Seeing the Membrane from Both Sides Now: Lipid Asymmetry and Its Strange Consequences

Milka Doktorova,[1] Ilya Levental,[1] and Frederick A. Heberle[2]

[1]Department of Molecular Physiology and Pharmacology, University of Virginia, Center for Membrane and Cell Physiology, Charlottesville, Virginia 22908, USA

[2]Department of Chemistry, University of Tennessee Knoxville, Knoxville, Tennessee 37996, USA

Correspondence: md4xm@virginia.edu; il2sy@virginia.edu; fheberle@utk.edu

Almost all biomembranes are constructed as lipid bilayers and, in almost all of these, the two opposing monolayers (leaflets) have distinct lipid compositions. This lipid asymmetry arises through the concerted action of a suite of energy-dependent enzymes that maintain living bilayers in a far-from-equilibrium steady-state. Recent discoveries reveal that lipid compositional asymmetry imparts biophysical asymmetries and that this dualistic organization may have major consequences for cellular physiology. Importantly, while transbilayer asymmetry appears to be an essential, near-ubiquitous characteristic of biological membranes, it has been challenging to reproduce in reconstituted or synthetic systems. Although recent methodological developments have overcome some critical challenges, it remains difficult to extrapolate results from available models to biological systems. Concurrently, there are few experimental approaches for targeted, controlled manipulation of lipid asymmetry in living cells. Thus, the biophysical and functional consequences of membrane asymmetry remain almost wholly unexplored. This perspective summarizes the current state of knowledge and highlights emerging themes that are beginning to make inroads into the fundamental question of why life tends toward asymmetry in its bilayers.

LIVING MEMBRANE ASYMMETRY: PAST AND PRESENT

One of the foundational facts of biology is that cells are enveloped and internally subdivided by fluid lipid bilayers. Shortly after the widespread acceptance of the elegantly named "fluid mosaic" model, cracks began to show in the tile, revealing that living membrane organization is much more intricate and variable than a homogenous two-dimensional patchwork. From lipidome complexity (Shevchenko and Simons 2010; Harayama and Riezman 2018) to lateral domains (Sezgin et al. 2017; Levental et al. 2020), to partitioning (Kusumi et al. 2012; Fujimoto and Parmryd 2016) and shaping (Bonifacino and Lippincott-Schwartz 2003; Simunovic et al. 2019) of membranes via proteins, cell membranes are textured and structured at nearly all length scales and involved in nearly all physiological functions. This perspective focuses on lipid asymmetry (i.e., distinct compositions of the two bilayer leaflets), a facet of membrane complexity that was initially described more than 50 years ago (Bretscher 1972; Verkleij et al. 1973), yet whose form and function remain mysterious to

Cite this article as *Cold Spring Harb Perspect Biol* doi: 10.1101/cshperspect.a041393

this day. Lipid asymmetry was originally discovered by experiments using enzymes or chemicals that could not penetrate the bilayer and thus could only modify external leaflet lipids (Bretscher 1972; Verkleij et al. 1973). These studies revealed that the outer leaflet in mammalian erythrocytes was composed nearly exclusively of relatively unreactive choline-headgroup lipids (phosphatidylcholine [PC] and sphingomyelin [SM]), whereas charged and amino-headgroup lipids (phosphatidylserine [PS], phosphatidylinositol [PI], and phosphatidylethanolamine [PE]) were confined to the inner, cytoplasmic plasma membrane (PM) leaflet (Fig. 1; Devaux 1991). These chemical/enzymatic approaches have been supported and complemented by immunolabeling of freeze-fractured cell membranes (Murate et al. 2015), together suggesting that all mammalian PMs, almost all eukaryote PMs (with some exceptions [Shiomi et al. 2021]), and likely most cell membranes have some degree of lipid asymmetry (Kobayashi and Menon 2018; Doktorova et al. 2020).

Since the 1980s, much of the effort in this field has focused on identifying and characterizing the protein machinery that establishes, maintains, and releases lipid asymmetry. We point the curious reader to a comprehensive recent review on these protein regulators (Sakuragi and Nagata 2023). Briefly, lipids are driven against their equilibrium (symmetric) distribution by ATP-dependent transporters known as "flippases" (which force lipids into the inner leaflet) and "floppases" (for outward translocation). There are likely many such enzymes with specificity for various headgroups. Asymmetry can then be released by relatively nonselective (Malvezzi et al. 2013) "scramblases," which—despite the name—are not enzymes but rather lipid channels that form a hydrophilic passage within the hydrophobic bilayer core through which lipid headgroups can quickly traverse and equilibrate between leaflets (Kobayashi and Menon 2018). Particularly notable among these is the TMEM16F scramblase (Suzuki et al. 2010; Malvezzi et al. 2013), which is gated by calcium and thus potentially implicates lipid scrambling among the many facets of calcium-associated signaling.

BOTH SIDES NOW: BIOPHYSICAL AND FUNCTIONAL ASYMMETRY

While the foundational facts of lipid asymmetry have been known for decades, the physiological purpose of highly asymmetric lipid distributions remains unknown. This may be a surprising claim, as there have been extensive reports and reviews on functional roles of lipid asymmetry (Op den Kamp 1979; Fadeel and Xue 2009; Bevers and Williamson 2016; Kobayashi and Menon 2018; Doktorova et al. 2020). However, in almost all such cases, the cellular function in question is mediated by exposure of PS onto

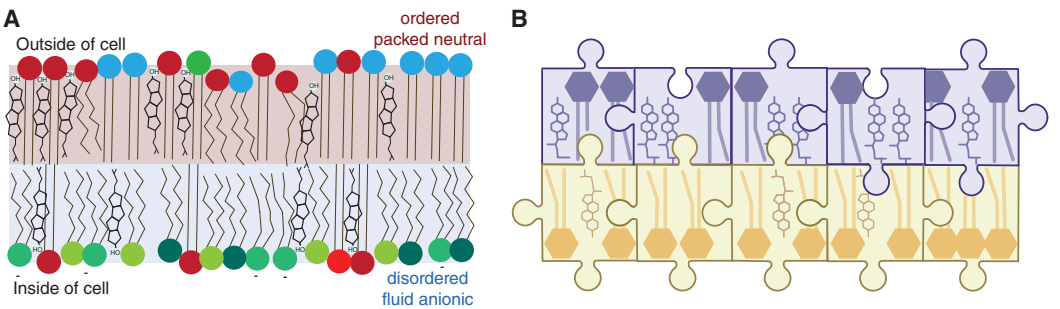

Figure 1. Organization of plasma membrane components. (*A*) Exoplasmic leaflets in mammalian plasma membranes are composed of phospholipids with relatively saturated acyl chains and uncharged lipid headgroups. This leads to more tightly packed and less mobile leaflets than their cytosolic counterparts, which are rich in anionic, unsaturated lipids. (*B*) Divergent results for cholesterol interleaflet distribution makes it challenging to piece together the precise organization of membrane components.

the outer leaflet (Bevers and Williamson 2016), that is, the loss of lipid asymmetry. PS exposure is a hallmark of apoptosis, required for coagulation, and involved in diverse functions including cell–cell fusion (Verma et al. 2018; Whitlock et al. 2018), viral infection (Husby et al. 2022), and synaptic pruning (Bevers and Williamson 2016). However, the notion that asymmetry exists to be released has a whiff of teleology. We argue that the significant expenditure of metabolic resources required to pump nearly all PM lipids far away from their equilibrium distribution suggests that asymmetry per se is likely functionalized by cells.

