



Regulation of membrane protein structure and function by their lipid nano-environment

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Abstract | Transmembrane proteins comprise ~30% of the mammalian proteome, mediating metabolism, signalling, transport and many other functions required for cellular life. The micro-environment of integral membrane proteins (IMPs) is intrinsically different from that of cytoplasmic proteins, 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 lipidomes of mammalian membranes, 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 we synthesize into a general framework termed the ‘functional paralipidome’ to understand the mutual regulation between membrane proteins and their surrounding lipid microenvironments.

Functional interactions between proteins and other biomolecules are fundamental to molecular cell biology. Constituting ~30% of the mammalian proteome¹ and 60% of all drug targets², integral membrane proteins (IMPs) 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 IMPs 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 hydrophobic³. Despite these 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 (that is, 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^{4,5}. Mammalian cells produce hundreds of distinct lipid species, and the specific lipid complements can vary dramatically depending on cell type^{6,7}, metabolic state⁸, disease state⁹ and external inputs (for example, from the diet)^{7,10}. 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 chain¹¹. 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 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

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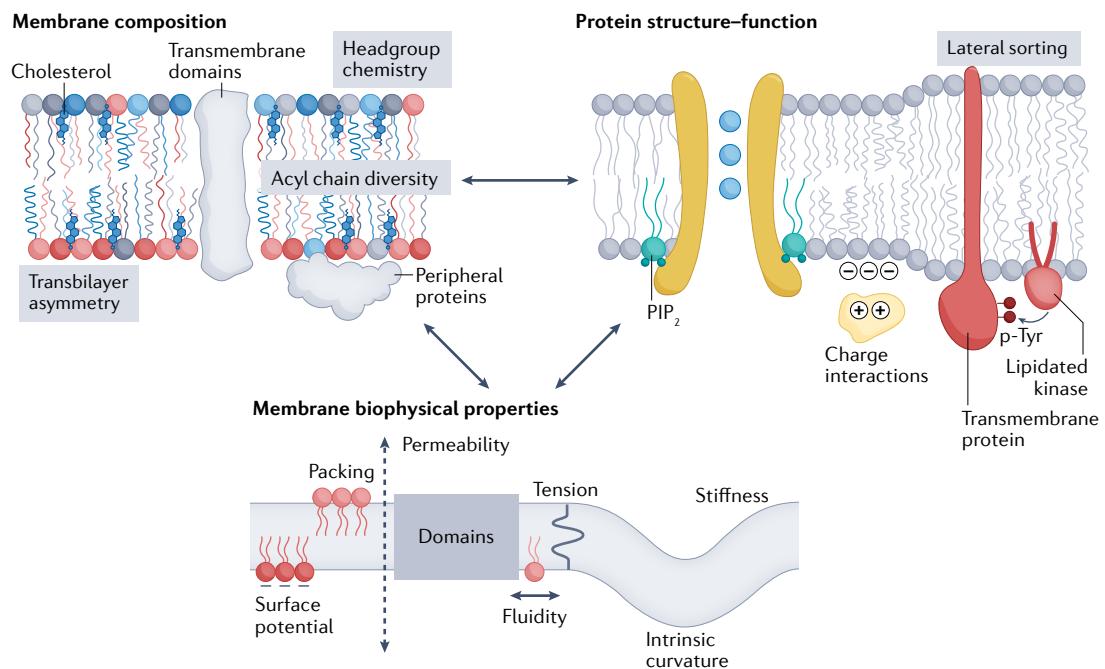


Fig. 1 | Inter-relationships between membrane lipidomes, protein structure–function and collective membrane physical properties. Individual lipid molecules can serve as specific cofactors for integral membrane proteins (IMPs) but lipids also collectively comprise the complex, dynamic solvent that determines the functional behaviour of IMPs. In turn, IMPs produce and transduce signals that can regulate membrane lipidomes, which in turn determine the biophysical properties of a given membrane.

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^{12,13}. Another broad area that will not be covered in this Review is 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^{14–19}) and protein–lipid interfaces as drug targets³, our intent is not to document an exhaustive list of such examples.

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 packing. At still larger length scales, membranes behave as thin-yet-robust elastic sheets that can be stretched, bent and shaped into the variety of morphologies required for cellular functions²⁰. Across these length scales, the membrane acts on membrane-embedded proteins to regulate their interactions, conformations, localizations and functions²¹ (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 lipid hydrophobic chains. These chains can vary from 12 up to 24 carbons in mammalian cells, suggesting a range of possible membrane thicknesses from ~3 to 4.5 nm (REF.²²). 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 membranes²³ (FIG. 3). More recently, direct images of membrane thickness variations in biomimetic and bioderived membranes^{22,24} have been obtained by cryogenic electron microscopy, with similar images of cryopreserved cells suggesting the feasibility of biomembrane thickness measurements *in situ*²⁵.

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 membranes²⁶ (FIG. 2a). Such ‘hydrophobic mismatch’ is energetically unfavourable because lipids must deform (for example, compress) to avoid exposure of hydrophobic regions to water (FIG. 2c). At the extremes, sufficient compression of the membranes allow lipids to scramble from one leaflet to the other²⁷. In a membrane of uniform thickness, misfit IMPs may cluster to minimize membrane distortion²⁸ (FIG. 2d) while coexisting domains of different thicknesses laterally sort proteins to their least-mismatched domain (FIG. 2b). It should be emphasized that these descriptions treat the membrane as a homogenous continuum, with energetic costs imposed by bending or stretching an elastic material. However, at the molecular level relevant for protein interactions, membranes are characterized

by complex biological lipidomes comprised of many different species capable of conforming to various protein shapes and sizes. Thus, in biological contexts, constraints imposed by membrane elastic properties must be considered alongside more local effects (for example, sorting of short lipids near short proteins)^{29,30} to describe the functional impact of the membrane environment on IMPs.

The energetics of hydrophobic mismatch are likely responsible for the conserved variation in TMD length between subcellular organelles, with TMDs of the endoplasmic reticulum (ER) and Golgi being shorter than those of proteins that reside in the plasma membrane³¹ (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 the hydrophobic thickness of organellar membranes suggests that proteins and lipids co-segregate based on their biophysical properties and that such segregation may facilitate subcellular trafficking³². 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 through³³. A recent report suggested that analogous domains in the mammalian ER may be organized by interactions between a multi-pass protein sigma 1 receptor (S1R) and cholesterol³⁴ and that such domains may mediate inter-organelle contact sites^{34,35}.

Just as hydrophobic mismatch 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 function²⁶ (FIG. 2d). This effect has been most clearly demonstrated for pumps and transporters, including the Na⁺/K⁺-ATPase³⁶, a bacterial aquaporin, the sarcoplasmic ER Ca²⁺-ATPase (SERCA)³⁷ and certain GPCRs³⁸. All of these proteins show clear variation in activity as a function of membrane thickness, with maximal activity in optimally matching membranes (for an in-depth discussion of hydrophobic mismatch, see REFS.^{14,39}). 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 system²⁹. Doping membranes with short-chain lipids that solvate this special surface reduces dimerization, demonstrating the critical importance of lipid context in the assembly of IMPs into functional complexes.

