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Perspective

The membrane surface as a platform that organizes cellular and biochemical processes

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SUMMARY

Membranes are essential for life. They act as semi-permeable boundaries that define cells and organelles. In addition, their surfaces actively participate in biochemical reaction networks, where they confine proteins, align reaction partners, and directly control enzymatic activities. Membrane-localized reactions shape cellular membranes, define the identity of organelles, compartmentalize biochemical processes, and can even be the source of signaling gradients that originate at the plasma membrane and reach into the cytoplasm and nucleus. The membrane surface is, therefore, an essential platform upon which myriad cellular processes are scaffolded. In this review, we summarize our current understanding of the biophysics and biochemistry of membrane-localized reactions with particular focus on insights derived from reconstituted and cellular systems. We discuss how the interplay of cellular factors results in their self-organization, condensation, assembly, and activity, and the emergent properties derived from them.

INTRODUCTION

Every cell is separated from the extracellular space by the plasma membrane. In addition, membranes encapsulate numerous subcellular compartments to separate biochemical reactions with semi-permeable boundaries while also providing extensive surfaces for proteins to bind to. For example, a typical hepatocyte cell has a volume of about 5,000 μm^3 and contains membranes with a total surface area of approximately 110,000 μm^2.[1] It is, therefore, not surprising that a plethora of biochemical reactions, assemblies, and processes are spatially and temporally coordinated on a membrane surface. The membrane accommodates signaling molecules that are permanently embedded in membranes, such as transmembrane proteins, and transiently associated, membrane-binding proteins that, together, permit both intercellular and intracellular communication. As such, membrane-localized reactions act as signal processing and molecular assembly hubs where information can be written, read, and erased with high fidelity in both space and time.

The membrane surface enables protein interactions that would otherwise be impossible in solution. In this review, we will first explain how the membrane functions as an active signaling scaffold that can initiate and control biochemical reactions. In the following sections, we will use examples to illustrate how membrane-localized reactions orchestrate complex cellular behavior by applying a paradigm of writers, readers, and erasers to help conceptualize what is arising at the membrane; namely, that enzymes pattern the membrane by adding, detecting, and removing marks on its surface.

HOW DOES CONFINEMENT TO A TWO-DIMENSIONAL SURFACE INFLUENCE BIOCHEMICAL REACTIONS?

Enzymes facilitate chemical reactions that, uncatalyzed, would have kinetics incompatible with life. Membranes influence the rates and specificity of these reactions by confining them within a defined volume (volume-confined reactions) or on a surface (surface-confined reactions). Eukaryotic cells are characterized by numerous membrane-encapsulated compartments in which volume-confined reactions take place under conditions distinct from the cytosolic milieu. The influence of volume confinement on biochemical reactions has been extensively reviewed elsewhere[2] and will not be the subject of this article. Here, we focus on how the membrane influences reactions confined to its surface.

Binding of proteins to membranes affects the thermodynamics and kinetics of biochemical interactions in fundamental ways (Figure 1). Assuming a cuboidal hepatocyte with a side length of 20 μm,[4] binding to the inner leaflet of the plasma membrane confines a protein to an area of around 2,400 μm^2 and into a thin layer of about 5 nm below the membrane. The accessible volume is, therefore, reduced from 8,000 μm^3 to only 12 μm^3, increasing the effective local concentration of reactants by a factor of more than 600 (Figure 1A). The presence of diffusion barriers or the formation of protein assemblies can laterally confine the reaction partners further. The implications for protein-protein interactions are significant. In solution, reactants are often present only at nanomolar concentrations, whereas their dissociation constants (K_D) are in the micromolar range. Confining soluble proteins in such a small volume dramatically increases the