Recently, a detailed analysis extended the previously established chemical asymmetry of hydrophilic headgroups to physical asymmetries associated with lipid acyl chains (Fig. 1A; Lorent et al. 2020). Specifically, the acyl chains of lipids comprising the outer PM leaflet were found to be largely saturated (i.e., containing few double bonds), whereas inner PM leaflets were mostly polyunsaturated, with four or more double bonds per lipid. Acyl chain saturation determines how tightly lipids can pack, which in turn determines how fast they can move (diffusion), how easily they deform (stiffness), and how readily stuff can slip between them (permeation). Thus, since the two PM leaflets have different degrees of saturation, they may also be expected to have different physical properties. This hypothesis was confirmed by several independent measurements that converged on the conclusion that inner leaflets are less tightly packed and more diffusive than outer leaflets (Fig. 1A; Gupta et al. 2020; Lorent et al. 2020). Intriguingly, this transbilayer lipid-packing asymmetry correlates with asymmetric structures of protein transmembrane domains (TMDs) (Sharpe et al. 2010; Lorent et al. 2020), which are on average bulkier in the loosely packed inner leaflet and slimmer in the tightly packed outer leaflet. Because TMD structures can be inferred directly from sequences, their asymmetries can be predicted for any organism for which genomic information is available. This approach was used to make the striking prediction that structural asymmetry may be a feature common to most eukaryote PMs (Lorent et al. 2020).

LIKE A ROLLING STONE: CHOLESTEROL ASYMMETRY

The transbilayer distributions of membrane phospholipids have been extensively investigated and comprise one of the core dogmas of membrane biology, contrasting sharply with the lack of consensus about the asymmetry of the mammalian PM's most abundant component (~40 mol% [Symons et al. 2021]): cholesterol (Fig. 1B; Steck and Lange 2018). This knowledge gap persists because sterols are chemically distinct from phospholipids in ways relevant for measuring their asymmetry. Specifically, the charged moieties of phospholipids prevent their rapid movement between leaflets (through the hydrophobic core), which is often called flip-flop and proceeds on the timescale of hours (Marquardt et al. 2017; Doktorova et al. 2019). Conversely, most membrane sterols have small uncharged "headgroups" (hydroxyl for cholesterol) and are flat and rigid, allowing them to easily and rapidly traverse the space between leaflets (Steck et al. 2002; Bennett et al. 2009; Bruckner et al. 2009; Steck and Lange 2012). In fact, this movement is so fast that there is little consensus about just how fast it is; most experiments are too slow to measure it accurately and estimates range from the microseconds to minutes timescale (Lange et al. 1981; Leventis and Silvius 2001; Steck et al. 2002; Bruckner et al. 2009; Steck and Lange 2012). This rapid equilibration between leaflets presents a unique difficulty in trying to measure cholesterol's residence in a single leaflet, since each cholesterol molecule samples both leaflets frequently and thus labeling with an external reagent (e.g., cholesterol oxidase) simply leads to all cholesterol being rapidly labeled (Lange et al. 1981).

Despite this daunting conundrum, many attempts have been made to measure cholesterol's asymmetric distribution in living PMs. Perhaps unsurprisingly, these attempts have yielded wildly divergent conclusions. As early as the 1970s, a report claimed up to three-fold enrichment in the outer leaflet (Fisher 1976), later contradicted by claims of the same degree of cholesterol enrichment, but in the cytoplasmic leaflet (Schroeder et al. 1991). Subsequent reports continued the discrepant trend, with convincing ev-

idence for cytoplasmic (Igbavboa et al. 1996; Mondal et al. 2009; Solanko et al. 2018), exoplasmic (Bittman and Rottem 1976; Hale and Schroeder 1982; Ingólfsson et al. 2014), or neither (Lange and Slayton 1982) leaflet being cholesterol enriched, and some attempts to justify the differences based on cell types (Hale and Schroeder 1982). This trend persists to recent years, with two high-profile papers again conflictingly claiming that cholesterol is either highly enriched in the inner PM leaflet (Courtney et al. 2018b) or almost exclusively (>90%) in the outer leaflet (Liu et al. 2017). The latter report used an elegant assay, relying on sensor proteins engineered to bind membranes with exquisite sensitivity to their cholesterol content. These probes were used to probe both PM outer leaflets (via external labeling by purified probe) and inner leaflets (by expressing in the cytoplasm) (Liu et al. 2017; Buwaneka et al. 2021). The same approach was also used to implicate possible regulators of cholesterol asymmetry, identifying the transporter ABCA1 and the Hedgehog-morphogen receptor Patched (Zhang et al. 2018). While this clever design appears straightforward, several possible contaminating effects were identified that cast doubt on the core conclusions (Courtney et al. 2018a; Steck and Lange 2018), although some of these were addressed in follow-up experiments (Buwaneka et al. 2021). Equally concerning to technical artifacts was the conceptual puzzle of how a molecule that rapidly flips between leaflets could possibly be so highly enriched in one of them. Our recent experiments (Doktorova et al. 2023) and clever theory and simulations (Varma and Deserno 2022) from the Deserno group provide several possible answers: (1) the two bilayer leaflets are not absolutely constrained to have the same number of phospholipids—if one leaflet has more than the other, cholesterol will naturally "flip to fill the gaps"; and (2) cholesterol prefers to interact with lipids that reside on outer leaflets (sphingolipids, saturated acyl chains) rather than inner leaflet lipids (often highly unsaturated). These factors (and others) combine to determine cholesterol steady-state distribution in cells. Ultimately, while several lines of evidence lead us to predict that cholesterol is likely enriched on the PM outer leaflet, the exact asymmetry of cholesterol in any given context remains a matter of debate.

Ch-ch-CHANGES: INTERLEAFLET COUPLING OF MEMBRANE PROPERTIES

Biophysical studies of synthetic membranes have played a key role in the evolution of PM models. The vast majority of such studies have relied on symmetric bilayers, mostly because of the historic unavailability of asymmetric model systems. Although methods for preparing and characterizing model asymmetric bilayers are seeing rapid development (Cheng and London 2011; Lin and London 2014; Doktorova et al. 2018; Enoki and Feigenson 2019), they still present technical challenges (Doktorova et al. 2018). Thus, there remain substantial gaps in our understanding of the biophysical consequences of lipid asymmetry. A key factor determining such consequences is interleaflet coupling, which (at the most basic level) reflects the extent to which properties of different monolayers are influenced by their mutual interactions in a bilayer. Put another way, interleaflet coupling is the physical manifestation of the bilayer's desire to achieve a minimum free energy by exploring available degrees of freedom within the individual leaflets. These can include various types of lipid deformations (e.g., stretching, splay, tilt, protrusion, etc.) as well as degrees of freedom associated with lipid mixing within and between leaflets.