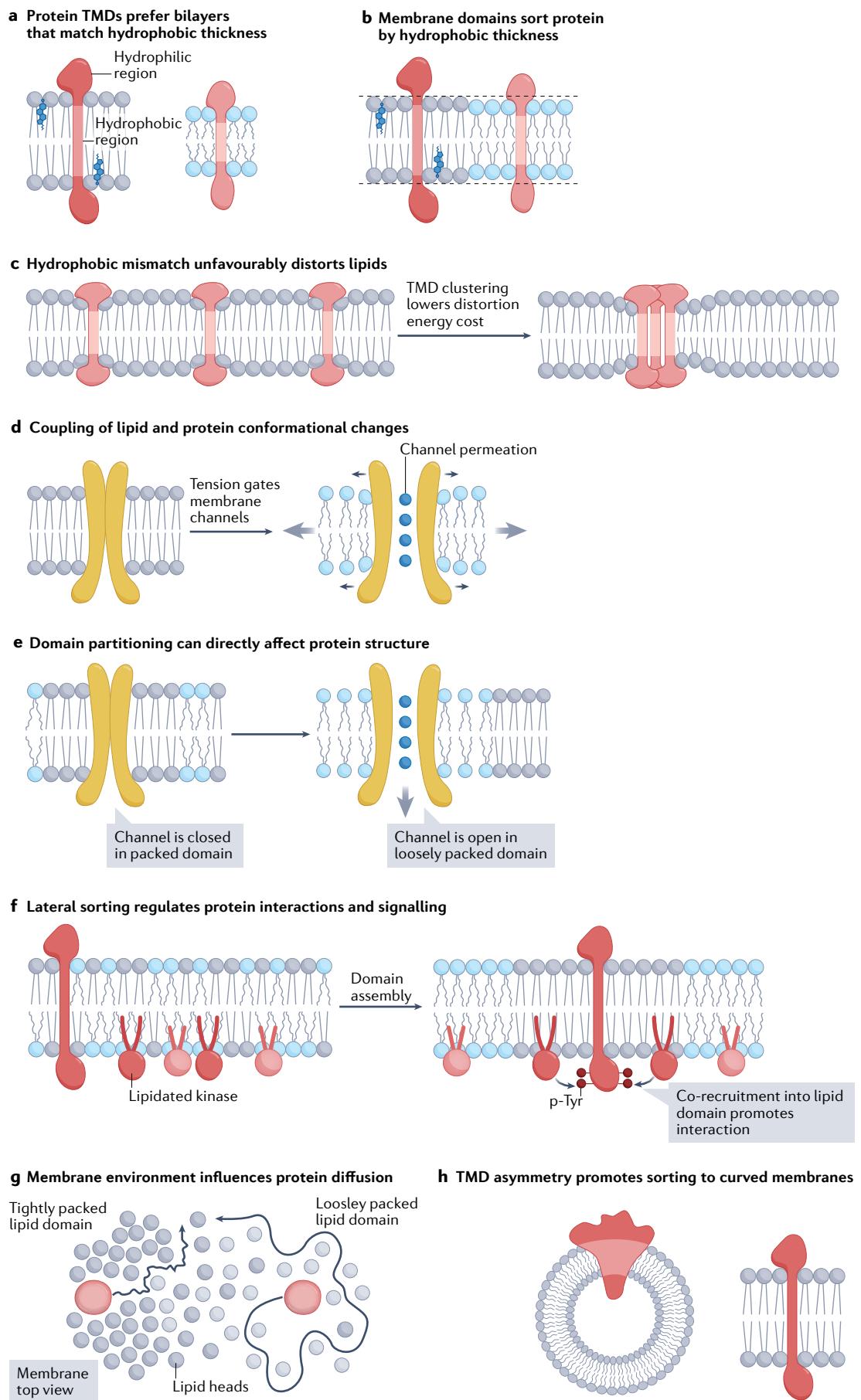
The notion that bilayer thickness regulates protein function suggests that this thickness must be sensed and regulated by cells. A prototype membrane thickness sensor is the bacterial ‘molecular calliper’ called DesK⁴⁰, which changes conformation as a function of membrane thickness to activate a *Bacillus subtilis* two-component signalling 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 membranes⁴¹. Such curvature selectivity is often accomplished by amphipathic helices that insert into one leaflet of the bilayer⁴². However, some transmembrane proteins also appear to sense curvature⁴³, preferentially enriching in highly curved membrane tubes (FIG. 2h). Other IMPs generate membrane curvature by repulsive interactions of their extramembrane domains^{44,45}. Finally, post-translational modifications near the transmembrane domain can affect the curvature preference of proteins, which may be used to sort proteins in the secretory pathway⁴⁶.

Sensing membrane fluidity and lipid packing by IMPs. 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 determines the lateral diffusion of IMPs and lipids and, therefore, the frequency of the protein–protein encounters that underlie signalling 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 chain⁴⁷.

The intrinsic capacity for cells to autonomously maintain their membrane fluidity has been recognized since the 1970s⁴⁸. This behaviour 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 remains unchanged despite changes in cell growth temperature via regulation of the lipidome (increased lipid unsaturation as growth temperature is lowered). This adaptation, termed homeoviscous adaptation⁴⁸, is in striking contrast to synthetic membranes, which (unable to modify their composition) become more viscous at lower temperature as hydrocarbon chains order, thus packing lipids closer together. Similar regulation of membrane fluidity in response to temperature variation has been reported across the tree of life⁴⁹, with examples in yeast, plants⁵⁰, fish⁵¹ and worms⁵². 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 diet¹⁰.

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 protein sensor of viscosity would have to integrate the dynamics of a membrane over time. For example, a change in viscosity might change the rate of conformational transitions of an IMP in a membrane, resulting in accumulation or depletion of a downstream signal. A mechanism like this was recently proposed for a yeast transmembrane protein



◀ Fig. 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 integral membrane proteins (IMPs) with longer hydrophobic transmembrane domains (vice versa for thinner membranes). **b** | In membranes containing domains of different thicknesses, proteins can be sorted by the length of their transmembrane domains (TMDs). **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** | The distinct compositions and physical properties of various membrane environments can directly regulate protein structure and activity. **f** | Sorting of IMPs via preferences for lipid domains can facilitate interactions with other domain residents or restrict collisions with domain-excluded components. **g** | Domains can affect IMP dynamics, with more ordered and tightly packed domains slowing protein diffusion. **h** | TMD shape can promote sorting to membrane subdomains of different curvature.

called Mga2 (REF.⁵³), a critical regulator of unsaturated lipid production (a good start for controlling viscosity). In this model, Mga2 dimerizes via its transmembrane domain but only certain dimer conformations lead to productive activation. The balance of productive versus non-productive dimers is controlled by the viscosity of the membrane. However, this dynamic explanation for the function of Mga2 has come under recent scrutiny^{54,55} — it appears that, rather than relying on dynamics controlled by viscosity, the conformational landscape of Mga2 dimerization is regulated by subtle changes in molecular lipid-packing density at a specific depth of the hydrocarbon membrane core.

These findings raise questions regarding exactly which membrane properties 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, appears to be activated by changes in membrane compressibility, that is, 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 initiate the stress response⁵⁶.

