HHS Public Access

Author manuscript

Nat Rev Mol Cell Biol. Author manuscript; available in PMC 2023 February 02.

Published in final edited form as:

Nat Rev Mol Cell Biol. 2023 February ; 24(2): 107–122. doi:10.1038/s41580-022-00524-4.

*correspondence to il2sy@virginia.edu and/or elyman@udel.edu.

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Regulation of membrane protein structure and function by their paralipidomes

Ilya Levental[1], **Ed Lyman**[2]

[1]Department of Molecular Physiology and Biological Physics, Center for Molecular and Cell Physiology, University of Virginia

[2]Department of Physics and Astronomy, Department of Chemistry and Biochemistry, University of Delaware

Abstract

Transmembrane proteins comprise ~30% of the mammalian proteome, mediating metabolism, signaling, transport, and many other functions required for cellular life. The microenvironment of integral membrane proteins (IMPs) is intrinsically different from cytoplasmic ones, with IMPs solvated by a compositionally and biophysically complex lipid matrix. These solvating lipids affect protein structure and function in a variety of ways, from stereospecific, high-affinity protein-lipid interactions to modulation by bulk membrane properties. Specific examples of functional modulation of IMPs by their solvating membranes have been reported for various transporters, channels, and signal receptors; however, generalizable mechanistic principles governing IMP regulation by lipid environments are neither widely appreciated nor completely understood. Here, we review recent insights into the inter-relationships between complex mammalian lipidomes, the membrane physicochemical properties resulting from such lipid collectives, and the regulation of IMPs by either or both. The recent proliferation of high-resolution methods to study such lipid-protein interactions has led to generalizable insights, which are synthesized into a general framework termed the “functional paralipidome” for understanding the mutual regulation between membrane proteins and their surrounding lipid microenvironments.

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INTRODUCTION

Functional interactions between proteins and other biomolecules are fundamental to molecular cell biology. Constituting ~30% of the mammalian proteome [1] and 60% of all drug targets [2], integral membrane proteins are solvated by complex mixtures of lipids that influence their structures, dynamics, and functions. However, in comparison to protein-protein and protein-nucleic acid interactions, the interactions between membrane proteins and their specific lipid nano-environments are not nearly as well characterized. For many IMP drug targets, the protein-lipid interface provides an important site through which pharmaceuticals enter the protein before accessing their binding sites, which are themselves often relatively hydrophobic [3]. Despite the obvious fundamental and biomedical impacts, knowledge gaps regarding lipid-mediated IMP regulation persist because both membrane proteins and their associated lipids are ill-suited to classical analytical and conceptual paradigms of molecular cell biology. Practically, both IMPs and lipids are hydrophobic and poorly soluble, and co-assemble into large structures (i.e. membranes) that are problematic for many common structural and biochemical approaches. Conceptually, lipids can serve simultaneously as solvents, substrates, and regulatory co-factors for membrane protein activity (Fig 1) and these roles are often entangled and overlapping.

The scope for protein regulation by membranes and their constituent lipids has been broadened by recent discoveries that reveal cellular lipidomes to be much more complex, diverse, and variable than is typically appreciated [4]. Mammalian cells produce hundreds of distinct lipid species, and the specific lipid complement can vary dramatically depending on cell type [5,6], metabolic state [7], disease state [8], and external inputs (e.g. from the diet) [6,9]. In some cases, a single specific lipid species appears to be required for regulating protein function, as was reported for the interaction between the transmembrane domain of the trafficking protein p24 and a sphingomyelin lipid containing an 18-carbon acyl chain (C18-SM) [10]. Such remarkable specificity combined with the complexity of mammalian lipidomes suggests the potential for a layer of regulation of IMPs by lipids that has been underappreciated.

Here, we discuss illustrative recent examples of protein regulation by interactions with their solvating membranes and their constituent lipids. We describe distinct roles of individual lipid molecules as specific protein cofactors versus collective properties like membrane thickness or packing. We focus specifically on transmembrane rather than peripheral or lipidated proteins, whose lipid interactions constitute an important, but separate, area of membrane biology [11,12] (for readability, integral membrane proteins will be referred to hereafter as IMPs). Another broad area that will not be covered in this review are the various enzymes involved in production, degradation, and regulation of the lipidome whose functionality inherently involves lipid interactions. Finally, while there have been extensive demonstrations of functionally relevant binding between membrane proteins and lipids (many expertly reviewed previously [13–18]) and protein-lipid interfaces as drug targets [3], our intent is not to document an exhaustive list of such examples. We instead present an experimentally informed and theoretically reinforced framework to rationalize the influence of lipids and bulk membrane properties on protein function.

IMP REGULATION BY MEMBRANE BIOPHYSICS

The combination of a cell's lipidome and its collection of IMPs produces a bilayer membrane with remarkable properties. On molecular scales, biomembranes are fluids whose lipids and proteins mix and interact via diffusion in two dimensions. At intermediate scales we can speak of collective properties such as membrane thickness and lipid tilt. At still larger length scales, the same materials behave as thin-yet-robust elastic sheets that can be stretched, bent, and shaped into the variety of morphologies required for cellular functions [19]. Across the length scales, the membrane acts on membrane-embedded proteins to regulate their interactions, conformations, localizations, and functions [20] (Fig 2).

Thickness mismatch can drive lateral and subcellular protein sorting

A fundamental structural feature of membranes is their thickness, which is largely determined by the length and order of lipids' hydrophobic chains. These chains can vary from 12 carbons up to 24 carbons in mammalian cells, suggesting a range of possible membrane thicknesses from ~3–4.5 nm [21]. While there have been few direct measurements of biomembrane thickness, X-ray scattering of purified membranes indicates that plasma membranes are >10% thicker than internal membranes [22] (Fig 3). More recently, direct images of membrane thickness variations in biomimetic and bioderived membranes [21,23] have been obtained by cryoEM, with similar images of cryopreserved cells suggesting feasibility of biomembrane thickness measurements in situ [24].

Variations in membrane thickness become relevant for proteins when the lengths of their hydrophobic membrane spanning regions (their transmembrane domains, TMDs) do not match the hydrophobic thickness of the surrounding membranes (Fig 2a)[25]. Such 'hydrophobic mismatch' is energetically unfavorable because lipids must deform (e.g. compress) to avoid exposure of hydrophobic regions to water (Fig 2b). At the extremes, sufficient compression of the membranes allow lipids to scramble from one leaflet to the other [26]. In a membrane of uniform thickness, misfit IMPs may cluster to minimize membrane distortion [27] (Fig 2c). In membranes with coexisting domains of different thicknesses, proteins are laterally sorted to their least-mismatched domain (Fig 2c). It should be emphasized that these descriptions treat the membrane as a homogenous continuum, with elastic costs imposed by bending or stretching. However, at the molecular level relevant for protein interactions, membranes are composed of discrete lipid species with complex biological lipidomes comprised of many different species capable of conforming to various shapes and sizes of proteins. Thus, in biological contexts, elastic considerations must be considered alongside more local effects (e.g. sorting of short lipids near short proteins)[28,29].

The energetics of hydrophobic mismatch are likely responsible for the conserved variation in TMD length between subcellular organelles, with TMDs of the endoplasmic reticulum and Golgi being shorter than those of proteins that reside in the plasma membrane (PM) [30] (Fig 3). These variations correlate closely with the presumed cholesterol gradients between those membranes, suggesting that cholesterol concentrations are important determinants of organellar membrane properties (Fig 3). The striking correlation between TMD length and hydrophobic thickness of organellar membranes suggests that proteins and lipids co-segregate based on their biophysical properties, and that such segregation may be a

prerequisite to proper subcellular trafficking [31]. Yeast use an analogous mechanism for sorting membrane proteins between the mother cell and the daughter bud. Here, a thick ER domain at the cleavage furrow appears to act as a filter, preferentially allowing long-TMD proteins to pass through [32]. A recent report suggested that analogous domains in the mammalian ER may be organized by interactions between a multipass protein sigma-1 receptor (S1R) and cholesterol [33], and that such domains may mediate intra-organelle contacts [33,34].

Just as hydrophobic mistmatch applies stress to bilayer lipids (Fig 2b), that stress is also propagated to the proteins. In some cases, this stress can change their conformation and function (Fig 2f)[25]. This effect has been most clearly demonstrated for pumps and transporters, including the Na+/K+ ATPase [35], a bacterial aquaporin, and the sarcoplasmic ER Ca2+ ATPase (SERCA) [36], and certain GPCRs [37], all of which show clear variation in activity as a function of membrane thickness, with maximal activity in optimally matching membranes (for in-depth discussion of hydrophobic mismatch, see [13,38]). More recently, the oligomeric assembly of a bacterial antiporter was shown to be driven by hydrophobic mismatch between the membrane and one protein surface that becomes buried upon homodimerization, lowering the overall free energy of the system [28]. Doping membranes with short-chain lipids that solvate this special surface reduces dimerization, demonstrating the critical importance of lipid context in IMP functional assembly.