A useful experimental strategy for investigating interleaflet coupling is to compare a particular property measured in an asymmetric bilayer to that measured in a symmetric bilayer of identical or similar overall composition, with the signal preferably isolated to a single leaflet. In this way, various groups have investigated how asymmetry influences lipid order (Cheng and London 2011), packing (Heberle et al. 2016; Frewein et al. 2022), and lateral diffusion (Chiantia et al. 2011; Chiantia and London 2012), membrane bending rigidity (Elani et al. 2015; Lu et al. 2016; Rickeard et al. 2020) and compressibility (Lu et al. 2016), and the line tension (Enoki et al. 2021) and thermal stability (Cheng et al. 2009) of ordered domains. Perhaps unsurprisingly, the picture that

emerges is complex and does not yield a simple recipe for predicting the behavior of asymmetric membranes. Three case studies exemplify the challenge of elucidating general rules for interleaflet coupling: (1) London and coworkers observed strong coupling of lipid diffusion in asymmetric vesicles with brain SM in the outer leaflet and mixed chain PC in the inner leaflet, evidenced by reduced lateral diffusion in both leaflets (Chiantia and London 2012). Curiously, the change in diffusion was not accompanied by increased order in the inner leaflet, suggesting that different properties can couple to different extents within the same bilayer. (2) The Ces group found anomalous stiffening of asymmetric giant vesicles: the bending rigidity of an asymmetric bilayer of DOPC (di18:1 PC) and POPC (16:0,18:1 PC) leaflets was more than twofold greater than that of either symmetric bilayer (Elani et al. 2015). (3) Eicher et al. discovered remarkable differences in coupling depending on the sidedness of the asymmetry. Using asymmetric liposomes in which the inner leaflet was either POPC or POPE and the outer leaflet a mixture of the two, strongly coupled leaflets (evidenced by a single melting transition and similar lipid packing in both leaflets) were observed when the inner leaflet was POPE, but an apparent lack of coupling (i.e., two independent melting transitions) was found when the inner leaflet was POPC (Eicher et al. 2018). Moreover, neither asymmetric bilayer showed evidence for coupling at higher temperatures, where the membrane was a fully melted fluid. If any robust conclusion can be drawn from these and other studies, it is that the extent of interleaflet coupling of membrane structural and material properties depends not only on lipid composition, but also other factors (e.g., temperature, curvature, relative packing).

PHASED AND CONFUSED: COUPLING OF PHASE BEHAVIOR

On timescales where lipid translational degrees of freedom are relevant (i.e., >microseconds for fluid phases), interleaflet coupling can also influence the spatial organization of lipids—and potentially induce phase changes—in either or both leaflets (Fig. 2). Because of the relevance of asymmetry and domain coupling to PM rafts, many experimental and theoretical studies of asymmetry have focused on a scenario involving leaflets that would exhibit disparate phase behaviors in symmetric bilayers. Such phase asymmetries could hypothetically be related to the steady-state arrangement of mammalian PMs (Lorent et al. 2020), whose cytoplasmic leaflet is composed largely of unsaturated glycerolipids (which would not be expected to form ordered phases) and whose exoplasmic leaflet composition—rich in SM, lower melting phosphocholine lipids, and cholesterol—would seem to fall squarely within a two-phase coexistence regime (Fig. 2; Veatch and Keller 2003; Heberle et al. 2010). Experimental studies of this sort have produced outcomes ranging from no apparent coupling of phase behavior, to induced lipid segregation in the model cytoplasmic leaflet, to suppressed lipid segregation in the model exoplasmic leaflet (Crane et al. 2005; Kiessling et al. 2006; Garg et al. 2007; Collins and Keller 2008; Wan et al. 2008; Lin and London 2015; Heberle et al. 2016; Wang and London 2018; Enoki and Feigenson 2019, 2022; Enoki et al. 2021). While the sheer variety of membrane geometries (i.e., supported and unsupported planar bilayers vs. vesicles of various sizes), leaflet compositions, and experimental techniques used in these studies makes it difficult to tease out systematic trends and organizing principles, it is clear that the full spectrum of coupling scenarios are possible in biomimetic membranes. These observations make an urgent call for studies of interleaflet coupling in which the lipid compositions of both leaflets are systematically varied. A further exciting direction would be evaluating the effects of bilayer spanning components (i.e., proteins), which would likely enhance coupling.

A key parameter determining interleaflet coupling of domains is the surface tension at the midplane interface of a mismatched or "anti-registered" domain (e.g., one with an L_o-like composition in one leaflet and an L_d-like composition in the other) (Fig. 2). This surface tension, often called mismatch free energy γ (units of k_BT/nm^2), is defined as the free energy cost per unit area to create an asymmetric do-

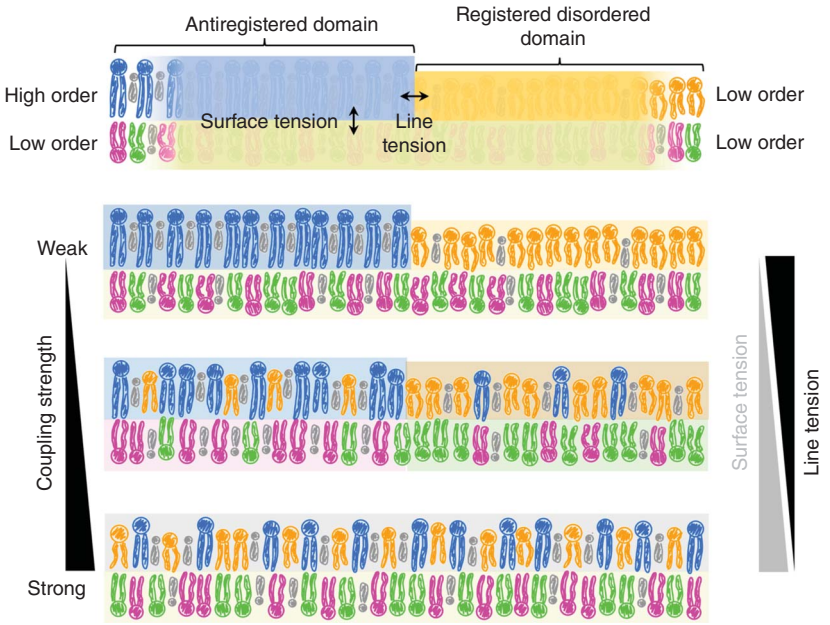

Figure 2. A thermodynamic model for interleaflet coupling of phase behavior. In mammalian plasma membranes (PMs), the outer leaflet contains a mixture of saturated and unsaturated lipids, while the inner leaflet contains polyunsaturated lipids (the transbilayer distribution of cholesterol is uncertain, but likely enriched in the outer leaflet). Symmetric models of outer leaflet mixtures have a strong tendency to form coexisting ordered and disordered domains, while inner leaflet models tend to form a uniform disordered phase. The magnitude of interleaflet coupling is determined by a balance between line tension that favors phase separation in the outer leaflet, and interleaflet surface tension that opposes it. If line tension dominates (i.e., weak coupling), lipid organization within each leaflet reflects that of cognate symmetric bilayers. If surface tension dominates, both leaflets are uniformly mixed. The lipid raft hypothesis may represent an intermediate between these extremes, with clustering or phase separation of outer leaflet lipids potentially inducing segregation of inner leaflet lipids.

main. For mismatched ordered/disordered liquid phases, γ has been estimated from heuristic arguments (Collins and Keller 2008), calculated from molecular mean-field theory (Putzel et al. 2011), and measured in both experiment (Blosser et al. 2015) and simulation (Risselada and Marrink 2008), culminating in a disparate set of values that span nearly two orders of magnitude. Although the underlying molecular origins of mismatch free energy are unclear, a reasonable assumption is that γ scales with the difference in acyl chain order of the opposing phases, such that large transbilayer differences in outer and inner leaflet order are energetically disfavored. In bilayers exhibiting asymmetric compositions and propensities to form domains (like the mammalian PM), the free energy minimum therefore reflects a compromise between line tension λ, which promotes domain formation in the outer leaflet, and midplane surface tension (γ) that opposes it. Large values of λ would thus favor mismatched raft domains in a sea of registered disordered phase, with γ serving to tune the outer/inner order difference and with it the partitioning of membrane components between raft and nonraft environments (Fig. 2).