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 bacterial molecular mechanosensors of the MscL and MscS families⁵⁷. Functioning as cellular osmoregulators, these channels open in response to tension applied to their surrounding membrane, as occurs during 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 path⁵⁸. A similar role has been ascribed to mechanosensitive mammalian

channels, known as Piezo1 and Piezo2 (REF.⁵⁹), that transduce touch and strain (for example, owing to arterial pressure). The mechanism of Piezo channel gating appears to involve their deformation of the surrounding membrane to form a dimple around the protein⁶⁰. When the membrane is stretched, the dimple is flattened and the channel opens. Other eukaryotic mechanosensors function via analogous mechanisms, suggesting that cellular mechanical stimuli may be generically sensed via membrane tension⁶¹. 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^{62,63}, which produces the remarkable cellular contraction required for auditory signal amplification in mammalian outer hair cells⁶⁴.

Lipid phases in protein organization. Lipids self-organize into membranes but can also spontaneously de-mix via liquid–liquid phase separation into coexisting domains with different lipid properties (that is, order and packing). This self-organizing capacity is functionalized by cells for lateral membrane organization into functional microdomains, often referred to as lipid rafts⁶⁵. After a cycle of excitement, confusion and controversy, the raft concept has emerged as an important paradigm in membrane biology⁶⁶. Building upon a mountain of biophysical insights in synthetic model membranes^{67–70}, a critical confirmation of the raft concept was the observation^{71,72} and biophysical characterization of lipid liquid–liquid phase separation in isolated mammalian plasma membranes^{73–76} and vacuoles of living yeast cells^{77,78}. While such microscopically observable rafts are only seen under special conditions, evidence from spectroscopic imaging^{79–81}, single-molecule tracking^{82,83}, and super-resolution microscopy⁸⁴ and spectroscopy⁸⁵ has revealed that dynamic, nanoscopic, lipid-driven domains are an important feature of the organization of mammalian membranes. The nature, compositions, functions and controversies surrounding membrane rafts have been extensively reviewed^{65,86} and will not be further discussed here.

The major function of membrane domains to sort proteins laterally via their preference for distinct lipid environments (FIG. 2b). The structural bases for these preferences have been characterized by quantitative measurements of protein and lipid partitioning in isolated vesicles derived from the plasma membrane (often called giant plasma membrane vesicles⁸⁷). For single-pass proteins, the TMD is the major determinant of affinity for raft microdomains. Long⁸⁸ and thin⁸⁹ TMDs, supported by post-translational palmitoylation⁹⁰, 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 domains⁹¹. The determinants for multi-pass transmembrane proteins are less clear, though recent observations suggest that most are excluded from the ordered phase of giant plasma membrane vesicles⁹². Together, these features support

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

MscL and MscS families
Bacterial mechanosensitive channels that open in response to membrane tension, for example, induced by osmotic shock.

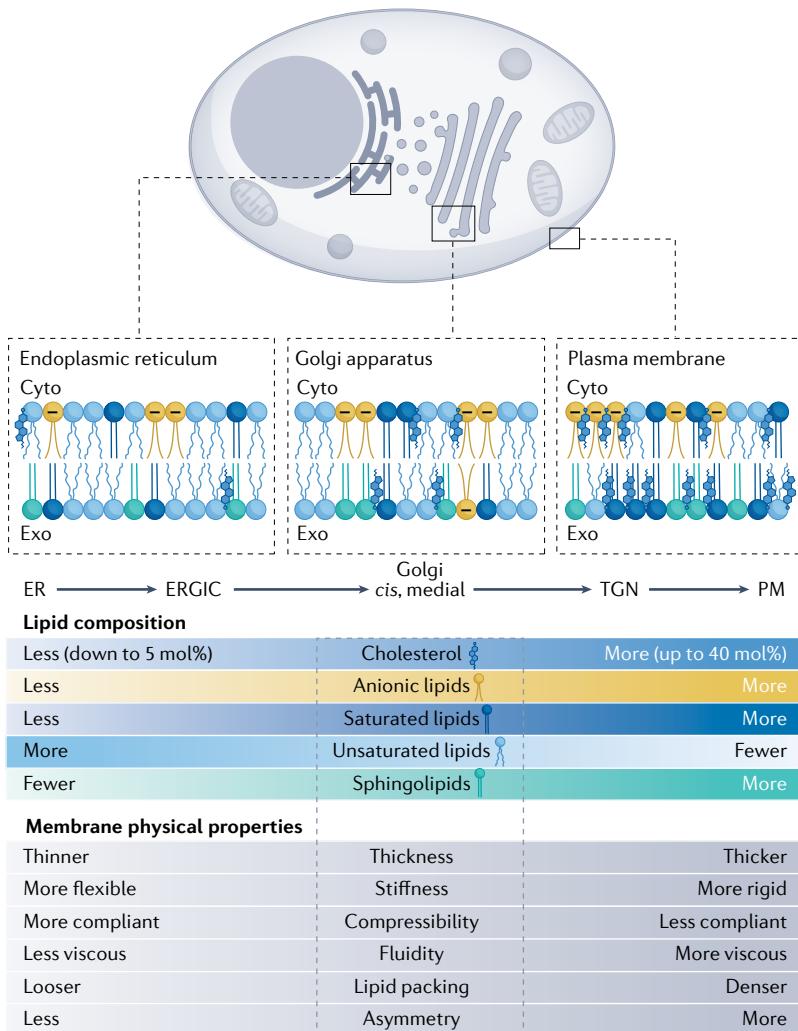


Fig. 3 | Physical and compositional variations in subcellular membranes. Membranes of various subcellular organelles can differ dramatically in their compositions and resulting physical properties. Most integral membrane proteins (IMPs) traffic through the secretory pathway as they are synthesized in the endoplasmic reticulum (ER), modified and sorted in the Golgi, and transported to the plasma membrane (PM). In their journey through these membranes, IMPs experience increasing cholesterol, sphingolipid 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 becoming more tightly packed and ordered, while the inner leaflet adopts greater negative charge owing to increasing concentrations of anionic lipids. As they progress through the secretory pathway, membranes also become less fluid, more rigid and thicker. In polarized cells, specialized regions of the plasma membrane, such as the apical membrane of epithelial cells^{23,181,182}, 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, transmembrane domains of IMPs resident in the plasma membrane are longer and more asymmetric (that is, thinner near the exoplasmic (Exo) leaflet, bulkier near the cytoplasmic (Cyto) leaflet) compared to those of IMPs functioning in the ER and Golgi^{31,170}. ERGIC, ER–Golgi intermediate compartment; TGN, trans-Golgi network.

raft partitioning, which allows the assembly of membrane proteins and lipids into signalling complexes⁸⁴ (FIG. 2f), enables transmembrane protein trafficking^{32,88} and facilitates assembly of complex multimolecular machines like viruses^{93,94}.