The notion that bilayer thickness regulates protein function suggests that this thickness must be sensed and regulated by cells. A prototype membrane thickness sensor is the bacterial “molecular caliper” called DesK [39], which changes conformation as a function of membrane thickness to activate a *B. subtilis* two-component signaling system. Intriguingly, the originally proposed function of this protein is for sensing temperature, with the rationale that colder temperatures lead to thicker membranes as lipids pack more tightly together and their acyl chains elongate.

A related but distinct principle is protein sorting by membrane curvature. An array of peripheral proteins, many containing banana-shaped features called BAR domains, are known to preferentially bind highly curved membranes and also to induce such curvature when applied to flat membranes [40]. Such curvature selectivity is often accomplished by amphipathic helices that insert into one leaflet of the bilayer [41]. However, some transmembrane proteins also appear to sense curvature [42], preferentially enriching in highly curved membrane tubes (Fig 2h). Other IMPs generate membrane curvature by repulsive interactions of their extramembrane domains [43,44]. Finally, post-translational modifications near the transmembrane domain can affect proteins’ curvature preference, which may be used to sort proteins in the secretory pathway [45].

Protein sensors of membrane fluidity and lipid packing

The fluidity of biomembranes is fundamental to their function, as it allows proteins and lipids to explore the cell surface and find interaction partners. The viscosity of this fluid largely determines the lateral diffusion of IMPs and lipids, and therefore the frequency of the encounters that underlie signaling and other cellular processes. An elegant recent demonstration of this principle linked cellular respiration rates to the fluidity of the inner

mitochondrial membrane via the intramembrane diffusion of quinones, key intermediates of the electron transport chain [46].

The intrinsic capacity for cells to autonomously maintain their membrane fluidity has been recognized since the 1970s [47]. This behavior was first identified in bacteria using fluorescent probes that measure membrane viscosity via their rotational motion, reported by the time-dependent loss of fluorescence polarization. These measurements revealed a remarkable homeostasis mechanism, wherein membrane viscosity remained unchanged despite changes in cells' growth temperature via regulation of the lipidome (increased unsaturation as growth temperature is lowered). This adaptation, termed homeoviscous adaptation [47], is in striking contrast to synthetic membranes, which (unable to modify their composition) become more viscous at lower temperature as hydrocarbon chains order, packing lipids closer together. Similar regulation of membrane fluidity in response to temperature variation has been reported across the tree of life [48], from yeast to plants [49], fish [50] and worms [51]. Recently, an analogous lipidomic and biophysical homeostasis has been described across various mammalian cell types whose membrane properties were challenged by lipid inputs from the diet [9].

Homeoviscous adaptation requires a mechanism for *sensing* membrane fluidity, which must then be linked to the machinery that regulates membrane composition. Viscosity is inherently a dynamical (rather than structural) property; thus a direct sensor of viscosity would have to integrate the dynamics of a IMP over time. For example, a change in viscosity might change the *rate* of conformational dynamics of a MP, resulting in accumulation or depletion of a downstream signal. A mechanism like this was recently proposed for a yeast transmembrane protein called Mga2 [52], a critical regulator of unsaturated lipid production (a good start for controlling viscosity). However, the dynamic explanation for Mga2's function has come under recent scrutiny [53,54] — it appears instead that dimerization of the TMD domain of Mga2 responds to subtle changes in lipid packing in the hydrocarbon core.

These findings raise questions regarding what, exactly, cells are aiming to maintain and what they can sense to do so. While membrane viscosity (as measured by probes embedded in the hydrophobic bilayer core) is clearly maintained in various settings, there remains the possibility that viscosity is an epiphenomenon that changes together with other membrane properties. For example, Ire1, an important regulator of the unfolded protein response (UPR), appears to be activated by changes in membrane compressibility, i.e. how much energy it takes to stretch or compress a bilayer. The TMD of Ire1 contains an amphipathic helix that "squeezes" the ER membrane. When the ER membrane becomes too saturated, it becomes more difficult to compress, causing the Ire1 TMD to oligomerize and creating a signal to produce the stress response [55].

Force from membranes regulates proteins

In addition to a plethora of biochemical inputs, cells must transduce mechanical stimuli such as membrane stretching/compression, changing elastic moduli, and localized stresses. For many of these functions, the membrane provides the medium that transmits mechanical information to protein sensors (Fig 2d), such as the molecular mechanosensors of the MscL and MscS families [56]. Functioning as cellular osmoregulators, these channels open in

response to tension applied to their surrounding membrane, as in the case of hypo-osmotic shock. The structural mechanisms of such lipid-mediated opening have recently been described, with lipids playing roles in both conducting mechanical force to the protein and direct occlusion of the water permeation path [57]. A similar role has been ascribed to mechanosensitive mammalian channels known as Piezo1 and Piezo2 [58], that transduce touch and strain (e.g. due to arterial pressure). The mechanism of Piezo gating appears to involve their deformation of the surrounding membrane to form a dimple around the protein [59]. When the membrane is stretched, the dimple is straightened, and the channel opens. Other eukaryotic mechanosensors function via analogous mechanisms, suggesting that cellular mechanical stimuli may be generically sensed via membrane tension [60]. Under special circumstances, this mechanism may also run in the opposite direction, with conformational changes in transmembrane proteins *producing* tension in membranes. This is the case for the electromotive protein Prestin [61,62], which produces the remarkable cellular contraction required for auditory signal amplification in mammalian outer hair cells [63].

Lipid phases in protein organization

Lipids self-organize into membranes but can also spontaneously demix into coexisting domains via liquid-liquid phase separation. This self-organizing capacity is functionalized by cells for lateral membrane organization into functional microdomains known as lipid rafts [64]. After a cycle of excitement, confusion, and controversy, the raft concept has emerged as an important paradigm in membrane biology [65]. Building upon a mountain of biophysical insights in synthetic model membranes [66–69], a critical confirmation of the raft concept was the observation [70,71] and biophysical characterization of lipid liquid-liquid phase separation in isolated mammalian plasma membranes [72–75] and vacuoles of living yeast cells [76,77]. The nature, compositions, functions, and controversies surrounding membrane rafts have been extensively reviewed [64,78] and will not be further discussed here.

Membrane domains function largely by sorting proteins laterally via their preference for distinct lipid environments (Fig 2c). The structural bases for these preferences have been characterized by quantitative measurements of protein and lipid partitioning in isolated PM vesicles (often called Giant Plasma Membrane Vesicles [79]). For single-pass proteins, the TMD is the major determinant of raft microdomain affinity. Long [80], thin [81] TMDs, supported by post-translational palmitoylation [82], tend to prefer ordered membrane domains. The principles governing lipid anchored protein partitioning are similarly well understood, with proteins anchored by sterols and saturated acyl chains preferring ordered domains while short, branched, or unsaturated lipid anchors drive affinity for disordered domains [83]. The determinants for multi-pass transmembrane proteins are less clear, though recent observations suggest that most are excluded from the ordered phase of GPMVs [84]. Together, these features allow the assembly of membrane proteins and lipids into signaling complexes [85] (Fig 2e), enable transmembrane protein trafficking [31,80], and support assembly of complex multimolecular machines like viruses [86,87].

Lipid microdomains may also affect protein function beyond simply reorganizing their lateral organization. First, it is likely that the large differences in lipid composition between various microdomains may influence protein conformation, as detailed below (Fig 2g).

In some cases, it has been shown directly that cholesterol-induced lipid ordering can meaningfully modulate a protein's conformation landscape, as for rhodopsin [88], other GPCRs [89], and potassium channels [90]. Another possibility is that membrane domains may regulate protein-protein interaction dwell times by decreasing their diffusivity (Fig 2f).

The most notable protein-lipid interaction implicated in microdomain formation exists within a subtype of ordered membrane microdomain called caveolae, which are small invaginations of the PM dependent on the protein caveolin and its interaction with cholesterol [91]. Historically, caveolae were often conflated with rafts because they share many biophysical, compositional, and functional qualities [92]. Although ordered lipid domains are clearly possible without caveolar proteins, caveolae likely represent a functionally important, stabilized, immobilized assembly of ordered lipids [93]. These stabilized caveolar domains dramatically enrich cholesterol compared to the surrounding PM [94] and their formation absolutely requires high levels of cholesterol [95], possibly mediated by putative cholesterol-binding sequences present in the unusual membrane-embedded domain of caveolin-1 [96]. Interestingly, caveolae have been shown to disassemble in response to membrane stretch [97], resulting in release of caveolar-sequestered lipids, which significantly change the surrounding lipid content [93]. This coupling suggests a functional interplay between lipid-mediated lateral organization and mechanical stresses on the membrane.