These ideas can be quantitatively explored with thermodynamic models, resulting in phase diagrams that predict the number and types of coexisting phases (i.e., registered and antiregistered) as a function of leaflet composition and the strength of in-plane versus midplane lipid interactions, represented, respectively, by λ and γ (Allender and Schick 2006; Wagner et al. 2007; Putzel and Schick 2008; Williamson and Olmsted 2015, 2018). Conceptually, these phase dia-

grams broadly recapitulate the more common framing of interleaflet coupling in terms of leaflet dominance: does a leaflet with a tendency to phase separate induce domains in an opposing leaflet that would otherwise mix uniformly? Or does the uniform leaflet instead impose its behavior and abolish phase separation in the opposing leaflet? Viewed through the lens of mean-field theory, the former corresponds to weak interleaflet coupling, in which γ is large enough to induce the lateral partitioning of inner leaflet components (perhaps including signaling molecules such as acylated proteins or phosphorylated lipids), but not so strongly unfavorable that it overwhelms outer leaflet line tension and destroys the raft entirely (i.e., inner leaflet dominance). This behavior has been observed in PM-mimetic asymmetric giant unilamellar vesicles (GUVs) that showed induced partitioning of a fluorescent probe in a leaflet where phase separation would not be expected (Lin and London 2015; Enoki and Feigenson 2019). An advantage of the mean-field framework is its emphasis on coupling strength as a continuous variable that can be controlled by the cell, which could result in a continuum of inner and outer leaflet lipid partitioning behaviors between the two extremes of leaflet dominance (Fig. 2).

For example, a resting cell PM with sufficiently large γ might reside in a region of the asymmetric phase space in which both leaflets are uniformly mixed, although with the outer leaflet poised to phase separate owing to a large λ. Any change in composition that would tend to make the inner leaflet more ordered or the outer leaflet more disordered could potentially induce raft formation by moving the system into a phase coexistence region. Lipid scrambling (for example, through the activation of calcium-dependent scramblases [Suzuki et al. 2010; Malvezzi et al. 2013]) could have that effect, while flippases and floppases would act to restore a larger order difference and abolish rafts. Molecules that localize to the midplane (i.e., between leaflets) may have their own unique effects by lowering the surface tension of the mismatched phase and expanding the phase coexistence region. Crossing a phase boundary can induce not only mismatched and disordered phases, but also phases in which both leaflets of the domain are relatively ordered, raising the possibility of multiple types of raft phases. Transmembrane proteins could have distinct preferences for these phases, which may in turn have unique functional consequences for the proteins. It is not difficult to imagine a scenario in which a protein is nonfunctional in the resting asymmetric phase but is functionalized upon partitioning into a biophysically distinct registered-disordered phase.

UNDER PRESSURE: FROM MODEL MEMBRANES TO CELLS

The chemical and functional complexity, and inherent collectivity, of biological membranes complicates investigation of specific mechanisms underlying their involvement in cellular phenomena. It is unsurprising, therefore, that the main historical approach to understanding membrane behavior has been to strip down their functional roles and simplify their underlying chemistry—in other words, to create and characterize model membranes. Until recently, two factors dominated the conceptual models underlying membrane biophysics: the structures of the constituent lipids and their in-plane interactions (Marsh 2013). Clearly demonstrated in symmetric bilayers, these two factors were analogously extended to asymmetric ones, where mixtures modeling the lipids of exoplasmic and cytoplasmic leaflets could be studied in isolation or combined to create synthetic asymmetric liposomes (St. Clair et al. 2017; Scott et al. 2021). In such asymmetric membranes, simple mixtures modeling the cellular exoplasmic leaflet affected, and were affected by, opposing leaflets composed of some disordered lipids but not others, and this applied to some properties (e.g., lipid diffusion) but not others (e.g., lipid order) (see above).

Importantly, recent discoveries highlight a key characteristic inherent to compositionally asymmetric bilayers that exists in parallel to the chemical composition of lipid membranes: resting stresses that accumulate in asymmetric leaflets and modulate their properties (Doktorova and Weinstein 2018; Hossein and Deserno 2020). Such leaflet tension was shown in simulations of simple (single-component) bilayers with mis-

matched numbers of lipids in the two leaflets: the overpopulated leaflet becomes compressed while the underpopulated one becomes tensed (Fig. 3A; Esteban-Martín et al. 2009; Park et al. 2015). Importantly, the stresses are equal and opposite in the two leaflets, thus the bilayer has no net tension. Similar behavior can occur in compositionally asymmetric bilayers, but with an unpredictable dependence on the relative lipid abundances in the two sides (Doktorova and Weinstein 2018). For example, both leaflets could have the same number of phospholipids, but the lipids of one leaflet may pack more tightly than the other (Fig. 3A). This phenomenon, now formally defined as differential stress (Hossein and Deserno 2020), refers to coexisting, perfectly balanced stresses within leaflets resulting from their equal-and-opposite suboptimal lipid packing densities in a bilayer. Analogous to the structural and dynamical effects of net bilayer tension, the properties of compositionally identical bilayers can vary dramatically depending on the amount of differential stress (tension or compression) in their leaflets.

While initially observed and characterized in computational models, differential stress is likely to also occur in synthetic and biological membranes. After all, for a compositionally asymmetric membrane, the truly tension-free bilayer is very much a special case: the preferred surface areas of all lipids in a given leaflet must perfectly balance those of a compositionally different one (Doktorova and Weinstein 2018). Such a fine-tuned balance seems unlikely to arise spontaneously; thus, many experimentally prepared asymmetric membranes may harbor differential stresses. For example, a combination of simulations and theory explained an initially puzzling experimental result—the anomalous stiffening of asymmetric giant vesicles (Elani et al. 2015; Hossein and Deserno 2020)—by invoking the presence of differential stress. Similar asymmetry-induced membrane stiffening was also reported in neutron spin-echo experiments of extruded vesicles prepared via cyclodextrin-mediated lipid exchange (Rickeard et al. 2020). Both observations suggest that, even in the ab-

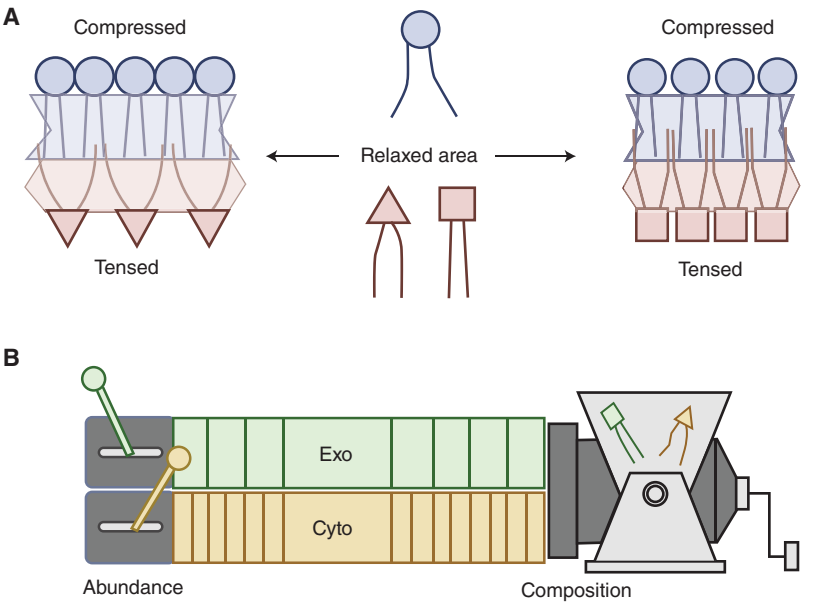

Figure 3. Regulation of differential stress. (*A*) Differential stress can arise in asymmetric membranes when one leaflet has a different preferred surface area than another. This situation can arise when one leaflet has more lipids than the other (*left*) and/or in leaflets with equal lipid numbers but very different packing preferences (*right*). (*B*) Cells can regulate the differential stress in their membranes by tuning the relative lipid compositions and total abundances in the two leaflets.

sence of external energy input (i.e., enzymes), lipid-only asymmetric bilayers can be trapped in relatively stable differentially stressed states.