Lipid microdomains may also affect protein function beyond simply influencing 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. 2e). In some cases, including rhodopsin⁹⁵, other GPCRs⁹⁶ and potassium channels⁹⁷, it has been directly shown that cholesterol-induced lipid ordering can meaningfully modulate a protein's conformation landscape, thereby regulating its propensity for activation and downstream signalling. Another possibility is that membrane domains may regulate protein–protein interaction dwell times by decreasing their diffusivity (FIG. 2g).

The most notable protein–lipid interaction implicated in microdomain formation exists within a subtype of ordered membrane microdomains called caveolae, which are small invaginations of the plasma membrane dependent on the protein caveolin and its interaction with cholesterol⁹⁸. Historically, caveolae were often conflated with rafts because they share many biophysical, compositional and functional qualities⁹⁹. Although ordered lipid domains are clearly possible without caveolar proteins, caveolae likely represent a functionally important, stabilized and immobilized assembly of ordered lipids¹⁰⁰. These stabilized caveolar domains dramatically enrich cholesterol compared to the surrounding plasma membrane¹⁰¹, and their formation absolutely requires high levels of cholesterol¹⁰², possibly mediated by putative cholesterol-binding sequences present in the unusual membrane-embedded domain of caveolin 1 (REF. 103). Interestingly, caveolae have been shown to disassemble in response to membrane stretching¹⁰⁴, resulting in release of caveolar-sequestered lipids, which significantly change the surrounding lipid content¹⁰⁰. This coupling suggests a functional interplay between lipid-mediated lateral organization and mechanical stresses on the membrane.

Interactions of IMPs 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^{12,13}, defining interactions between IMPs and lipids is conceptually more difficult. Several models for such interactions have been proposed, from specificity for a single lipid species¹¹, to a stably associated, selective lipid ring¹⁰⁵, to non-selective transmembrane domain solvation¹⁰⁶. However, these apparently distinct interaction modes differ only in degree of specificity and affinity, rather than the nature of the interaction. This continuum of possible interactions presents a challenge to the interpretation of experimental data as different experimental (and simulation) techniques are typically restricted to a relatively narrow spatio-temporal 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 later in the section Interpreting protein–lipid interactions, after discussing early evidence that IMPs recruit particular lipid environments, as well as subsequent structural evidence for specific interactions.

The concept of annular lipids. The first direct evidence for lipid–protein interactions was obtained by electron paramagnetic resonance (EPR) spectroscopy (BOX 1) (expertly reviewed in REF.¹⁰⁵). Experiments in the early 1970s using spin-labelled lipids observed a spectrally distinct fraction of lipids around bovine cytochrome oxidase, interpreted as a layer of bound lipids around the protein’s perimeter¹⁰⁷ (FIG. 4a). Later analysis of this ‘bound’ fraction using conventional equilibrium binding models suggested a single type of ‘binding site’¹⁰⁸. Modelling of EPR signals then permitted the determination of lipid exchange rates between the protein surface ‘binding sites’ and the bulk membrane, finding them to be about 10^{-7} s^{-1} (or a lifetime at the protein surface of 100 ns)¹⁰⁹.

These experiments indicated that lipids in the immediate environment (first solvation shell) of a protein are different from lipids in the bulk membrane — their dynamics are reduced and they are 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. 4a).

However, as the EPR signal is averaged over the entire protein surface, it affords no insight into the localization of lipid–protein interactions within the annulus, which must be obtained by other techniques. For example, the selectivity of anionic lipid interactions with a potassium channel was reported using brominated lipids to quench Trp residues on lipid-facing domains of KcsA¹¹⁰. Overall, these data suggested that IMPs may organize their local nano-environment by associating with a distinct lipid shell.

Ligand-like, specific protein–lipid binding. What about 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^{111–113}. A lipid surviving the process of solubilization, purification and crystallization suggested a

Box 1 | Methods to study dynamics of lipids — integral membrane protein interactions

Electron paramagnetic resonance spectroscopy

Classical studies that established the concept of distinct lipid properties in the annulus surrounding a transmembrane protein relied on lipids labelled with a stable free-radical (for example, nitroxide). The dynamics of such ‘spin probes’ report on the fluidity and polarity (that is, hydration) of the membrane nano-environment^{53,183}.

A subset of spin-labelled lipids is slowed by the presence of transmembrane proteins¹⁰⁵, 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 behaviour of these systems can be provided by computational simulations (reviewed in REF.¹⁷). 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-rates¹⁴⁶, binding energetics¹⁴⁹ and preferential solvation by specific lipid subtypes¹⁵⁵.

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 pulldown¹⁸⁴. Higher resolution and quantification is achieved via spectroscopic methods, for example, fluorescence resonance energy transfer (FRET) between a fluorescent lipid and a neighbouring protein, identifying potential protein–lipid interactions¹¹ (see figure, part a). Similarly, single-molecule techniques can reveal changes in protein conformation or assembly induced by membrane environments¹⁸⁵. 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 analogues of both phospholipids¹⁸⁴ and sterols¹⁸⁶ have been developed and characterized.

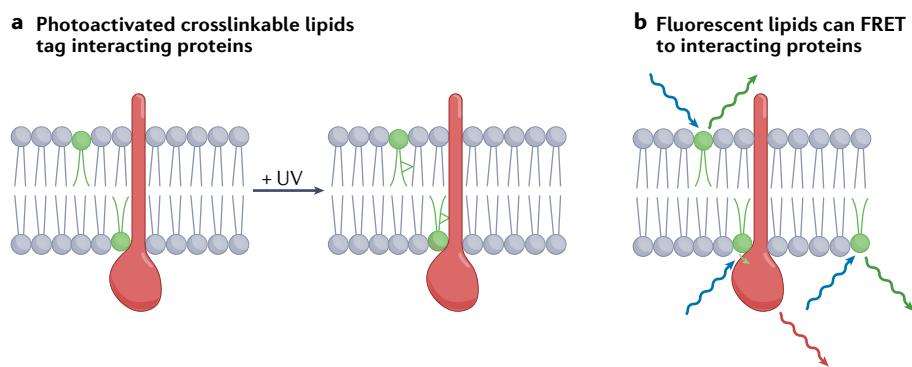


Photo-activatable lipids

Lipids can be engineered to contain small moieties that allow photo-conversion between a stable chemical bond (for example, diazirine) and a highly reactive one (for example, carbene free radical). Upon UV-activation, these lipids rapidly react with their neighbouring molecules, including other lipids and transmembrane proteins (see figure, part b). Inclusion of fluorescence or radioactivity into such photo-activated lipids allows detection of protein–lipid crosslinks by standard biochemical methods (for example, electrophoresis or chromatography)^{16,187}. Several of the above moieties can be combined to make multi-functional lipid analogues, containing photo-labile caging, cross-linking and fluorescent groups for powerful, versatile and temporally resolved analysis of protein–lipid dynamics^{188,189}.

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^{118,190}. Alternatively, similar techniques can be applied to patches of reconstituted liposomes¹⁹¹, or even whole giant liposomes¹⁹², whose lipid composition can be precisely controlled.

PIP₂

Phosphatidylinositol 4,5-bisphosphate, a highly charged and tightly regulated lipid type that can associate tightly with integral membrane proteins through both electrostatic and stereospecific interactions.

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^{17,114}).

