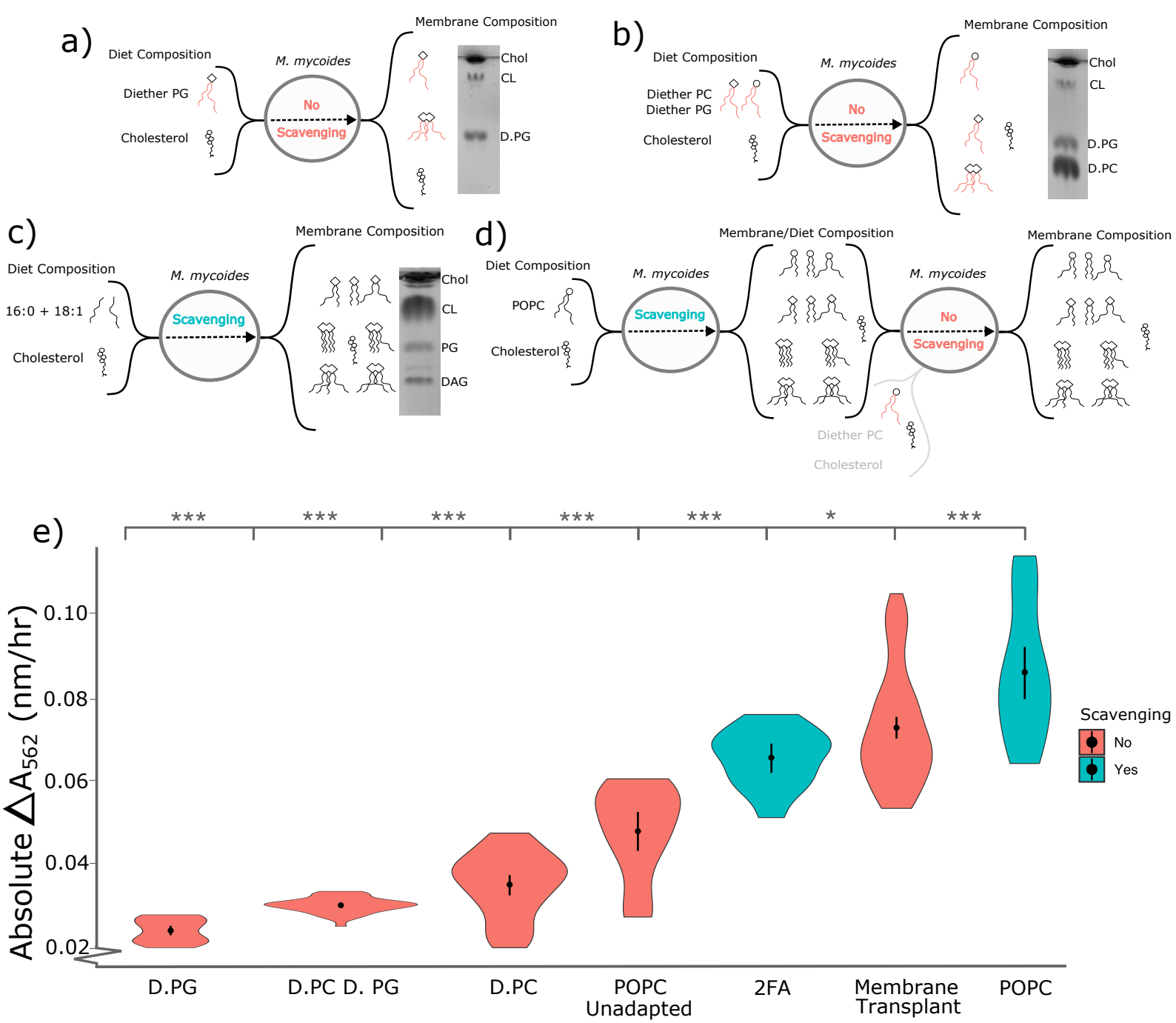

Figure 3: Living Membranes with Tunable Lipid Class and Acyl Chain Complexity

a) 16:0-18:1 diether PG diets results in the synthesis of Cardiolipin with fully ether-linked hydrocarbon chains, increasing head group, but not acyl chain complexity.. **b)** A diet of both Diether PC and PG restores the headgroup, but not acyl chain, comlplexity of the POPC diet. **c)** A diet of free fatty acids, restores the acyl chain, but not head group, complexity of the POPC diet. **d)** A diet derived from a total lipid extract of cells grown on the POPC diet (a "transplant" diet) restores the full complexity of the POPC. Growth on this transplanted diet was performed after the first passage (p = 1) to the new diet, before cells begin to scavenge acyl chains from exogenous phospholipids. **e)** Reintroducing acyl chain complexity is more effective than headgroup complexity for rescuing growth (relative to cells adapted to a POPC diet), but even when full complexity is restored acyl chain scavenging capacity still improves growth.