There is also evidence to suggest that biological membranes may carry significant differential stresses. The complexity of live cell lipidomes and the intricate, dynamic, and interwoven biosynthetic networks that produce them would seem to preclude perfectly balanced leaflet areas. And indeed, experimental measurements of the relative packing densities of the two PM leaflets in mammalian cells show large differences between exoplasmic and cytoplasmic monolayers (Gupta et al. 2020; Lorent et al. 2020). Moreover, the mammalian PM contains a high concentration of cholesterol. While cholesterol may be expected to buffer tension via its fast transbilayer redistribution (Miettinen and Lipowsky 2019), preferential interactions with saturated lipids can dominate over those tendencies and drive its accumulation in a particular leaflet, thereby generating differential stresses (Doktorova and Levental 2022; Varma and Deserno 2022). The exoplasmic leaflet in mammalian PMs is much more saturated than the cytoplasmic one (Lorent et al. 2020), which likely serves to recruit cholesterol (Steck and Lange 2018; Buwaneka et al. 2021) and produce a relative compression therein. Thus, the enormous configuration space available by varying relative lipid abundances and compositions between leaflets may provide cells with yet-unexplored flexibility in regulating differential stresses and the molecular processes affected by them (Fig. 3B).

Considering these converging lines of evidence, we argue that deep characterization of differential stress in asymmetric membranes represents a critical need in membrane biology and biophysics. While currently experimentally inaccessible, the presence of differential stress can be inferred from observables including changes in membrane morphology, bending rigidity, packing density, and phase transitions (Foley et al. 2023). Combining simulations with wet-lab approaches can help reveal the molecular details, magnitude, and effects of differential stress. It should also be emphasized that the differential stress in an asymmetric membrane cannot be approximated from the lipid compositions of its leaflets alone (Scott et al. 2021; Foley et al. 2023). Other essential determinants are the mixing behavior of the lipids, lipid abundance imbalances between leaflets, and the yet-unknown effects of interleaflet coupling. Thus, at present, it is not possible to predict or directly measure—and very difficult to even infer—the differential stress in any experimental system.

Ultimately, robust understanding of differential stress is essential for designing, executing, and interpreting manipulations of lipid composition and distribution in cells. Quantifying the actual magnitude of the differential stress in a living cell PM is not yet achievable, and it likely varies within and between cells. However, other related questions may be more generalizable and within reach: How can cells alter differential stress? How is leaflet tension coupled to lipid composition and interleaflet interactions? How do the properties of the membrane leaflets change when differential stress is varied, and how can those changes affect biology at the PM? Addressing these questions will begin to reveal the versatile functional roles of actively maintained PM asymmetry in cells.

STILL HAVEN'T FOUND WHAT WE'RE LOOKING FOR: OUTLOOK AND PERSPECTIVE

While many questions remain about the structure and function of cell membranes, one fact is clear: the asymmetric distribution of their lipids cannot be ignored. An asymmetric membrane has many more degrees of freedom, providing access to a wider range of biophysical properties. In that respect, both the steady-state organization of the membrane and any deviations from it can be integrated into cellular function. There exists a large variety of lipids in the PM, and actively maintaining the asymmetry of some may drive the asymmetry of others (Girard and Bereau 2023). A clear example is cholesterol, which can redistribute in response to changes in phospholipid composition and abundance (Varma and Deserno 2022). Further, lipid asymmetry can be released (scrambled), sometimes completely (Segawa and Nagata 2015) (as in apoptosis) but sometimes also in more local-

ized and transient ways (Doktorova et al. 2020). Notably, this release of asymmetry may involve intermediate asymmetric states that have functional consequences (Hammill et al. 1999). Characterizing the compositional states accessible by cells in different contexts and understanding the biophysical principles governing the emerging behavior of asymmetric membranes are critical next steps for deciphering the enigma of PM lipid organization.

ACKNOWLEDGMENTS

We acknowledge funding from the National Institutes of Health, National Institute for General Medical Sciences (NIH/NIGMS) R01GM138887 (F.A.H.), R35GM134949 (I.L.), and F32GM134704 (M.D.). We acknowledge Dr. Joseph Lorent for design of Figure 1A.

REFERENCES

Allender DW, Schick M. 2006. Phase separation in bilayer lipid membranes: effects on the inner leaf due to coupling to the outer leaf. *Biophys J* **91:** 2928–2935. doi:10.1529/biophysj.106.086868

Bennett WD, MacCallum JL, Hinner MJ, Marrink SJ, Tieleman DP. 2009. Molecular view of cholesterol flip-flop and chemical potential in different membrane environments. *J Am Chem Soc* **131:** 12714–12720. doi:10.1021/ja903529f

Bevers EM, Williamson PL. 2016. Getting to the outer leaflet: physiology of phosphatidylserine exposure at the plasma membrane. *Physiol Rev* **96:** 605–645. doi:10.1152/physrev.00020.2015

Bittman R, Rottem S. 1976. Distribution of cholesterol between the outer and inner halves of the lipid bilayer of mycoplasma cell membranes. *Biochem Biophys Res Commun* **71:** 318–324. doi:10.1016/0006-291X(76)90285-0

Blosser MC, Honerkamp-Smith AR, Han T, Haataja M, Keller SL. 2015. Transbilayer colocalization of lipid domains explained via measurement of strong coupling parameters. *Biophys J* **109:** 2317–2327. doi:10.1016/j.bpj.2015.10.031

Bonifacino JS, Lippincott-Schwartz J. 2003. Coat proteins: shaping membrane transport. *Nat Rev Mol Cell Biol* **4:** 409–414. doi:10.1038/nrm1099

Bretscher MS. 1972. Asymmetrical lipid bilayer structure for biological membranes. *Nat New Biol* **236:** 11–12. doi:10.1038/newbio236011a0

Bruckner RJ, Mansy SS, Ricardo A, Mahadevan L, Szostak JW. 2009. Flip-flop-induced relaxation of bending energy: implications for membrane remodeling. *Biophys J* **97:** 3113–3122. doi:10.1016/j.bpj.2009.09.025

Buwaneka P, Ralko A, Liu SL, Cho W. 2021. Evaluation of the available cholesterol concentration in the inner leaflet of the plasma membrane of mammalian cells. *J Lipid Res* **62:** 100084. doi:10.1016/j.jlr.2021.100084

Cheng HT, London E. 2011. Preparation and properties of asymmetric large unilamellar vesicles: interleaflet coupling in asymmetric vesicles is dependent on temperature but not curvature. *Biophys J* **100:** 2671–2678. doi:10.1016/j.bpj.2011.04.048