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. However, occasionally, specific lipids are clearly identifiable, and these often act as essential regulators of protein activity. A prominent example is direct binding and activation of the inward rectifying potassium channel Kir2.2 by the phosphorylated lipid PIP₂ (phosphatidylinositol 4,5-bisphosphate)¹¹⁵ (FIG. 4b). This mechanism may be used to dynamically regulate Kir channels, as PIP₂ can be rapidly produced, consumed or sequestered by various cytoplasmic proteins¹¹⁶. A non-exclusive alternative is that PIP₂-binding signals the arrival of potassium

channels on the plasma membrane (where PIP₂ is enriched; FIG. 3), preventing ion leakage during biosynthesis or intracellular trafficking. An intriguing complication is that Kir channels also interact with another lipid characteristic of the plasma membrane: cholesterol¹¹⁷. However, while PIP₂ activates the channel, cholesterol appears to suppress the open state¹¹⁸. 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^{97,118}. Other, weaker lipid association sites on Kir2.2 have also been reported, with a complex interplay and competition between them¹¹⁹.

The example of Kir channels is instructive, as their regulation by PIP₂ and cholesterol appears to be characteristic of many cell-surface ion channels¹²⁰, transporters¹²¹ and receptors¹¹⁴, reported by either structural or biochemical evidence. 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

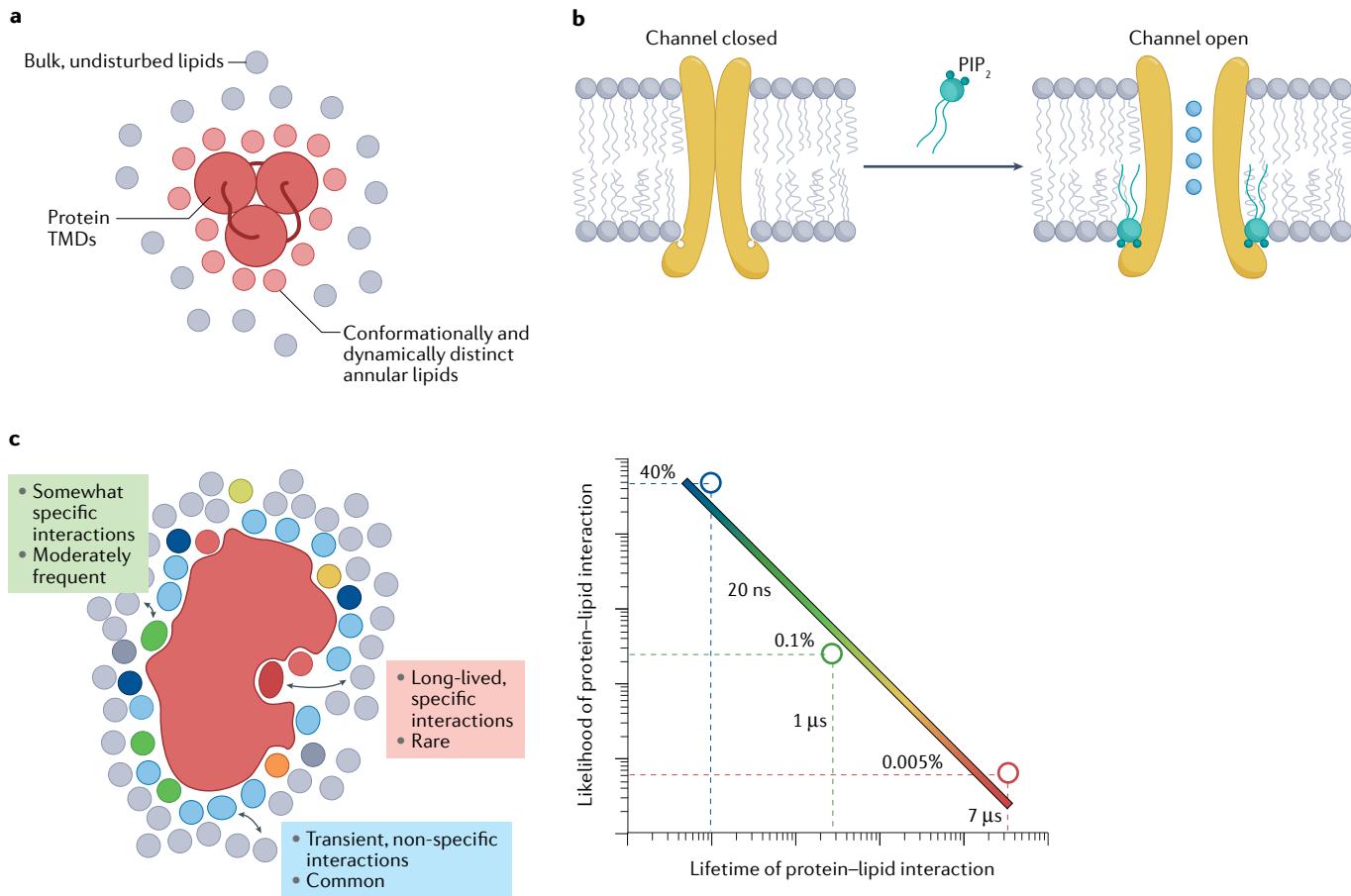


Fig. 4 | Possible modes of lipid interactions with transmembrane proteins. **a** | A shell of annular lipids stably associates with transmembrane protein domains (TMDs). The exchange rate of these annular lipids with the bulk is slowed, such that these lipids can be conceptualized as an extended part of the protein. **b** | Charged and large lipids, such as PIP₂, can be tightly and specifically bound by protein domains to introduce conformational changes. In these cases, lipids act as allosteric ligands for protein function. **c** | Interactions between integral membrane proteins (IMPs) and individual lipid molecules (coloured) are in constant competition with those from bulk lipids (grey). The affinity of a protein for a particular lipid species determines

how likely it is to occupy a particular binding site on the IMP, 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 (represented by the different colours) from relatively high affinity interactions, which are rare, to very common short-lived interactions (in the nanosecond range), which indicate rapid lipid exchange that is not influenced by protein binding. These observations imply that there is no single characteristic range for protein–lipid interactions but rather that specific, semi-selective and more solvent-like interactions could potentially be simultaneously relevant for an IMP.

headgroup that can be coordinated by positively charged binding pockets. PIP₂ abundance, lateral distribution and localization are tightly regulated by enzymatic control, while its confinement to the inner leaflet of the plasma membrane 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 unabundant, highly localized anionic lipids, including other phosphatidylinositides, cardiolipin¹²² and phosphatidic acid¹²³.

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 plasma membrane (30–40%), where it produces unique biophysical properties (higher lipid packing, ordered domains, increased thickness; FIG. 3). However, the structure and dynamics of cholesterol 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, that is, its range of possible conformations is far less restricted by the presence of a protein than for a phospholipid. These features perhaps contribute to the many examples of cholesterol binding to and occasionally functionally regulating membrane proteins, including multi-pass receptors^{124–131}, channels^{132,133}, transmembrane oligomers^{131,134} and even single-pass transmembrane domains¹³⁵. Interestingly, despite the ubiquity of reported cholesterol interaction sites, their structural determinants remain ambiguous. While cholesterol recognition/interaction amino acid consensus sites have been widely reported, the predictiveness and structural basis of this motif has been questioned¹³⁶.