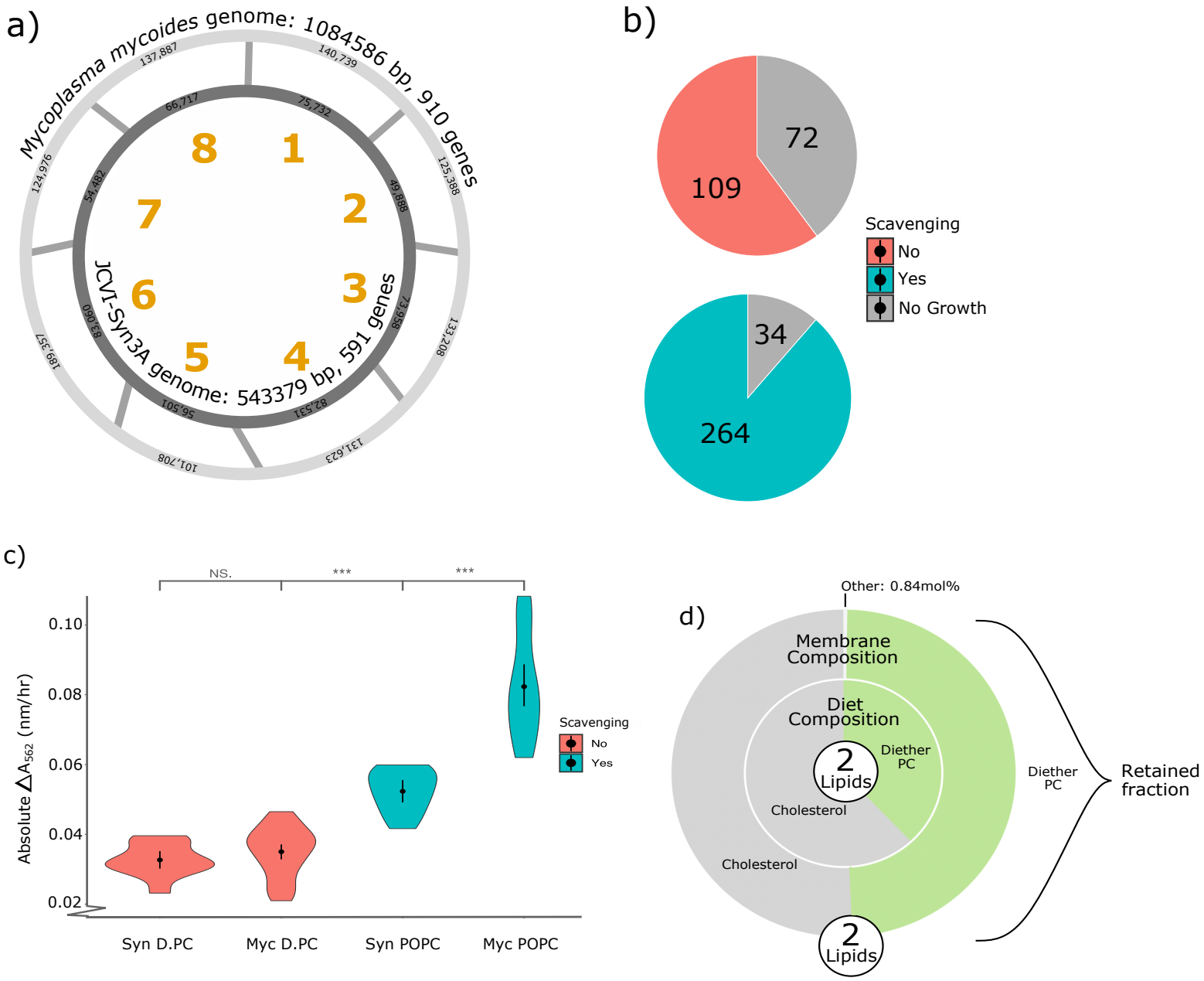

Figure 4:JCVI-Syn3A is a Simpler Cell with a More Fragile Membrane

a) JCVI-Syn3A is minimal synthetic cell analog of *M. mycoides*. Adapted from Hutchison et. al, 2016. **d)** JCVI-Syn3A exhibits no growth on a number of lipid diets, especially where acyl chain scavenging is restricted. **c)** JCVI-Syn3A grows significantly slower than *M. mycoides* on the same diets. **d)** JCVI-Syn3A is viable grown on a Diether PC + Cholesterol diet with 99.16 mol% of the membrane comprised of only two lipids.