INTERACTIONS OF MEMBRANE PROTEINS WITH LIPIDS

In addition to being potential binding partners and functional cofactors, lipids are also the solvating medium for membrane proteins. While many peripheral proteins are recruited to membranes by tight binding to specific lipid headgroups [11,12], defining interactions between IMPs and lipids is conceptually more difficult. Several models for such interactions have been proposed, from specificity for a single lipid species [10], to a stably-associated, selective lipid ring [98], to non-selective transmembrane domain solvation [99]. These apparently distinct interaction modes, however, differ only in degree, not quality. This continuum of possible interactions presents a challenge to the interpretation of experimental data, since different experimental (and simulation) techniques are typically restricted to a relatively narrow dynamic range, and therefore only reveal a correspondingly narrow window of protein-lipid interactions. Moreover, the apparent affinity of a particular lipid-protein interaction will depend on the specific mixture of lipids in which the protein finds itself. We return to this problem below in the section on "Interpreting Protein-Lipid Interaction", after discussing early evidence that IMPs recruit particular lipid environments, and then structural evidence for specific interactions.

Early evidence for annular lipids

The first direct evidence for lipid protein interactions was obtained by EPR spectroscopy (expertly reviewed in [98]). Experiments in the early 1970s using spin-labeled lipids observed a spectrally distinct fraction of lipids around bovine cytochrome oxidase, interpreted as a layer of bound lipids around the protein's perimeter [100] (Fig 5a). Later analysis of this 'bound' fraction using conventional binding isotherms suggested a single type of "binding site" [101]. Modeling of EPR lineshapes then permitted determination of lipid exchange rates

between the protein surface and the bulk, finding them to be about 10^{-7} sec^{-1} (or a lifetime at the protein surface of 100 nsec) [102].

These experiments indicated that lipids in the immediate environment (first solvation shell) of a protein are different from lipids in the bulk – dynamically restricted and slower to exchange. This set of observations was later synthesized into the concept of a "lipid annulus," with properties and composition distinct from the bulk membrane (Fig 5a). However, since the EPR signal is averaged over the entire protein surface, it affords no insight into localization of lipid-protein interactions *within* the annulus, which must be obtained by other techniques. For example, selectivity of anionic lipid interactions with a potassium channel was reported using brominated lipids to quench Trp residues on lipid-facing domains of KcsA [103].

Ligand-like, specific protein-lipid binding

What about even more localized, more specific lipid interactions? Structurally detailed information on lipid-protein binding is sometimes obtained in high resolution structures, at the cost of removing the protein from its native environment. Early advances in structural methods for membrane proteins led to several well-resolved transmembrane protein structures, with some of these revealing electron densities consistent with lipid molecules [104–106]. A lipid surviving the process of solubilization, purification, and crystallization suggested a significantly tighter binding than the few kT obtained for lipid-protein interactions from EPR measurements, raising doubts about the presence of a single population of "annular lipids," and leading to the concept of specific lipid binding sites. (A comprehensive list of structural data is beyond the scope of this review, and would anyway make for tedious reading.) The curious reader is directed to several excellent recent reviews [16,107].

While specific lipid structures are often poorly resolved, suggesting that the lipids do not adopt a specific conformation or that a variety of different lipids may be bound, occasionally specific lipids are clearly identifiable and are then found to act as essential regulators of protein activity. A famous example is direct binding and activation of the inward rectifying potassium channel Kir2.2 by the phosphorylated lipid PIP2 (phosphatidyl inositol 4,5 bisphosphate) [108] (Fig 5b). This mechanism may be used to dynamically regulate Kir channels, as PIP2 can be rapidly produced, consumed, or sequestered by various cytoplasmic proteins [109]. A non-exclusive alternative is that PIP2-binding signals arrival of the potassium channels on the PM (where PIP2 is enriched, Fig 3), preventing ion leakage during biosynthesis or trafficking. An intriguing wrinkle is that Kir channels also interact with another lipid characteristic of the PM, cholesterol [110]. However, while PIP2 activates the channel, cholesterol appears to suppress the open state [111]. This modulation of Kir activity has prompted the intriguing hypothesis that translocation to cholesterol-rich lipid rafts may be a mechanism for functional regulation of these channels [90,111]. Other, weaker lipid association sites on Kir2.2 have also been reported, with a complex interplay and competition between them [112].

The example of Kir channels is instructive, as their regulation by PIP2 and cholesterol appears to be characteristic of many cell-surface ion channels [113], transporters [114], and

receptors [107]. Quite why these particular lipids are so over-represented in membrane protein structures is somewhat mysterious. The case for PIP2 may be clearer: it has a large, highly charged, stereospecific headgroup that can be coordinated by positively charged binding pockets. PIP2 composition and localization are tightly regulated by enzymatic control, while its confinement to the inner leaflet of the PM may make PIP2 a useful coincidence detector for cell surface localization. Because of these features, it is easy to imagine how and why specific PIP2 binding has evolved. Similar arguments could be made for other relatively inabundant, highly localized anionic lipids, including other phosphatidylinositides, cardiolipin [115], and phosphatidic acid [116].

Cholesterol, on the other hand, has none of these features, being deeply membrane embedded, almost entirely hydrophobic, and present in many cellular membranes. Perhaps its most outstanding characteristic is its high concentration in the PM (30–40%), with the many biophysical sequalae associated with cholesterol-rich membranes (higher lipid packing, ordered domains, increased thickness – see Fig 3). However, cholesterol's structure and dynamics are entirely distinct from other membrane lipids, containing five rigidly coupled rings rather than long, floppy tails. This relative rigidity means that cholesterol pays a smaller entropic price for being bound to protein surfaces. These features perhaps contribute to the many examples of cholesterol binding to, and occasionally functionally regulating, membrane proteins, including multipass receptors [117–124], channels [125,126], transmembrane oligomers [124,127], and even single-pass transmembrane domains [128]. Interestingly, despite the ubiquity of reported cholesterol interaction sites, their structural determinants remain ambiguous. While "Cholesterol Recognition/interaction Amino acid Consensus (CRAC)" sites have been widely reported, the predictiveness and structural basis of this motif has been questioned [129].

In addition to the many reports of cholesterol and PIP2 binding are more rare cases of other lipids interacting with and regulating membrane proteins. A notable category are glycosylated sphingolipids, which are often ligands for soluble protein binding (e.g. for bacterial toxins [130,131] or viruses [132]), but also can interact in cis (i.e. on same membrane) with membrane proteins [133,134].

INTERPRETING PROTEIN-LIPID INTERACTION

Despite the tremendous technological advances across various structural and biophysical techniques, there remain conceptual shortcomings in our understanding of the mutual regulation between membrane proteins and their associated lipids. These ambiguities remain because of challenges in interpreting measurements of protein-lipid interactions, and because of limitations in molecular simulation techniques.

For example, densities *consistent with* lipids have often been identified in crystal and cryoEM structures of membrane proteins and such observations are often taken as *bona fide* evidence of protein-lipid binding. However, it should be noted that lipid structures are rarely well-resolved, leaving doubt to their specific identity. In some cases, the poor resolution may be due to lipids' inherent flexibility. Another possibility is that the lipid component may be intrinsically heterogenous, with a variety of potential lipids filling a given hydrophobic

cavity within a protein. Similarly, an amphiphilic detergent molecule may fill the niche that would have been occupied by lipids *in situ*. These possibilities imply that while *some* lipid is an essential component of a membrane protein's structure, there may be very weak selectivity for any particular lipid type. Such sites would be more akin to solvating water molecules for soluble proteins rather than specific ligands.

On the other hand, evidence for more selective interactions has come from some of the most elegant lipid-IMP binding experiments to date, which used native mass spectrometry to directly reveal protein-lipid coupling and the structural and functional consequences thereof [135] (Fig 4d). These experiments generally show that structurally diverse lipids bind with similar affinities [136,137], though with some important exceptions as in the bacterial transporter LeuT, where phosphatidylglycerol appears to be essential for dimerization [138].

Lipids bearing photoactivated crosslinking groups are a powerful technology for assaying protein-lipid interactions (Box 1, Fig 4b). However, interpretations of such experiments must account for the likelihood of labeling without specific interactions. This is because reactive lipids present in the membrane at any reasonable abundance will have access to most IMPs. Consider a moderately-sized membrane protein, such as a GPCR with a diameter ~3 nm, which would be surrounded at all times by ~40 bilayer lipids [139] (e.g. Fig 5c). Assuming no selectivity for any set of lipids, a cross-linker or other labeled lipid present at 1 mol% would be present in the first interacting shell of ~40% of the protein ensemble. In principle, it is possible that more specific interactions could be captured above such a high background of non-specific labeling, but it is an inescapable conclusion that highly abundant lipids (e.g. cholesterol in the PM) are encountering most membrane proteins most of the time. Specificity in such experiments is often demonstrated through identification of the putative lipid-binding site, whose mutation then reduces labeling. While such observations support specificity, it is essential to verify that mutations do not dramatically affect the lipid-facing interface of the protein (e.g. by disrupting TMD oligomers) or change a protein's subcellular localization. In the latter case, the different lipid compositions of various subcellular membranes (Fig 3) would be expected to affect the likelihood of labeling. Similar issues may affect FRET-based experiments (Fig 4c).