Additional 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 is glycosylated sphingolipids, which are often ligands for soluble protein binding (for example, for bacterial toxins^{137,138} or viruses¹³⁹) but can also interact in *cis* (that is, on same membrane) with membrane proteins^{140,141}.

Interpreting protein–lipid interactions

Despite the tremendous technological advances across 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 limitations in molecular simulation techniques.

For example, as mentioned in the previous section, densities consistent with lipids have often been identified in crystal and electron microscopy 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 the inherent

conformational flexibility of lipids. Another possibility is that the lipid component may be intrinsically heterogeneous, 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 the structure of a membrane protein, 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.

Nevertheless, there is some evidence for more selective interactions of IMPs with lipids, generated by 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 thereof¹⁴² (BOX 2). These experiments generally show that structurally diverse lipids bind with similar affinities^{143,144}, though with some important exceptions, as in the bacterial transporter LeuT, where phosphatidylglycerol appears to be essential for dimerization¹⁴⁵.

Lipids bearing photoactivated crosslinking groups are a powerful technology for assaying protein–lipid interactions (BOX 1). However, interpretations of such experiments must account for the likelihood of labelling 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 of ~3 nm, which would be surrounded, at all times, by ~40 bilayer lipids¹⁴⁶ (see FIG. 4c). Assuming no selectivity for any set of lipids, a cross-linker or other labelled 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 nonspecific interaction but it is an inescapable conclusion that highly abundant lipids (for example, cholesterol in the plasma membrane) 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 labelling. While such observations support specificity, it is essential to verify that mutations do not dramatically affect the lipid-facing interface of the protein (for example, by disrupting TMD oligomers) or change the subcellular localization of a protein. In the latter case, the different lipid compositions of various subcellular membranes (FIG. 3) would be expected to affect the likelihood of labelling. Similar issues may affect FRET-based experiments (BOX 1).

Given the difficulties in directly identifying lipid–IMP 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 IMPs owing to the difficulty of achieving sufficient dilution over a wide range of concentrations. This barrier has been recently overcome by elegant experiments

Sphingolipids

Lipid with sphingoid backbones rather than glycerol. Lipids of this class (for example, sphingomyelin) are common components of eukaryote plasma membranes, interact well with sterols and bind strongly to some proteins.

Native mass spectrometry

A mass spectrometric technique capable of measuring molecular weights of large macromolecules (that is, proteins and their complexes) without fragmentation.

Box 2 | Structural methods to study interactions between lipids and integral membrane proteins

Structural biology

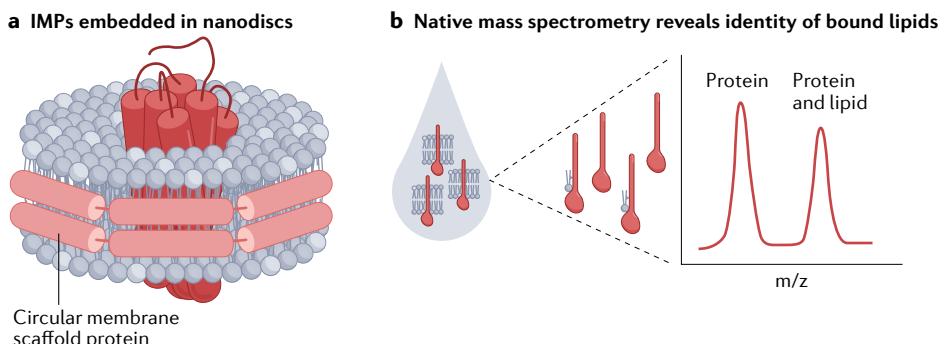
X-ray crystallography and, more recently, cryogenic electron microscopy have been extensively applied to membrane proteins, with thousands of structures already solved¹⁹³ and more being rapidly produced. In almost all cases, native lipids are replaced by detergents during the purification procedure. However, supplementing protein crystals or crystallization solutions with lipids can sometimes reveal lipid binding sites and, more importantly, demonstrate structural and functional effects of bound lipids, as for PIP₂ in gating of the inward rectifying potassium channel Kir2.2 (REF.¹¹⁵).

Electron crystallography

A specialized application of crystallography for the analysis of lipid–protein interactions relies on electron diffraction to achieve atomic-level resolution of the structure of both proteins and lipids. This method is applicable to 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 environment¹⁹⁴ to reveal the intimate integration of lipids within the transmembrane domain bundle and between tightly packed oligomers¹⁹⁵. 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 (disordered) and compositionally flexible (different lipid species can be accommodated at the same location). The apotheosis of this technique was a $<2\text{ \AA}$ resolution structure of the water channel aquaporin 0 (AQP0)¹⁰⁶, 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 nanodiscs¹⁵⁶ (see box figure, part a). The intrinsic bilayer-stabilizing properties of apolipoproteins enabled the



design of a family of membrane scaffolding proteins. When mixed with lipids and membrane proteins of interest, these membrane scaffolding proteins facilitate the formation of ~10 nm discs in which the protein target is solvated by a semi-native lipid bilayer. Combined with cryogenic electron microscopy, nanodiscs have propelled an explosion of structural insights into membrane protein structure¹⁵⁶. More recently, synthetic polymer scaffolds have proven capable of producing similar protein–lipid nanodiscs¹⁵⁷. 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 proteins¹⁵³.

Native MS

The development of ‘soft ionization’ methods like electron spray ionization has facilitated mass spectrometry (MS) of intact complex biomolecules. Electron spray ionization-MS was an enabling technology for shotgun lipidomics, allowing identification and quantification of hundreds of lipid species without the need for chromatographic separation or fragmentation¹⁹⁶. More recently, similar methodology was applied to membrane proteins¹⁹⁷, revealing that intact folded proteins could be stripped of their detergent and analysed for oligomerization, lipid binding and, most importantly, the structural and functional consequences of lipid–protein interactions¹⁴² (see box figure, part b).

IMP, integral membrane proteins.

on the lipid dependence of the dimerization of a chloride transporter, showing that lipid dependence of IMP function is not well described by a classic binding and linkage model²⁹. Instead, at low concentrations of a short-chain lipid (which would serve as a putative ‘ligand’), the dimerization equilibrium depends logarithmically on concentration, indicating a preferential solvation effect first described in the context of soluble protein folding¹⁴⁷. This effect is conceptually distinct from either ligand-like activity or regulation by bulk membrane properties, rather suggesting that minor membrane components can preferentially associate with, and stabilize, TMD regions that do not interact favourably with their surrounding membranes.

Moreover, changing membrane composition during a titration experiment also changes collective membrane properties, which can influence protein structure and function independent from specific interactions as discussed above (FIG. 1). Cholesterol is especially problematic, as 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 on the IMP 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 dilute¹⁴⁸. 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 REF.¹⁴⁹), revealing a broad spectrum of lipid–protein interactions¹²⁹ (FIG. 4c). Conceptualizing 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

Shotgun lipidomics

A mass spectrometric technique for identifying and quantifying the lipid components of a complex sample (for example, cell membrane) without prior chromatographic separation.