Cheng HT, Megha, London E. 2009. Preparation and properties of asymmetric vesicles that mimic cell membranes: effect upon lipid raft formation and transmembrane helix orientation. *J Biol Chem* **284:** 6079–6092. doi:10.1074/jbc.M806077200

Chiantia S, London E. 2012. Acyl chain length and saturation modulate interleaflet coupling in asymmetric bilayers: effects on dynamics and structural order. *Biophys J* **103:** 2311–2319. doi:10.1016/j.bpj.2012.10.033

Chiantia S, Schwille P, Klymchenko AS, London E. 2011. Asymmetric GUVs prepared by MβCD-mediated lipid exchange: an FCS study. *Biophys J* **100:** L1–L3. doi:10.1016/j.bpj.2010.11.051

Collins MD, Keller SL. 2008. Tuning lipid mixtures to induce or suppress domain formation across leaflets of unsupported asymmetric bilayers. *Proc Natl Acad Sci* **105:** 124–128. doi:10.1073/pnas.0702970105

Courtney KC, Fung KY, Maxfield FR, Fairn GD, Zha X. 2018a. Comment on "Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol." *eLife* **7:** e38493. doi:10.7554/eLife.38493

Courtney KC, Pezeshkian W, Raghupathy R, Zhang C, Darbyson A, Ipsen JH, Ford DA, Khandelia H, Presley JF, Zha X. 2018b. C24 sphingolipids govern the transbilayer asymmetry of cholesterol and lateral organization of model and live-cell plasma membranes. *Cell Rep* **24:** 1037–1049. doi:10.1016/j.celrep.2018.06.104

Crane JM, Kiessling V, Tamm LK. 2005. Measuring lipid asymmetry in planar supported bilayers by fluorescence interference contrast microscopy. *Langmuir* **21:** 1377–1388. doi:10.1021/la047654w

Devaux PF. 1991. Static and dynamic lipid asymmetry in cell membranes. *Biochemistry* **30:** 1163–1173. doi:10.1021/bi00219a001

Doktorova M, Levental I. 2022. Cholesterol's balancing act: defying the status quo. *Biophys J* **121:** 3771–3773. doi:10.1016/j.bpj.2022.08.036

Doktorova M, Weinstein H. 2018. Accurate in silico modeling of asymmetric bilayers based on biophysical principles. *Biophys J* **115:** 1638–1643. doi:10.1016/j.bpj.2018.09.008

Doktorova M, Heberle FA, Eicher B, Standaert RF, Katsaras J, London E, Pabst G, Marquardt D. 2018. Preparation of asymmetric phospholipid vesicles for use as cell membrane models. *Nat Protoc* **13:** 2086–2101. doi:10.1038/s41596-018-0033-6

Doktorova M, Heberle FA, Marquardt D, Rusinova R, Sanford RL, Peyear TA, Katsaras J, Feigenson GW, Weinstein H, Andersen OS. 2019. Gramicidin increases lipid flip-flop in symmetric and asymmetric lipid vesicles. *Biophys J* **116:** 860–873. doi:10.1016/j.bpj.2019.01.016

Doktorova M, Symons JL, Levental I. 2020. Structural and functional consequences of reversible lipid asymmetry in living membranes. *Nat Chem Biol* **16:** 1321–1330. doi:10.1038/s41589-020-00688-0

Doktorova M, Symons JL, Levental KR, Wang H, Zhang K, Heberle FA, Lyman E, Levental I. 2023. Number asymmetry: the hidden dimension of mammalian plasma membranes. *Biophys J* **122:** 229a. doi:10.1016/j.bpj.2022.11.1352

Eicher B, Marquardt D, Heberle FA, Letofsky-Papst I, Rechberger GN, Appavou MS, Katsaras J, Pabst G. 2018. Intrinsic curvature-mediated transbilayer coupling in asymmetric lipid vesicles. *Biophys J* **114:** 146–157. doi:10.1016/j.bpj.2017.11.009

Elani Y, Purushothaman S, Booth PJ, Seddon JM, Brooks NJ, Law RV, Ces O. 2015. Measurements of the effect of membrane asymmetry on the mechanical properties of lipid bilayers. *Chem Commun (Camb)* **51:** 6976–6979. doi:10.1039/C5CC00712G

Enoki TA, Feigenson GW. 2019. Asymmetric bilayers by hemifusion: method and leaflet behaviors. *Biophys J* **117:** 1037–1050. doi:10.1016/j.bpj.2019.07.054

Enoki TA, Feigenson GW. 2022. Improving our picture of the plasma membrane: rafts induce ordered domains in a simplified model cytoplasmic leaflet. *Biochim Biophys Acta Biomembr* **1864:** 183995. doi:10.1016/j.bbamem.2022.183995

Enoki TA, Wu J, Heberle FA, Feigenson GW. 2021. Investigation of the domain line tension in asymmetric vesicles prepared via hemifusion. *Biochim Biophys Acta Biomembr* **1863:** 183586. doi:10.1016/j.bbamem.2021.183586

Esteban-Martín S, Risselada HJ, Salgado J, Marrink SJ. 2009. Stability of asymmetric lipid bilayers assessed by molecular dynamics simulations. *J Am Chem Soc* **131:** 15194–15202. doi:10.1021/ja904450t

Fadeel B, Xue D. 2009. The ins and outs of phospholipid asymmetry in the plasma membrane: roles in health and disease. *Crit Rev Biochem Mol Biol* **44:** 264–277. doi:10.1080/10409230903193307

Fisher KA. 1976. Analysis of membrane halves: cholesterol. *Proc Natl Acad Sci* **73:** 173–177. doi:10.1073/pnas.73.1.173

Foley SL, Varma M, Hossein A, Deserno M. 2023. Elastic and thermodynamic consequences of lipid membrane asymmetry. *Emerg Top Life Sci* **7:** 95–110. doi:10.1042/ETLS20220084

Frewein MPK, Piller P, Semeraro EF, Batchu KC, Heberle FA, Scott HL, Gerelli Y, Porcar L, Pabst G. 2022. Interdigitation-induced order and disorder in asymmetric membranes. *J Membr Biol* **255:** 407–421. doi:10.1007/s00232-022-00234-0

Fujimoto T, Parmryd I. 2016. Interleaflet coupling, pinning, and leaflet asymmetry—major players in plasma membrane nanodomain formation. *Front Cell Dev Biol* **4:** 155.