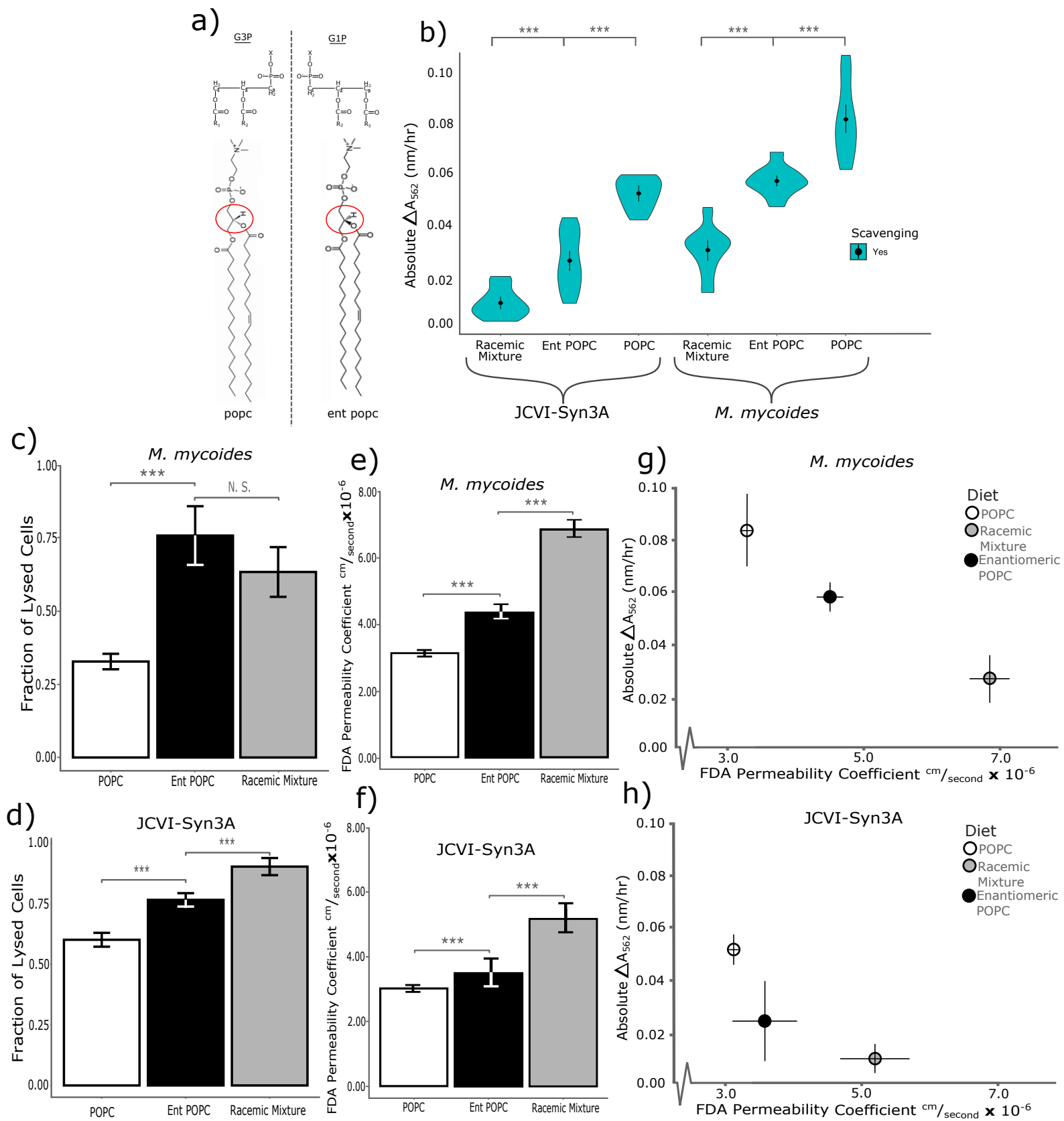

Figure 5: Enantiomeric Lipid Diets Negatively Affect Cell Growth and Membrane Properties

a) Enantiomeric (Ent) POPC is a synthetic chiral POPC with the head group in the SN1 position, rather than SN3 position. **b)** Lipid diets with enantiomeric POPC present significantly hindered growth compared to the POPC diet. **c)** *M. mycoides* and **d)** JCVI-Syn3A cells are more fragile to hypoosmotic shock when grown on diets with enantiomeric POPC present. **e)** *M. mycoides* and **d)** JCVI-Syn3A cells are