Nanodiscs

An experimental construct containing an integral membrane protein, lipids and a scaffold that solubilizes them. The scaffold can be a protein (MSP) or synthetic polymer (for example, styrene maleic acid).

Gramicidin A

An antibiotic peptide that assembles to form transmembrane pores, dependent on its membrane environment.

broad, decaying as a power law in both atomistic¹⁴⁶ and coarse-grained¹²⁹ simulations (FIG. 4c), covering the entire dynamic range of the simulation — longer than 30 μs; similarly broad lifetime distributions have been reported for rhodopsin¹⁵⁰. These observations suggest that lipid–protein interaction strengths are very heterogeneous and, therefore, describing only average lipid–protein affinities, or simplistic binary classifications, is likely to yield misleading conclusions.

The functional paralipidome model

While details of specific lipid–protein interactions, solvent-like effects, and/or effects of material properties (like membrane thickness) can sometimes explain functional regulation of IMPs by their membrane nano-environments, the functional consequences of lipids on proteins can be conceptualized without reliance on a specific mechanism¹⁵¹. The illustration of this basic concept is provided by an IMP that has two different conformations (A and B; for example, B might be the open state of an ion channel while A is the closed state), each of which selects a different local lipid environment owing to the distinct lipid interaction interfaces of each conformation (FIG. 5a,b). The function of this protein depends on the fraction of the conformational ensemble in conformation A versus conformation B. The relative population of the two states depends on the free energy difference between states A and B, which includes contributions from the membrane, as detailed below. 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 that differ in A and B, ΔG^{P-L} is the change in lipid–protein interactions in A versus B, and ΔG^{L-L} is the contribution from the bulk lipid environment.

The effect of the local lipid environment — which we term here the ‘paralipidome’ — is described by ΔG^{P-L} . The model is agnostic regarding the origins of ΔG^{P-L} , which can encompass specific lipid binding favoured in one conformation over the other or may represent the accumulation of many weaker, ‘solvent-like’ interactions¹⁵⁰ or changes in protein shape and lipid environment that minimize thickness mismatches or other stresses^{30,152}. As the conformational landscape — that is, the distributions of possible conformations — of an IMP may depend on all such interactions, this ‘paralipidome model’ offers a way to rationalize their effects without recourse to (often undetermined) particulars (FIG. 5b,c).

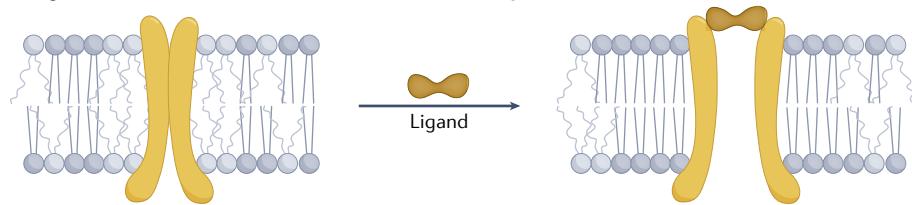
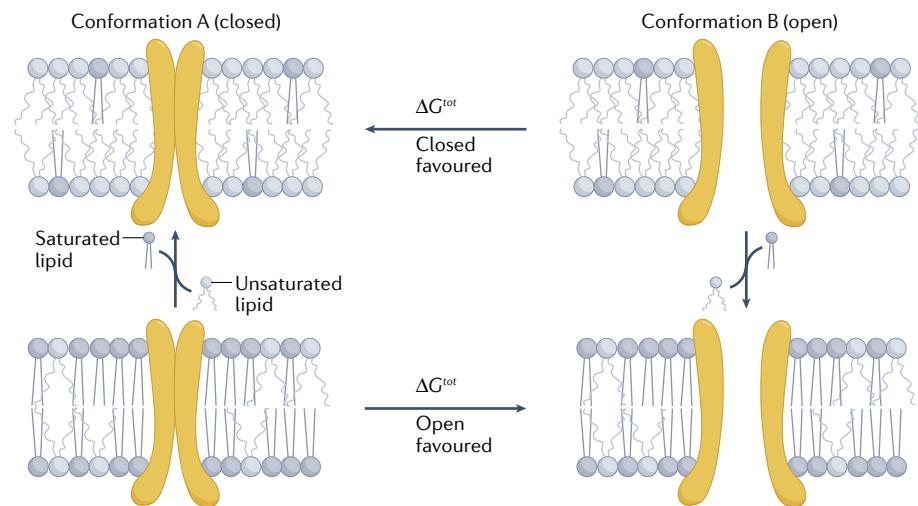
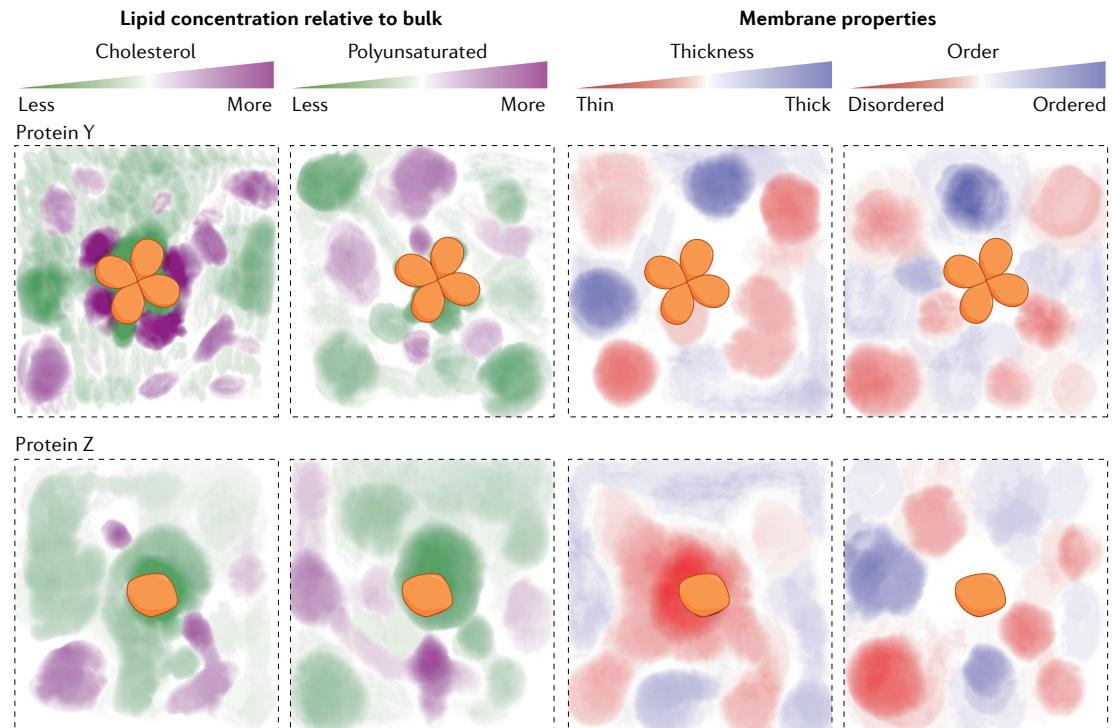
The model is relatively simple, requiring only that (1) IMPs recruit a local lipid environment (paralipidome) that is distinct in composition from the bulk and that (2) different conformational states recruit distinct paralipidomes (FIG. 5); there is extensive evidence for both aspects. The ability of proteins to recruit selective paralipidomes has been experimentally documented^{153,154} (see also discussion in sections above) 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 lipid ‘fingerprint’, defined by subtle-yet-clear enrichment of specific lipid subtypes. These enrichments were spatially defined, with hotspots for various headgroups, lipid saturations and sterols^{129,155} (FIG. 5c). Experimentally, nanodiscs have become an important tool for interrogating and controlling the local lipid environment (BOX 2). Originally constructed from modified apolipoproteins and widely used for structural biology¹⁵⁶, 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 IMPs¹⁵⁷. This approach has already been used to identify enrichment of certain lipid species in the paralipidomes of proteins¹⁵³. 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 proteins¹⁵⁸. It is important to emphasize here that neither protein-scaffolded nor polymer-scaffolded nanodiscs extract the ‘native’ paralipidome of a given IMP¹⁵⁹ nor necessarily recapitulate the native properties of a membrane¹⁶⁰. How much these issues will limit their usefulness for studies of paralipidomes remains to be seen.