Garg S, Rühe J, Lüdtke K, Jordan R, Naumann CA. 2007. Domain registration in raft-mimicking lipid mixtures studied using polymer-tethered lipid bilayers. *Biophys J* **92:** 1263–1270. doi:10.1529/biophysj.106.091082

Girard M, Bereau T. 2023. Induced asymmetries in membranes. *Biophys J* **122:** 2092–2098. doi:10.1016/j.bpj.2022.12.004

Gupta A, Korte T, Herrmann A, Wohland T. 2020. Plasma membrane asymmetry of lipid organization: fluorescence lifetime microscopy and correlation spectroscopy analysis. *J Lipid Res* **61:** 252–266. doi:10.1194/jlr.D119000364

Hale JE, Schroeder F. 1982. Asymmetric transbilayer distribution of sterol across plasma membranes determined by fluorescence quenching of dehydroergosterol. *Eur J Biochem* **122:** 649–661. doi:10.1111/j.1432-1033.1982.tb06488.x

Hammill AK, Uhr JW, Scheuermann RH. 1999. Annexin V staining due to loss of membrane asymmetry can be reversible and precede commitment to apoptotic death. *Exp Cell Res* **251:** 16–21. doi:10.1006/excr.1999.4581

Harayama T, Riezman H. 2018. Understanding the diversity of membrane lipid composition. *Nat Rev Mol Cell Biol* **19:** 281–296. doi:10.1038/nrm.2017.138

Heberle FA, Wu J, Goh SL, Petruzielo RS, Feigenson GW. 2010. Comparison of three ternary lipid bilayer mixtures: FRET and ESR reveal nanodomains. *Biophys J* **99:** 3309–3318. doi:10.1016/j.bpj.2010.09.064

Heberle FA, Marquardt D, Doktorova M, Geier B, Standaert RF, Heftberger P, Kollmitzer B, Nickels JD, Dick RA, Feigenson GW, et al. 2016. Subnanometer structure of an asymmetric model membrane: interleaflet coupling influences domain properties. *Langmuir* **32:** 5195–5200. doi:10.1021/acs.langmuir.5b04562

Hossein A, Deserno M. 2020. Spontaneous curvature, differential stress, and bending modulus of asymmetric lipid membranes. *Biophys J* **118:** 624–642. doi:10.1016/j.bpj.2019.11.3398

Husby ML, Amiar S, Prugar LI, David EA, Plescia CB, Huie KE, Brannan JM, Dye JM, Pienaar E, Stahelin RV. 2022. Phosphatidylserine clustering by the Ebola virus matrix protein is a critical step in viral budding. *EMBO Rep* **23:** e51709. doi:10.15252/embr.202051709

Igbavboa U, Avdulov NA, Schroeder F, Wood WG. 1996. Increasing age alters transbilayer fluidity and cholesterol asymmetry in synaptic plasma membranes of mice. *J Neurochem* **66:** 1717–1725. doi:10.1046/j.1471-4159.1996.66041717.x

Ingólfsson HI, Melo MN, van Eerden FJ, Arnarez C, Lopez CA, Wassenaar TA, Periole X, de Vries AH, Tieleman DP, Marrink SJ. 2014. Lipid organization of the plasma membrane. *J Am Chem Soc* **136:** 14554–14559. doi:10.1021/ja507832e

Kiessling V, Crane JM, Tamm LK. 2006. Transbilayer effects of raft-like lipid domains in asymmetric planar bilayers measured by single molecule tracking. *Biophys J* **91:** 3313–3326. doi:10.1529/biophysj.106.091421

Kobayashi T, Menon AK. 2018. Transbilayer lipid asymmetry. *Curr Biol* **28:** R386–R391. doi:10.1016/j.cub.2018.01.007

Kusumi A, Fujiwara TK, Chadda R, Xie M, Tsunoyama TA, Kalay Z, Kasai RS, Suzuki KG. 2012. Dynamic organizing principles of the plasma membrane that regulate signal transduction: commemorating the fortieth anniversary of Singer and Nicolson's fluid-mosaic model. *Annu Rev Cell DevBiol* **28:** 215–250. doi:10.1146/annurev-cellbio-100809-151736

Lange Y, Slayton JM. 1982. Interaction of cholesterol and lysophosphatidylcholine in determining red cell shape. *J Lipid Res* **23:** 1121–1127. doi:10.1016/S0022-2275(20)38048-2

Lange Y, Dolde J, Steck TL. 1981. The rate of transmembrane movement of cholesterol in the human erythrocyte. *J Biol*

Chem **256:** 5321–5323. doi:10.1016/S0021-9258(19)69200-8

Levental I, Levental KR, Heberle FA. 2020. Lipid rafts: controversies resolved, mysteries remain. *Trends Cell Biol* **30:** 341–353. doi:10.1016/j.tcb.2020.01.009

Leventis R, Silvius JR. 2001. Use of cyclodextrins to monitor transbilayer movement and differential lipid affinities of cholesterol. *Biophys J* **81:** 2257–2267. doi:10.1016/S0006-3495(01)75873-0

Lin Q, London E. 2014. Preparation of artificial plasma membrane mimicking vesicles with lipid asymmetry. *PLoS ONE* **9:** e87903. doi:10.1371/journal.pone.0087903

Lin Q, London E. 2015. Ordered raft domains induced by outer leaflet sphingomyelin in cholesterol-rich asymmetric vesicles. *Biophys J* **108:** 2212–2222. doi:10.1016/j.bpj.2015.03.056

Liu SL, Sheng R, Jung JH, Wang L, Stec E, O'Connor MJ, Song S, Bikkavilli RK, Winn RA, Lee D, et al. 2017. Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol. *Nat Chem Biol* **13:** 268–274. doi:10.1038/nchembio.2268

Lorent JH, Levental KR, Ganesan L, Rivera-Longsworth G, Sezgin E, Doktorova M, Lyman E, Levental I. 2020. Plasma membranes are asymmetric in lipid unsaturation, packing and protein shape. *Nat Chem Biol* **16:** 644–652. doi:10.1038/s41589-020-0529-6

Lu L, Doak WJ, Schertzer JW, Chiarot PR. 2016. Membrane mechanical properties of synthetic asymmetric phospholipid vesicles. *Soft Matter* **12:** 7521–7528. doi:10.1039/C6SM01349J

Malvezzi M, Chalat M, Janjusevic R, Picollo A, Terashima H, Menon AK, Accardi A. 2013. Ca^{2+}-dependent phospholipid scrambling by a reconstituted TMEM16 ion channel. *Nat Commun* **4:** 2367. doi:10.1038/ncomms3367

Marquardt D, Heberle FA, Miti T, Eicher B, London E, Katsaras J, Pabst G. 2017. ^{1}H NMR shows slow phospholipid flip-flop in gel and fluid bilayers. *Langmuir* **33:** 3731–3741. doi:10.1021/acs.langmuir.6b04485

Marsh D. 2013. *Handbook of lipid bilayers*. CRC, Boca Raton, FL.

Miettinen MS, Lipowsky R. 2019. Bilayer membranes with frequent flip-flops have tensionless leaflets. *Nano Lett* **19:** 5011–5016. doi:10.1021/acs.nanolett.9b01239

Mondal M, Mesmin B, Mukherjee S, Maxfield FR. 2009. Sterols are mainly in the cytoplasmic leaflet of the plasma membrane and the endocytic recycling compartment in CHO cells. *Mol Biol Cell* **20:** 581–588. doi:10.1091/mbc.e08-07-0785

Murate M, Abe M, Kasahara K, Iwabuchi K, Umeda M, Kobayashi T. 2015. Transbilayer distribution of lipids at nano scale. *J Cell Sci* **128:** 1627–1638.