Given the difficulties in directly identifying lipid-MP interactions, why not look for titratable effects of a given lipid on protein structure or function? For soluble small molecules, such dose-dependence and saturability are usually accepted as *bona fide* evidence of specific biomolecular interactions. Unfortunately, such experiments can be quite difficult to perform rigorously for MPs, owing to the difficulty of achieving sufficient dilution over a wide range of concentrations. This barrier has been recently overcome by elegant experiments on the lipid dependence of the dimerization of a chloride transporter, showing that lipid dependence of IMP function is not well described by a simple binding isotherm, as would be predicted by the classic binding and linkage model [28]. Instead, at low concentration of a short chain lipid (which would serve as a putative "ligand"), the dependence of the dimerization equilibrium depends *logarithmically* on concentration, indicating a preferential solvation effect, first described in the context of soluble protein folding [140]. This effect is conceptually very different from either a ligand-like activity or regulation by bulk membrane properties.

Moreover, changing membrane composition during a titration experiment also changes collective membrane properties, which can influence protein structure and function independently from specific interactions, as discussed above (Fig 1). Cholesterol is especially problematic, since increasing membrane cholesterol will also increase membrane thickness and has a strong effect on the chemical potential of other lipids, especially sphingomyelin. As an example, consider an IMP with a binding site for cholesterol in two different membranes: (1) a polyunsaturated phospholipid with 30 mol% cholesterol versus (2) a saturated sphingomyelin with the same fraction of cholesterol. Because cholesterol interacts preferentially with sphingolipids and saturated lipids, its chemical activity is much lower in the second case and therefore the occupancy of the cholesterol binding site will be lower compared to the unsaturated/cholesterol mixture. While similar effects may complicate binding equilibria for soluble proteins, they are much more significant for lipid-protein interactions, where the solvent is crowded and is almost never dilute [141]. Such collective effects are often complex, dose-dependent, and saturable, complicating simple interpretations of titration experiments.

Driven partly by these challenges, simulations have become an important complement to experimental measurements of lipid binding (recently reviewed in [142]), revealing a broad spectrum of lipid-protein interactions [122](Fig 5c). Imagining lipid-protein interactions to be either tightly bound and ligand-like or transient and solvent-like has been the dominant paradigm since the early EPR measurements described above, guiding experimental design and interpretation of data. However, evidence from computer simulations suggests that such binary classification is misguided. The distribution of first solvation shell lifetimes for lipids around a GPCR is very broad, decaying as a power law in both atomistic [139] and coarse-grained [122] simulations (Fig 5c), covering the entire dynamic range of the simulation — longer than 30 μsec. Similarly broad lifetime distributions have been reported for rhodopsin [143]. These observations suggest that lipid-protein interaction strengths are very heterogeneous, and therefore describing lipid-protein affinities only on the average is likely to yield misleading conclusions.

THE FUNCTIONAL PARALIPIDOME MODEL

While details of specific lipid-protein interactions, and/or solvent-like effects, and/or effects of material properties (like membrane thickness) can sometimes explain functional regulation of IMPs, the functional consequences of lipids on proteins can be conceptualized without reliance on a specific mechanism, as recently described [144]. The basic concept is illustrated in Figure 6a–b, which shows an IMP that has two different conformations (A and B), each of which selects a different local lipid environment due to the distinct lipid interaction interfaces of each conformation.

The function of this protein depends on the fraction of the conformational ensemble in conformation A versus conformation B — for example, “B” might be the open state of an ion channel while “A” is the closed state. The relative population of the two states depends on the free energy difference between states A and B, *which includes contributions from the membrane*. The total free energy difference ΔG^{tot} between states A and B is a sum of different types of interaction

$$\Delta G^{tot} = \Delta G^{P-P} + \Delta G^{P-L} + \Delta G^{L-L},$$

where ΔG^{P-P} is the contribution from interactions within the protein which differ in A and B, ΔG^{P-L} are changes in lipid-protein interactions in A versus B, and ΔG^{L-L} are the contributions from the bulk lipid environment.

The effect of the local lipid environment, or "paralipidome," is determined by ΔG^{P-L}. The model is agnostic regarding the origins of ΔG^{P-L}, which can encompass specific lipid binding favored in one conformation over the other, or may represent the accumulation of many weaker, "solvent-like" interactions [143], or changes in protein shape and lipid environment that minimize thickness mismatches or other stresses [29,145]. Since the conformational equilibrium of an IMP (and therefore its function) may depend on all such interactions, the paralipidome model offers a way to rationalize their effects without recourse to (often undetermined) particulars (Fig 6b–c).

The model is relatively simple, requiring only that (a) IMPs recruit a local lipid environment (paralipidome) that is distinct in composition from the bulk, and (b) different conformational states recruit distinct paralipidomes (Fig 6a–c). There is extensive evidence for both aspects. Proteins' recruitment of selective paralipidomes has been experimentally documented [146,147], but is perhaps most clearly evident in simulations. For example, in a compositionally complex model of a mammalian plasma membrane, ten different membrane proteins each recruited a unique "fingerprint" of membrane nano-environments, defined by subtle-yet-clear enrichment of specific lipid subtypes. These enrichments were spatially defined, with hotspots for various headgroups, lipid saturations, and sterols [122,148] (Fig 6c). Experimentally, nanodiscs have become an important tool for interrogating and controlling the local lipid environment (Fig 4a). Originally constructed from modified apolipoproteins and widely used for structural biology [149], protein-scaffolded nanodiscs required detergent solubilization of target proteins, which limits their utility for exploring *native* paralipidomes. More recently, synthetic polymers have replaced proteins as nanodisc-stabilizing scaffolds, with the advantage that these do not require pre-solubilization of IMPs [150]. This approach has already been used to identify enrichment of certain lipid species in the paralipidomes of proteins [146]. Remarkably, some proteins appear to modulate the physical properties of their surrounding lipids, as was elegantly shown for leaflet-selective lipid ordering by gap junction channels composed of connexin proteins [151]. It is important to emphasize here that neither protein- nor polymer-scaffolded nanodiscs extract the "native" paralipidome of a IMP [152], nor necessarily recapitulate the native properties of a membrane [153]. How much these issues will limit their usefulness for studies of paralipidomes remains to be seen.

There is also substantial evidence from simulations for recruitment of different lipid environments depending on conformation. Coarse-grained simulations of the A_{2A} adenosine receptor revealed lipid-dependent partitioning of the receptor between regions of distinct composition [154]. All atom simulations of the same protein showed state-dependent recruitment of distinct lipid environments [144]; similar results were obtained for rhodopsin [143]. Although direct detection of lipid environments is challenging experimentally (as discussed above), coupling of receptor state and lipid composition has been observed many

times, from classic experiments on gramicidin A [29,155,156] to more recent measurements of cholesterol-dependent GPCR activation [157–159].

CONCLUSIONS AND PERSPECTIVES

Returning to the central question of this review: how do membrane proteins interact with lipids? Do lipids comprise a stable annulus, traveling with a protein like a greasy tutu (Fig 5a)? Or do lipids bind selectively like soluble ligands, allosterically modulating protein function (Fig 5b)? Or is there no specificity at all, just a random sea of lipid solvent for transmembrane domains? All three scenarios are likely relevant in various contexts. On the one hand, lipid dynamics and conformations are clearly affected by proximity to a protein — that is, lipids near a protein are different than those far from it. On the other hand, few of these lipids are bound in any thermodynamically meaningful sense, in that they are rapidly replaced by other, often different, lipids from the bulk. Some lipids (cholesterol, PIP2) have potential for relatively high-affinity interactions, while most others interact with similar affinity and are therefore largely interchangeable (from a protein's perspective). Functionally, strongly binding lipids can act as ligands or co-factors, driving conformation and oligomerization, while other proteins maybe be entirely agnostic about their solvating lipids. We submit that the combination of these factors drives the formation of a local paralipidome with a unique set of compositional and biophysical features (Figs 5c & 6c). Paralipidome features can, in turn, be modulated by external factors to affect structural and functional changes in IMPs. These changes could be physical (e.g. membrane tension) or biochemical, including enzymatic conversion of lipid species or metabolic changes to bulk lipidomes (Fig 6a–b).

This hypothesis makes an urgent call for methods to detail protein paralipidomes. While computational measurements are providing deep insights, these must be supported by experimental approaches. Native-MS [147] and nanodiscs will be critically important approaches moving forward, though an emphasis on avoiding detergent solubilization of IMPs is needed. In cellular systems, super-resolution spectroscopy [160] and single-particle tracking [161] can be used to quantitatively evaluate lipid-protein interactions in situ. High resolution structures will continue to be important, perhaps soon obtained *in situ*. A major advance in this realm could be identification of multiple structures within a single preparation and modulation of this conformational landscape by lipid components, as recently suggested for the temperature-sensing channel TRPV3 [162].