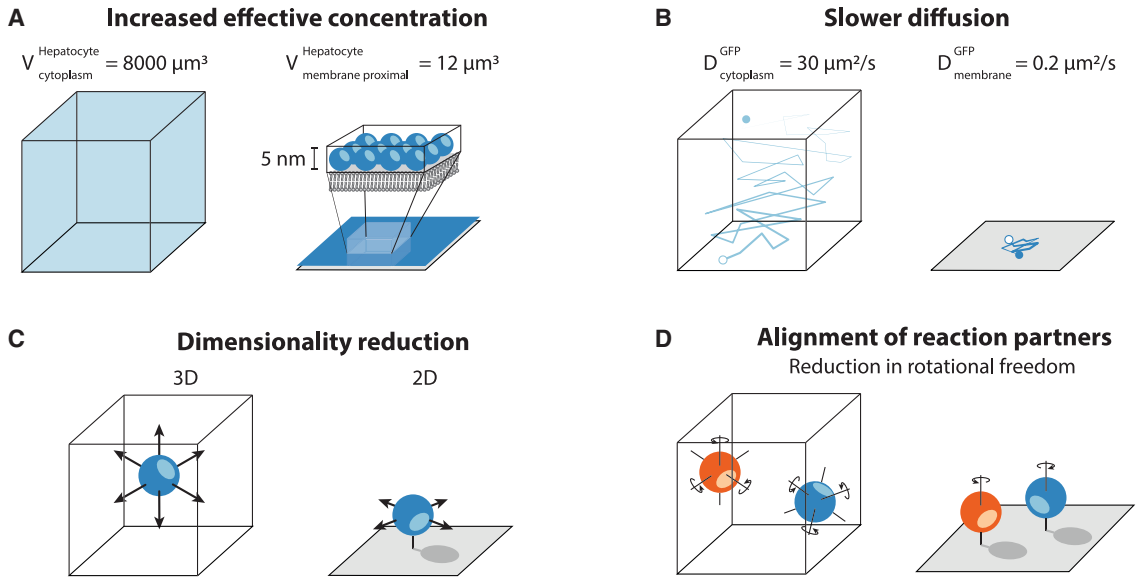


Figure 1. How does confinement to a surface influence biochemical reactions?
(A) By confining a protein into a thin layer ~5 nm from the surface, membrane binding dramatically increases the concentration of reactants.
(B) Although the characteristic diffusion coefficient of GFP in the eukaryotic cytoplasm is approximately 30 $\mu m^2/s$,[3] it is reduced by ~100-fold on the membrane.
(C) Membrane binding not only reduces lateral mobility, but also limits the rotational degrees of freedom.
(D) The combination of spatial and rotational confinements helps to align reaction partners and, thereby, lowers the entropic cost of their interactions.

apparent affinity and specificity, enabling protein interactions impossible in solution.[5]

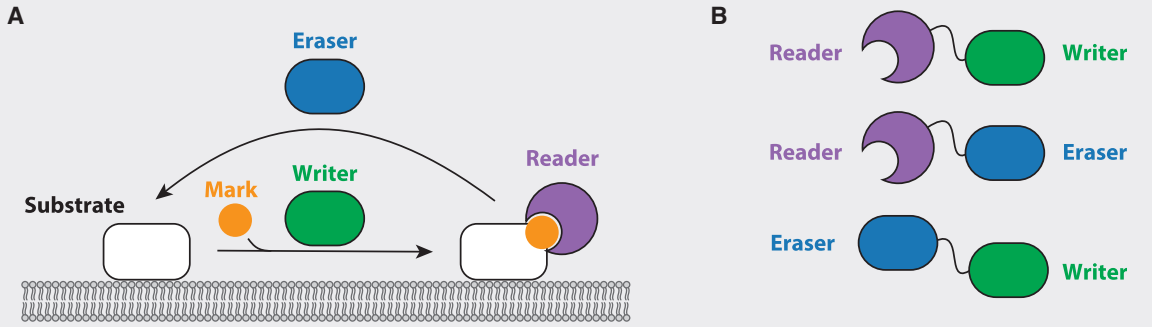
The reduction of dimensionality can speed up biochemical reactions not only due to an increase in the thermodynamic stability of protein complexes, but also because the encounter probability between proteins is density dependent, which can be significantly enhanced on a membrane surface (Figure 1B).[6] In fact, there is a chance that mobile reactants never run into each other in three dimensions, whereas they will eventually encounter each other if bound to the same two-dimensional (2D) surface (unless they are separated by physical barriers).[7,8] At the same time, membrane binding typically slows down protein diffusion by a factor of 100, reducing encounter rates. In extreme cases, reactants could even be effectively immobilized on the membrane and the reaction rate will tend to zero.[5] An enhancement of the encounter rate is, therefore, unlikely to be the only reason for faster reactions. In addition to increased local concentrations, membrane confinement also accelerates biochemical reactions by aligning reaction partners.[6] As the rotational motion of a protein is significantly restricted to the axis perpendicular to the membrane, the entropic cost of protein interactions is significantly lowered (Figures 1C and 1D).