more permeable to non-chiral fluorescein diacetate (FDA) when grown on diets with enantiomeric POPC present **g)** *M. mycoides* and **h)** JCVI-Syn3A cell growth and membrane permeability are inversely correlated.

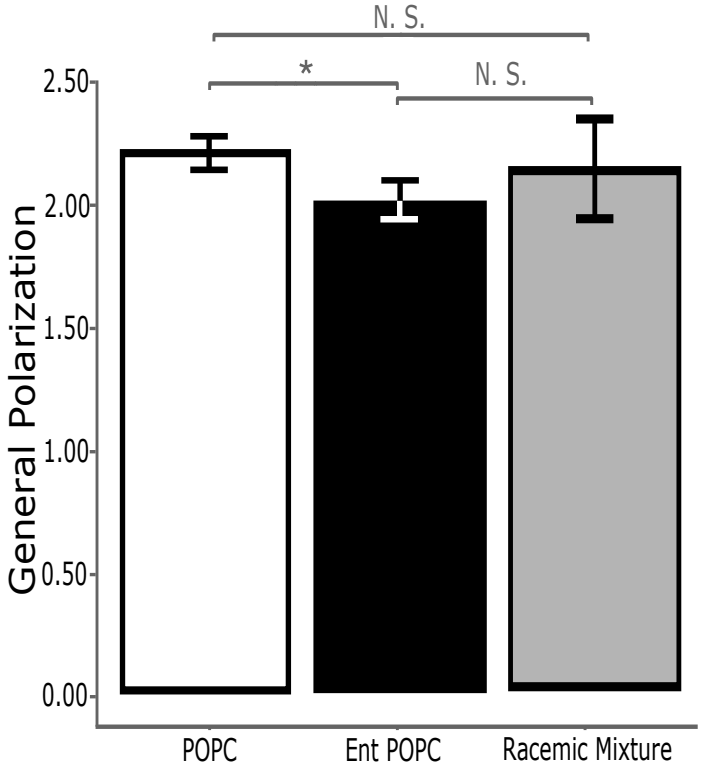

Figure 6: Enantiomeric Lipids Have no Significant Effect on Membrane Order

Lipid vesicles reconstituted from total lipid extracts of *M. mycoides* cells had a similar membrane order (measured as the general polarization of the membrane using C-laurdan) for all three diets with POPC enantiomers.