In what cellular contexts might changes in local lipid complements (i.e. paralipidomes) be relevant for regulating protein function? Perhaps the simplest argument can be made for sub-cellular localization. Each organellar membrane supports unique bilayer characteristics, often with major divergences between them (see Fig 3). For example, eukaryote PMs are very rich in sterols, with mammalian PMs containing up to 40 mol% [163]. This high concentration stands in contrast to most intracellular organelles [164]. It is plausible that lipid-determined conformational equilibria may restrict protein activity to only particular membranes within a cell. A protein's paralipidome might also be regulated by recruiting it to a distinct lipid subdomain via reversible post-translational modifications. For example, palmitoylation (i.e. addition of a long, saturated acyl chain) is an essential driver of a

protein's affinity for ordered microdomains, is rapidly reversible [165,166], and is present on hundreds of PM proteins [167]. Ordered phases in synthetic systems can be enriched by up to 10-fold in saturated lipids and 3–5-fold in cholesterol [168,169] with qualitatively similar enrichments reported in isolated PMs [75,170,171]. Finally, large-scale metabolic activity may, in special cases, drive sufficient lipidomic changes to affect protein structures, e.g. the enzymatic conversion of sphingomyelin to ceramide during apoptosis or incorporation of exogenous fatty acids into phospholipids [9].

Perhaps the most significant feature of biomembranes omitted from the discussions above is their compositional and biophysical asymmetry [172]. Most well studied has been the mammalian plasma membrane, where the cytoplasmic leaflet is rich in unsaturated, charged, loosely packed, amino-headgroup lipids opposite an external-facing leaflet that is uncharged, more saturated, and tightly packed [163]. Similar asymmetries are likely present in other organelles, e.g. endosomes [163] and the ER [173]. An under-appreciated and low-energy way of rapidly and dramatically changing proteins' lipid environments is by releasing this lipid asymmetry via scramblase channels [174], which likely induces major changes in the composition of both leaflets [172].

ACKNOWLEDGEMENTS

Major acknowledgement goes to Dr Kandice Levental for insightful feedback and for generating the beautiful and informative figures for the manuscript. We also acknowledge the members of the Levental and Lyman labs for critical reading and enlightening discussions that have polished the ideas in the manuscript. This work was supported by NIH/National Institute of General Medical Sciences (GM134949, GM124072, GM120351), the Volkswagen Foundation (grant 93091), and the Human Frontiers Science Program (RGP0059/2019).

Glossary

Unfolded protein response
a conserved cellular response to various stresses to the protein folding and secretory systems, can also be induced by lipid perturbations

Shotgun lipidomics
a mass spectrometric technique for identifying and quantifying the lipid components of a complex sample (e.g. cell membrane) without prior chromatographic separation

PIP2
Phosphatidyl inositol (4,5) bisphosphate, a highly charged and tightly regulated lipid type that can associate tightly with IMPs through both electrostatic and stereospecific interactions

Nanodisc
an experimental construct containing an IMP, lipids, and a scaffold that solubilizes them. The scaffold can be a protein (MSP) or synthetic polymer (SMA)

Native mass spectrometry
a mass spectrometric technique capable of measuring molecular weights of large macromolecules (i.e. proteins and their complexes) without fragmentation

Paralipidome

the preferred lipid nanoenvironments, and their resulting membrane properties, that solvate transmembrane proteins

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Box 1:

Structural methods to study interactions between lipids and integral membrane proteins

- Structural biology: X-ray crystallography and, more recently, cryogenic electron microscopy (cryo-EM) have been extensively applied to membrane proteins, with thousands of structures already solved [177] and more being rapidly produced. In almost all cases, native lipids are replaced by detergents during the purification procedure. However, supplementing proteins crystals or crystallization solutions with lipids can sometimes reveal lipid binding sites, and, more importantly, demonstrate structural and functional effects of bound lipids, as is the case for PIP2's gating of the inward rectifying potassium channel Kir2.2 [108].
- Electron crystallography: A specialized application of crystallography for analysis of lipid-protein interactions relies on electron diffraction to achieve atomic-level resolution of structure of both protein and lipids for specialized membrane proteins that can form two-dimensional crystals embedded within a lipid matrix. For example, the seven-pass channel bacteriorhodopsin was imaged within its native membrane environment [178] to reveal intimate integration of lipids within the transmembrane domain bundle and between tightly packed oligomers [179]. Notably, despite the unprecedented resolution, the specific structures of these lipids could not be identified, likely because they are not specific. That is, they are both conformationally flexible (i.e. disordered) and compositionally flexible (different lipid species can be accommodated at the same location). The apotheosis of this technique was a <2 A resolution structure of the water channel aquaporin 0 (AQP0) [99], complete with surrounding lipids and water molecules in the pore. Here again, no stereospecific lipid interactions were observed.
- Nanodiscs: an important addition to structural biology of protein-lipid interactions has been the development of nanodiscs [149] (Fig 4a). The intrinsic bilayer-stabilizing properties of apolipoproteins enabled the design of a family of membrane scaffolding proteins (MSP). When mixed with lipids and membrane proteins of interest, these MSPs facilitate formation of ~10 nm discs in which the protein target is solvated by a semi-native lipid bilayer. Combined with cryoEM, nanodiscs have propelled an explosion of structural insights into membrane protein structure [149]. More recently, synthetic polymer scaffolds have proven capable of producing similar protein/lipid nanodiscs [150]. Importantly, these can extract proteins directly from cellular membranes, obviating the need for detergents and suggesting the potential for isolating the native paralipidomes of membrane proteins [146].
- Native mass spectrometry (MS): the development of "soft ionization" methods like Electron Spray Ionization (ESI) has facilitated mass spectrometry of intact complex biomolecules. ESI-MS was an enabling

technology for shotgun lipidomics, allowing identification and quantification of hundreds of lipid species without the need for chromatographic separation or fragmentation [180]. More recently, similar methodology was applied to membrane proteins [181], revealing that intact folded proteins could be stripped of their detergent and analyzed for oligomerization, lipid binding, and most importantly, the structural and functional consequences of lipid-protein interactions [135] (Fig 4d).

Box 2:

Methods to study lipids - integral membrane protein interaction dynamics

- Electron paramagnetic resonance (EPR) spectroscopy: classical studies that established the concept of distinct lipid properties in the annulus surrounding a transmembrane protein relied on lipids labeled with a stable free-radical (e.g. nitroxide). The dynamics of such "spin probes" report on the fluidity and polarity (i.e. hydration) environment of the membrane nano-environment [52,182]. A subset of spin-labeled lipids is slowed by the presence of transmembrane proteins [98], providing evidence for protein-lipid interactions.
- Molecular dynamics simulations: structural techniques can provide high-resolution information about protein conformation and lipid binding sites, but the picture is usually static, obscuring the eternal, jiggling dance of biomolecules at biological temperatures. Insights into the dynamic behavior of these systems can be provided by computational simulations (reviewed in [16]). With increasing computational power, large and complex systems like membrane-embedded proteins can now be simulated over relevant time-scales to measure protein-lipid on/off-rates [139], binding energetics [142], and preferential solvation by specific lipid subtypes [148].
- Fluorescence microscopy and spectroscopy: complementary to structural and computational methods, protein-membrane interactions can be investigated *in situ* using fluorescent lipids. Interactions can be identified via microscopic co-localization or fluorescent lipid detection after protein-pulldown [183]. Higher resolution and quantitation is achieved via spectroscopic methods, e.g. fluorescence resonance energy transfer (FRET) between a fluorescent lipid and protein [10] (Fig 4c). Similarly, single-molecule techniques can reveal changes in conformation or assembly induced by membrane environments [184]. An important consideration is that fluorescent tags are often large on the scale of a lipid and can therefore change essential aspects of lipid structure and interactions. To address this limitation, structurally and functionally similar analogs of both phospholipids [183] and sterols [185] have been developed and characterized.
- Photo-activatable lipids: lipids can be engineered to contain small moieties that allow photo-conversion between a stable chemical bond (e.g. diazirine) and a highly reactive one (e.g. carbene free radical). Upon UV-activation these lipids rapidly react with their neighboring molecules, including other lipids and transmembrane proteins (Fig 4b). Inclusion of fluorescence or radioactivity into such photo-activated lipids allows detection of protein-lipid crosslinks by standard biochemical methods (e.g. electrophoresis or chromatography) [15,186]. Several of the above moieties can be combined to make multi-functional lipid analogs, containing photo-labile caging, cross-linking, and fluorescent groups for powerful, versatile, and temporally resolved analysis of protein-lipid dynamics [187,188].

- Electrophysiology: one of the few methods capable of directly testing membrane protein function in different lipid environments takes advantage of the fact that many such proteins conduct ions across a membrane, and therefore their activity can be assayed by electrical signals. Practically, this is often accomplished via a patch clamp, wherein a patch of membrane forms the seal between two electrically isolated compartments and flow of ions through individual channels or transporters is detected as current. These membrane patches are usually derived from cells, whose lipid composition can be manipulated [111,189]. Alternatively, similar techniques can be applies to patches of reconstituted liposomes [190], or even whole giant liposomes [191], whose lipid composition can be precisely controlled.