Op den Kamp JA. 1979. Lipid asymmetry in membranes. *Annu Rev Biochem* **48:** 47–71. doi:10.1146/annurev.bi.48.070179.000403

Park S, Beaven AH, Klauda JB, Im W. 2015. How tolerant are membrane simulations with mismatch in area per lipid between leaflets? *J Chem Theory Comput* **11:** 3466–3477. doi:10.1021/acs.jctc.5b00232

Putzel GG, Schick M. 2008. Phase behavior of a model bilayer membrane with coupled leaves. *Biophys J* **94:** 869–877. doi:10.1529/biophysj.107.116251

Putzel GG, Uline MJ, Szleifer I, Schick M. 2011. Interleaflet coupling and domain registry in phase-separated lipid bilayers. *Biophys J* **100:** 996–1004. doi:10.1016/j.bpj.2011.01.021

Rickeard BW, Nguyen MHL, DiPasquale M, Yip CG, Baker H, Heberle FA, Zuo X, Kelley EG, Nagao M, Marquardt D. 2020. Transverse lipid organization dictates bending fluctuations in model plasma membranes. *Nanoscale* **12:** 1438–1447. doi:10.1039/C9NR07977G

Risselada HJ, Marrink SJ. 2008. The molecular face of lipid rafts in model membranes. *Proc Natl Acad Sci* **105:** 17367–17372. doi:10.1073/pnas.0807527105

Sakuragi T, Nagata S. 2023. Regulation of phospholipid distribution in the lipid bilayer by flippases and scramblases. *Nat Rev Mol Cell Biol* doi:10.1038/s41580-023-00604-z

Schroeder F, Nemecz G, Wood WG, Joiner C, Morrot G, Ayraut-Jarrier M, Devaux PF. 1991. Transmembrane distribution of sterol in the human erythrocyte. *Biochim Biophys Acta* **1066:** 183–192. doi:10.1016/0005-2736(91)90185-B

Scott HL, Kennison KB, Enoki TA, Doktorova M, Kinnun JJ, Heberle FA, Katsaras J. 2021. Model membrane systems used to study plasma membrane lipid asymmetry. *Symmetry (Basel)* **13:** 1356. doi:10.3390/sym13081356

Segawa K, Nagata S. 2015. An apoptotic "eat me" signal: phosphatidylserine exposure. *Trends Cell Biol* **25:** 639–650. doi:10.1016/j.tcb.2015.08.003

Sezgin E, Levental I, Mayor S, Eggeling C. 2017. The mystery of membrane organization: composition, regulation and roles of lipid rafts. *Nat Rev Mol Cell Biol* **18:** 361–374. doi:10.1038/nrm.2017.16

Sharpe HJ, Stevens TJ, Munro S. 2010. A comprehensive comparison of transmembrane domains reveals organelle-specific properties. *Cell* **142:** 158–169. doi:10.1016/j.cell.2010.05.037

Shevchenko A, Simons K. 2010. Lipidomics: coming to grips with lipid diversity. *Nat Rev Mol Cell Biol* **11:** 593–598. doi:10.1038/nrm2934

Shiomi A, Nagao K, Yokota N, Tsuchiya M, Kato U, Juni N, Hara Y, Mori MX, Mori Y, Ui-Tei K, et al. 2021. Extreme deformability of insect cell membranes is governed by phospholipid scrambling. *Cell Rep* **35:** 109219. doi:10.1016/j.celrep.2021.109219

Simunovic M, Evergren E, Callan-Jones A, Bassereau P. 2019. Curving cells inside and out: roles of BAR domain proteins in membrane shaping and its cellular implications. *Annu Rev Cell Dev Biol* **35:** 111–129. doi:10.1146/annurev-cellbio-100617-060558

Solanko LM, Sullivan DP, Sere YY, Szomek M, Lunding A, Solanko KA, Pizovic A, Stanchev LD, Pomorski TG, Menon AK, et al. 2018. Ergosterol is mainly located in the cytoplasmic leaflet of the yeast plasma membrane. *Traffic* **19:** 198–214. doi:10.1111/tra.12545

St. Clair JR, Wang Q, Li G, London E. 2017. Preparation and physical properties of asymmetric model membrane vesicles. In *The biophysics of cell membranes* (ed. Epand R, Ruysschaert JM), pp. 1–27. Springer, Singapore.

Steck TL, Lange Y. 2012. How slow is the transbilayer diffusion (flip-flop) of cholesterol? *Biophys J* **102:** 945–946; author reply 947–949. doi:10.1016/j.bpj.2011.10.059

Steck TL, Lange Y. 2018. Transverse distribution of plasma membrane bilayer cholesterol: picking sides. *Traffic* **19:** 750–760. doi:10.1111/tra.12586

Steck TL, Ye J, Lange Y. 2002. Probing red cell membrane cholesterol movement with cyclodextrin. *Biophys J* **83:** 2118–2125. doi:10.1016/S0006-3495(02)73972-6

Suzuki J, Umeda M, Sims PJ, Nagata S. 2010. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* **468:** 834–838. doi:10.1038/nature09583

Symons JL, Cho KJ, Chang JT, Du G, Waxham MN, Hancock JF, Levental I, Levental KR. 2021. Lipidomic atlas of mammalian cell membranes reveals hierarchical variation induced by culture conditions, subcellular membranes, and cell lineages. *Soft Matter* **17:** 288–297. doi:10.1039/D0SM00404A

Varma M, Deserno M. 2022. Distribution of cholesterol in asymmetric membranes driven by composition and differential stress. *Biophys J* **121:** 4001–4018. doi:10.1016/j.bpj.2022.07.032

Veatch SL, Keller SL. 2003. Separation of liquid phases in giant vesicles of ternary mixtures of phospholipids and cholesterol. *Biophys J* **85:** 3074–3083. doi:10.1016/S0006-3495(03)74726-2

Verkleij AJ, Zwaal RF, Roelofsen B, Comfurius P, Kastelijn D, van Deenen LL. 1973. The asymmetric distribution of phospholipids in the human red cell membrane. A combined study using phospholipases and freeze-etch electron microscopy. *Biochim Biophys Acta* **323:** 178–193. doi:10.1016/0005-2736(73)90143-0

Verma SK, Leikina E, Melikov K, Gebert C, Kram V, Young MF, Uygur B, Chernomordik LV. 2018. Cell-surface phosphatidylserine regulates osteoclast precursor fusion. *J Biol Chem* **293:** 254–270. doi:10.1074/jbc.M117.809681

Wagner AJ, Loew S, May S. 2007. Influence of monolayer-monolayer coupling on the phase behavior of a fluid lipid bilayer. *Biophys J* **93:** 4268–4277. doi:10.1529/biophysj.107.115675

Wan C, Kiessling V, Tamm LK. 2008. Coupling of cholesterol-rich lipid phases in asymmetric bilayers. *Biochemistry* **47:** 2190–2198. doi:10.1021/bi7021552

Wang Q, London E. 2018. Lipid structure and composition control consequences of interleaflet coupling in asymmetric vesicles. *Biophys J* **115:** 664–678. doi:10.1016/j.bpj.2018.07.011

Whitlock JM, Yu K, Cui YY, Hartzell HC. 2018. Anoctamin 5/TMEM16E facilitates muscle precursor cell fusion. *J Gen Physiol* **150:** 1498–1509. doi:10.1085/jgp.201812097

Williamson JJ, Olmsted PD. 2015. Registered and antiregistered phase separation of mixed amphiphilic bilayers. *Biophys J* **108:** 1963–1976. doi:10.1016/j.bpj.2015.03.016

Williamson JJ, Olmsted PD. 2018. Effects of passive phospholipid flip-flop and asymmetric external fields on bilayer phase equilibria. *Biophys J* **115:** 1956–1965. doi:10.1016/j.bpj.2018.10.003

Zhang Y, Bulkley DP, Xin Y, Roberts KJ, Asarnow DE, Sharma A, Myers BR, Cho W, Cheng Y, Beachy PA. 2018. Structural basis for cholesterol transport-like activity of the Hedgehog receptor patched. *Cell* **175:** 1352–1364.e14. doi:10.1016/j.cell.2018.10.026