There is also substantial evidence from simulations of recruitment of different paralipidomes depending on IMP conformation. Coarse-grained simulations of the A_{2A} adenosine receptor revealed that certain lipid subtypes can change preferences of the receptor for certain membrane domains¹⁶¹. All-atom simulations of the same protein showed conformation-dependent recruitment of distinct lipid environments¹⁵¹; similar results were obtained for rhodopsin¹⁵⁰. Although direct detection of lipid environments is challenging experimentally (as discussed above), coupling of receptor state and local lipid composition has been observed many times, from classic experiments on gramicidin A^{30,162,163} to more recent measurements of cholesterol-dependent GPCR activation^{164–166}.

Conclusions and perspectives

Returning to the central question of this Review: how do membrane proteins interact with lipids? Do lipids comprise a stable annulus, travelling with a protein like a ‘greasy tutu’ (FIG. 4a)? Or do lipids bind selectively like soluble ligands, allosterically modulating protein function (FIG. 4b)? Or is there no specificity at all, just a random sea of lipidic solvent for the TMDs? 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 behave differently 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 membrane. Some lipids (cholesterol, PIP₂) have potential for relatively high-affinity interactions, while most others interact with similar affinity and are therefore largely interchangeable (from the perspective of a protein). Functionally, strongly binding lipids can

a Ligand-induced conformations can select different lipid environments**b Changing lipid environments can influence conformational equilibria****c**

act as ligands or co-factors driving changes in protein conformation and oligomerization, while other proteins maybe be entirely agnostic about their solvating lipids. We propose that the combination of these interactions between lipids and proteins drives the formation of a local paralipidome for each IMP that is characterized by a unique set of compositional and biophysical features (FIGS. 4c and 5c). Paralipidome features can, in turn, be

modulated by external factors to affect structural and functional changes in IMPs. These changes could be physical (for example, membrane tension) or biochemical, including enzymatic conversion of lipid species or metabolic changes to bulk lipidomes (FIG. 5a,b).

This hypothesis makes an urgent call for methods to detail protein paralipidomes. While computational measurements of paralipidomes are providing deep

◀ Fig. 5 | **The functional paralipidome.** **a** Integral membrane proteins (IMPs) can selectively interact with 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 (light grey) into its paralipidome, the open state will be favoured in membranes rich in unsaturated lipids (that is, those in which the chemical activity of unsaturated lipids is higher; top panels). Membranes rich in saturated lipids (dark grey), which preferentially solvate or bind the open conformation (B), will tend to favour the open state. Importantly, these effects depend not on absolute compositions but rather on relative lipid availability (that is, chemical activity). For example, inclusion of other lipids (for example, cholesterol) into the bulk may influence the chemical activity of saturated lipids (as these strongly interact with cholesterol), preventing their interaction with IMPs and influencing their conformational equilibria (bottom panels). ΔG^{tot} , total free energy difference. **c** The emerging view is that the local protein–lipid interactions across membranes are highly versatile: different IMPs have unique preferences for their local membrane nano-environment where the lipid composition and consequent biophysical parameters are tuned to the subtleties of protein conformation and dynamics. We term these membrane fingerprints ‘functional paralipidomes’ and suggest that changes to the paralipidome (composition and/or structure) can serve as important regulatory mechanisms for IMP functionality.

insights, these must be supported by experimental approaches. Native mass spectrometry¹⁵⁴ and nanodiscs (BOX 2) are likely to be critically important approaches moving forward, though an emphasis on avoiding detergent solubilization of IMPs is needed. In cellular systems, super-resolution spectroscopy¹⁶⁷ and single-particle tracking⁸³ 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 the identification of multiple structures within a single preparation and linking this conformational landscape to lipid components, as recently suggested for the temperature-sensing channel TRPV3 (REF¹⁶⁸).

A key feature of biological membranes is the compositional and biophysical asymmetry between the leaflets¹⁶⁹. 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 packed¹⁷⁰. Similar asymmetries are likely present in other organelles, for example, endosomes¹⁷⁰ and the ER¹⁷¹. This lipidomic and biophysical membrane asymmetry¹⁷⁰ prompts a mostly unexplored hypothesis

that protein paralipidomes are similarly asymmetric, with different compositions and physical properties solvating the outer and inner halves of IMP TMDs. An under-appreciated and low-energy way of rapidly and dramatically changing the lipid environments of proteins is by releasing this lipid asymmetry via scramblase channels¹⁷². This lipid re-shuffling likely induces major changes in the composition of both leaflets¹⁶⁹, which would in turn affect the asymmetric paralipidomes.

In what cellular contexts might changes in local lipid 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 (FIG. 3). For example, eukaryote plasma membranes are very rich in sterols, with mammalian plasma membranes containing up to 40 mol%¹⁷⁰. This high concentration stands in contrast to most intracellular organelles¹⁷³. It is plausible that lipid-determined conformational equilibria may restrict protein activity to only particular membranes within a cell. The paralipidome of a protein might also be regulated by recruiting it to a distinct lipid subdomain via reversible post-translational modifications. For example, palmitoylation (addition of a long, saturated acyl chain) is an essential driver of the affinity of a protein for ordered microdomains, is rapidly reversible^{174,175} and is present on hundreds of plasma membrane proteins¹⁷⁶. Ordered phases in synthetic systems can be enriched by up to tenfold in saturated lipids and threefold to fivefold in cholesterol^{177,178}, with qualitatively similar enrichments reported in isolated plasma membranes^{76,179,180}, suggesting that recruitment of IMPs to ordered domains may dramatically change their paralipidome. Finally, large-scale metabolic activity may, in special cases, drive sufficient lipidomic changes to affect protein structures, for example, the enzymatic conversion of sphingomyelin to ceramide during apoptosis or incorporation of exogenous fatty acids into phospholipids¹⁰. How these lipidomic perturbations affect the paralipidomes and how these, in turn, regulate IMP structure and function are questions forming the frontier of current research in membrane biology.

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