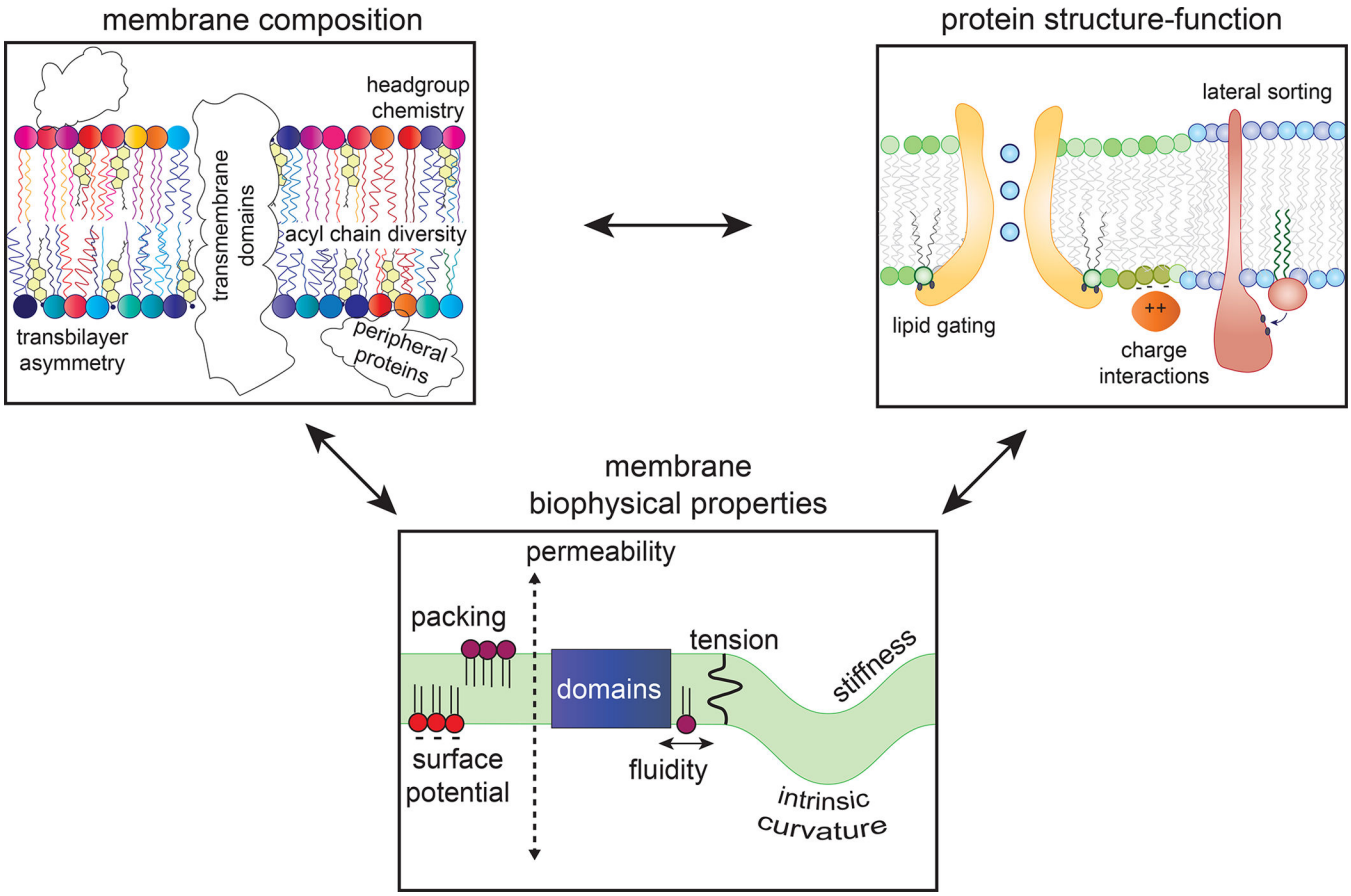

Figure 1. Inter-relationships between membrane lipidomes, protein structure/function, and collective membrane physical properties.

Individual lipid molecules can serve as specific cofactors for MPs, but lipids also collectively comprise the complex, dynamic solvent that determines the functional behavior of MPs. In turn, IMPs produce and transduce signals that can regulate membrane lipidomes, which themselves determine the biophysical properties of a given membrane.

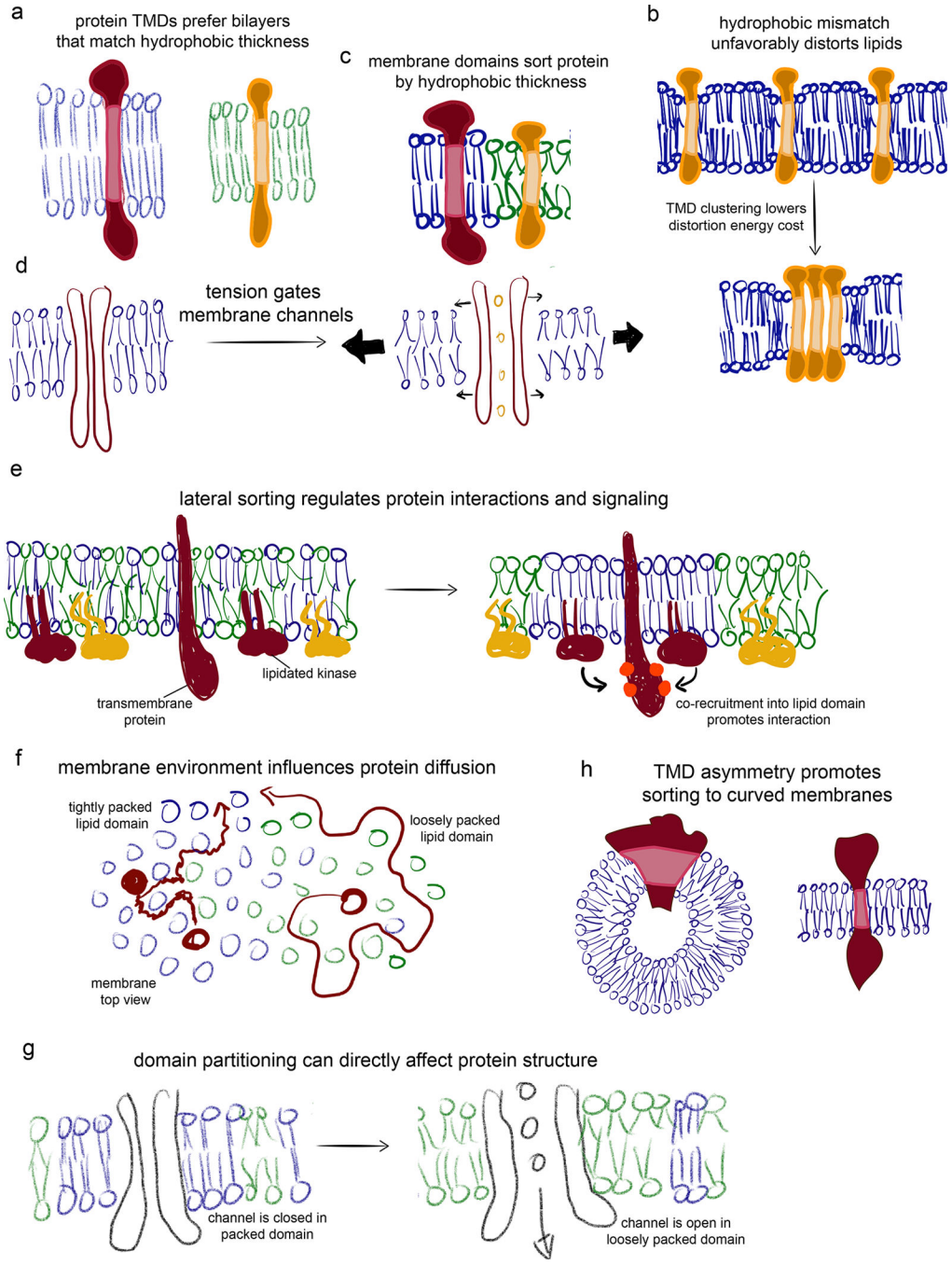

Figure 2. How collective membrane properties can affect protein organization and function.

(a) Membranes comprised of lipids with long acyl chains and/or cholesterol have thicker hydrophobic cores and thus prefer IMPs with longer hydrophobic transmembrane domains (*vice versa* for thinner membranes). (b) In membranes containing domains of different thicknesses, proteins can be sorted by their TMD length. (c) Hydrophobic mismatches create disturbances in optimal lipid configurations. These can be minimized by clustering of misfit TMDs. (d) Mechanical tension applied to a membrane decreases lipid packing, thins the membrane, and disorders lipid acyl chains. These effects can be transduced by

transmembrane channels to sense touch and pressure. (e) IMPs sorting via preferences for lipid domains can facilitate interactions with other domain residents or restrict collisions with domain-excluded components. (f) Domains can affect IMP dynamics, with more ordered and tightly packed domains slowing protein diffusion. (g) The distinct compositions and physical properties of various membrane environments can directly regulate protein structure and activity. (h) TMD shape can promote sorting to membrane subdomains of different curvature.

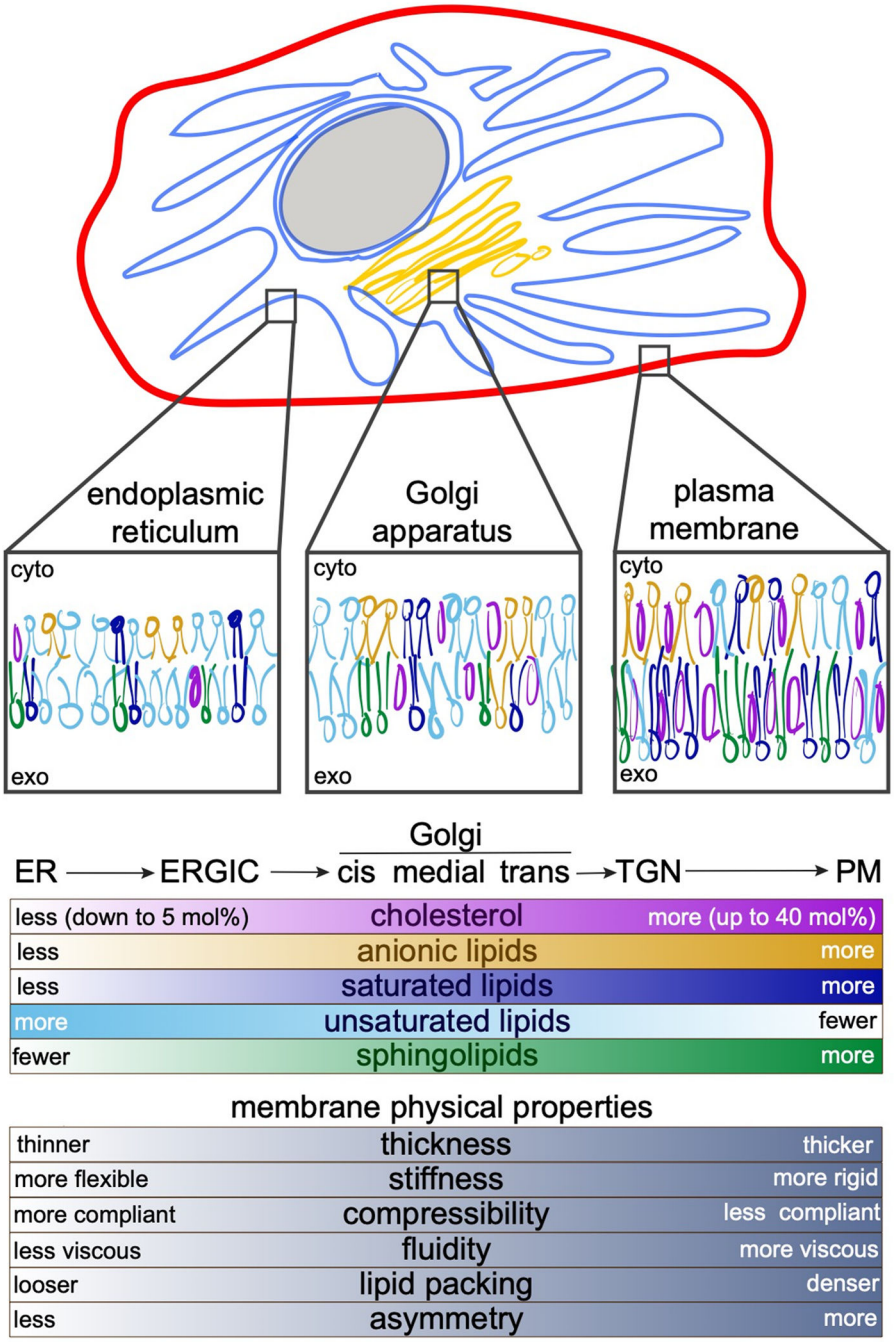

Figure 3. Physical and compositional variations in subcellular membranes.

Membranes of various subcellular organelles can differ dramatically in their compositions and resulting physical properties. The secretory pathway is experienced by most MPs, because they are synthesized in the ER, modified and sorted in the Golgi, and function at the PM. In their journey through these membranes, IMPs experience increasing cholesterol, sphingolipids, and saturated lipid concentrations. The membranes likely become more asymmetric with respect to the lipid compositions of the two bilayer leaflets, with the outer leaflet become more tightly packed and ordered, while the inner leaflet adopts

greater negative charge due to increasing concentrations of anionic lipids. As they progress through the secretory pathway, membrane also become less fluid, more rigid, and thicker. In polarized cells, specialized regions of the PM like the apical PM of epithelial cells [22,175,176] may be especially rich in tightly packing lipids and therefore thicker, less permeable, and more viscous. These features can be used to sort proteins between organelles and regulate their function within them. For example, TMDs of IMPs in the PM are longer and more asymmetric (i.e. bulkier near the exoplasmic leaflet, thinner near the cytoplasmic) compared to those of the ER and Golgi [30,163].

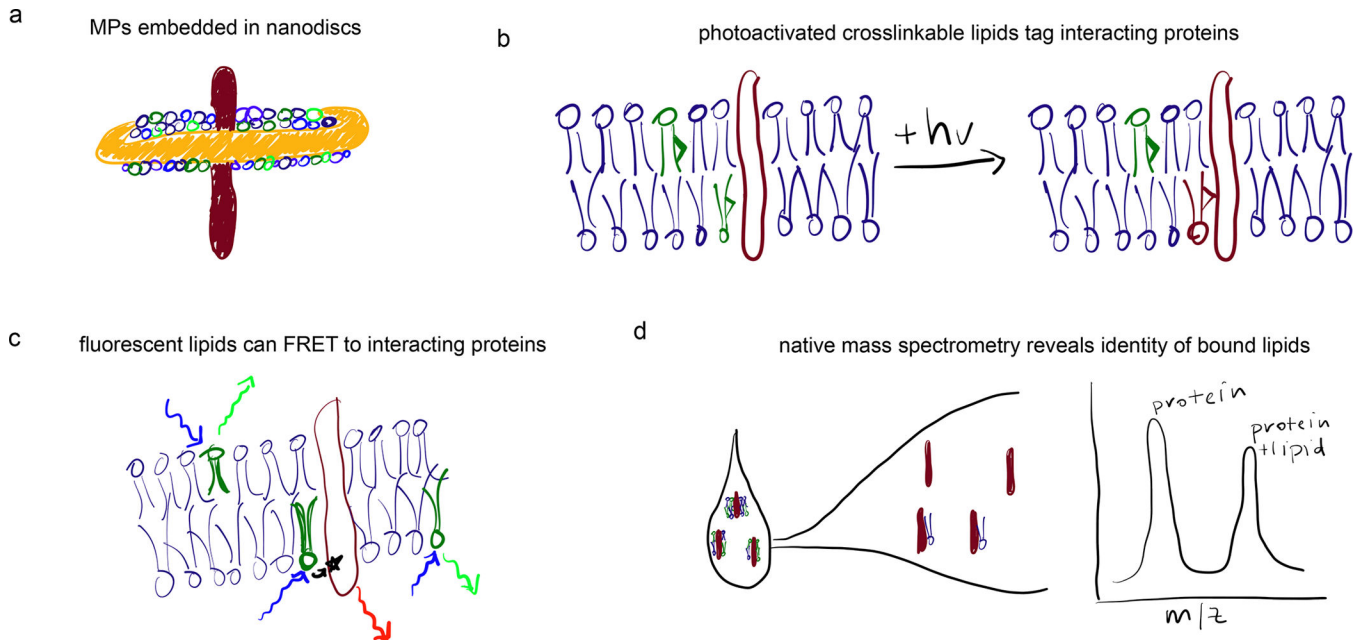

Figure 4. Methods to study MP-lipid interactions.

(a) Nanodiscs supported via amphiphilic protein or polymer scaffolds provide a native-like membrane environment for MPs. (b) Photoactivatable crosslinks can be introduced into lipid acyl chains, facilitating identification of protein-lipid interactions *in situ*. (c) FRET between fluorescently labeled lipids and proteins can report on interaction potentials. (d) Mass spectrometry of undigested proteins extracted from cells can reveal exact molecular identities and stoichiometries of bound lipids.

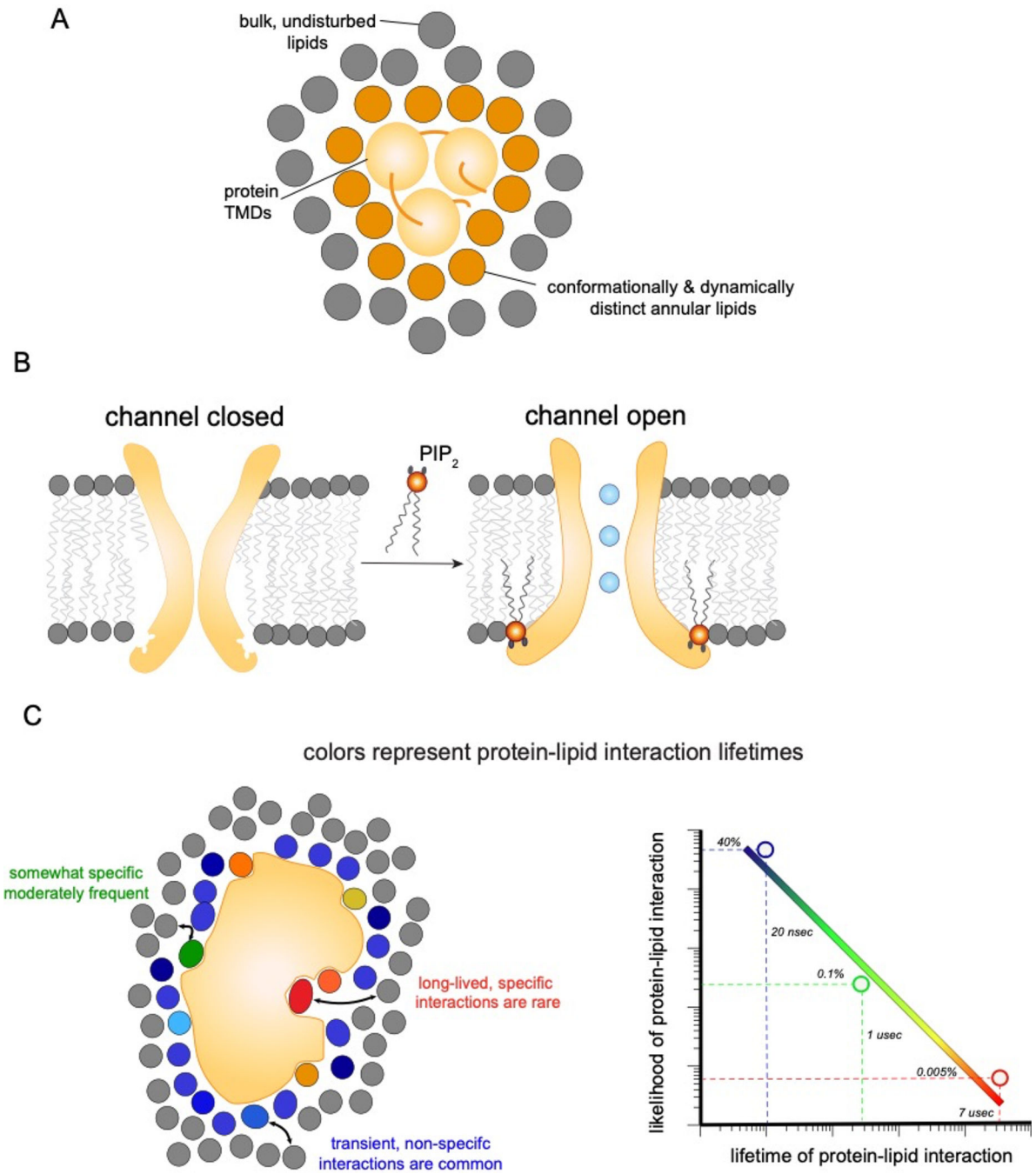

Figure 5. Possible modes of lipid interactions with transmembrane proteins.

(a) A shell of annular lipids stably associates with transmembrane protein regions. The exchange rate of these annular lipids with the bulk is slow, such that these lipids can be conceptualized as an extended part of the protein. (b) Charged and large lipids like PIP2 (orange), can be tightly and specifically bound by protein domains to introduce large scale conformational changes. In these cases, lipids act as allosteric ligands for protein function. (c) Interactions between IMPs and individual lipid molecules (colored) are in constant competition with those from bulk lipids (gray). The affinity for a particular lipid species

determines how likely it is to occupy a particular site, relative to its bulk concentration. The lifetime of those interactions is directly related to affinity. Simulations suggest that protein-lipid interactions span a range of lifetimes from relatively high affinity interactions (which are rare) to very common short-lived interactions (nanosecond range), which indicate rapid lipid exchange not influenced by protein binding. These observations imply that there is no single characteristic scale for protein-lipid interactions, but rather that specific, semi-selective, and bulk solvent-like effects could potentially be simultaneously relevant.

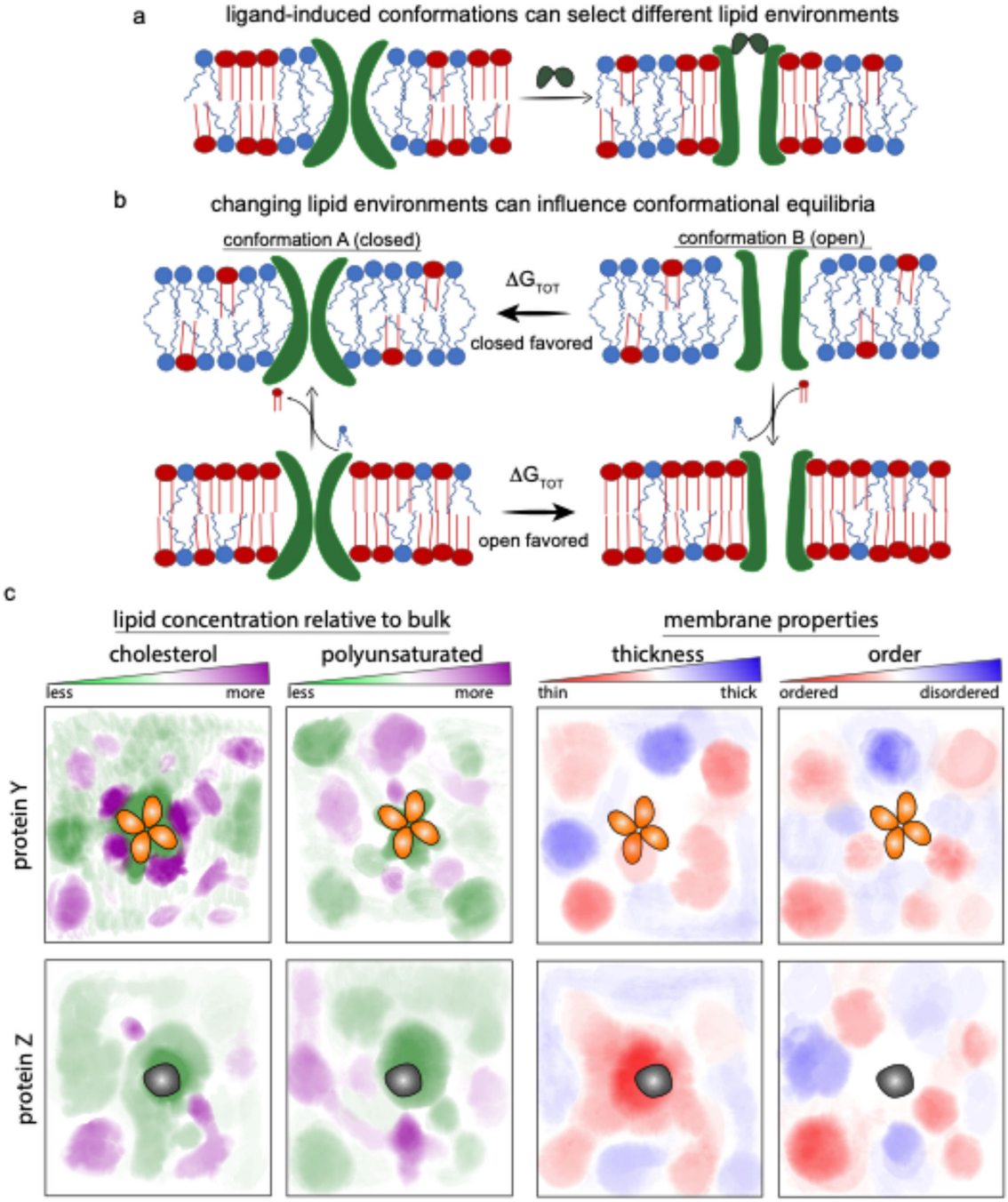

Figure 6. The functional paralipidome.

(a) IMPs can prefer distinct lipid environments in different conformations. (b) Conversely, the conformational equilibrium of a protein is determined in part by the lipid environment. If the closed state of an ion channel (A) recruits unsaturated lipids (blue) into its paralipidome (top left), the open state will be favored in membranes rich unsaturated lipids (i.e. those in which the chemical activity of unsaturated lipids is higher; top panels). (bottom panels) Membranes rich in saturated lipids, which preferentially solvate or bind the open conformation (B), will tend to favor the open state. Importantly, these effects depend

not on absolute compositions, but rather on chemical activities. For example, inclusion of other lipids (e.g. cholesterol) into the bulk may influence the chemical activity of saturated lipids and thereby change the conformational equilibria. (c) various IMPs have unique preferences of their local membrane nano-environment (i.e. membrane fingerprints), selecting both paralipidomes and consequent biophysical parameters based on subtleties of their conformation and dynamics.