If reaction partners reversibly bind to the membrane, the kinetic enhancement also depends on the time the proteins spend on the membrane: for fast binding rates, the combination of dimensionality reduction and an increase in effective concentration allows proteins to react more quickly, whereas the rate of membrane association can become rate limiting for slow binding reactions.[9]

Membrane binding also affects the emergent properties of biochemical networks, in which many proteins interact to exhibit collective behavior. Examples of soluble enzymes acting on membrane-bound substrates include kinases, phosphatases, guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). As these enzymes can be retained on the membrane, the outcome of the biochemical reaction can significantly change. First, localization to a 2D surface can facilitate processive enzymatic activity, where an individual enzyme catalyzes the modification of many substrates.[10–12] Second, enzymes might be recruited by their membrane-bound product via a secondary binding site. In this case, membrane recruitment not only promotes processivity but also gives rise to a positive feedback loop that promotes ultrasensitive behavior.[10,13,14] Here, small changes in enzyme concentration can give rise to a dramatic, switch-like transition between two different activity states. For example, a biochemical system composed of a reversibly binding protein and its regulatory enzymes can collectively switch between its off-state, where most proteins are in solution, and its on-state, where the activating enzyme dominates the system and most proteins are membrane bound. Strong, nonlinear positive feedback can also give rise to bi-stability, in which the system has two stable steady states and shows hysteretic behavior (see glossary in Supplemental Information). In extreme cases, the biochemical network can give rise to irreversible transitions, where the system is trapped in one activity state.[10,15–17] In this case, the positive feedback is so strong that it cannot be outcompeted by the enzyme that catalyzes the reverse reaction.[18] These kind of emergent properties of biochemical networks on membrane surfaces likely play an important role in determining the directionality of vesicle trafficking pathways.[19]

By contrast, in the case of multi-step phosphorylation cascades, membrane localization normally suppresses bi-stability.[20–24] The decreased mobility and high density of the substrate on the membrane facilitate enzyme rebinding, promoting a processive phosphorylation mechanism, which is incompatible with bi-stability.[25] However, as suggested for the multisite phosphorylation of the CD3ξ subunit of the T cell receptor

Box 1. The Writer/Reader/Eraser paradigm for signaling systems



(A) In signaling systems, writers add marks to substrates, such as other proteins or lipids. These post-translational modifications are recognized by reader proteins and can be subsequently removed by erasers. The opposing activities of writers and erasers can generate spatially defined and time-limited biochemical patterns on the cell membrane that are recognized and interpreted by readers. In GTPase signaling, writers, readers, and erasers have also been referred to as activators, effectors, and inactivators, respectively.

(B) The writer, reader, and eraser activities are localized to individual protein domains, which can be combined together in a particular protein or within a protein complex. Functional coupling of activities can result in complicated biochemical networks with myriad inputs and feedback control mechanisms.

(TCR) by the Src family kinase Lck, a switch-like transition in signaling cascades is still possible on membrane surfaces.[26] This theoretical study has proposed that a short refractory period of the enzyme after substrate phosphorylation could maintain the distributed, ultrasensitive mechanism. How membrane binding actually affects the emerging properties of signaling networks must, therefore, be tested empirically.

Spatiotemporal protein patterns commonly emerge from the highly cooperative binding of proteins and their reduced mobility on the membrane surface. For example, the bacterial cell division proteins MinD and MinE self-organize into protein oscillations *in vivo*[27,28] and traveling waves *in vitro*.[27] These spatiotemporal patterns can only emerge because the mobility of membrane-bound proteins is sufficiently decreased. By contrast, their fast diffusion in solution rapidly disperses the unbound proteins.[27,28] Similarly, the polarized distribution of activated Cdc42 necessitates slow diffusion on the plasma membrane to constrain proteins within a small surface area.[29]

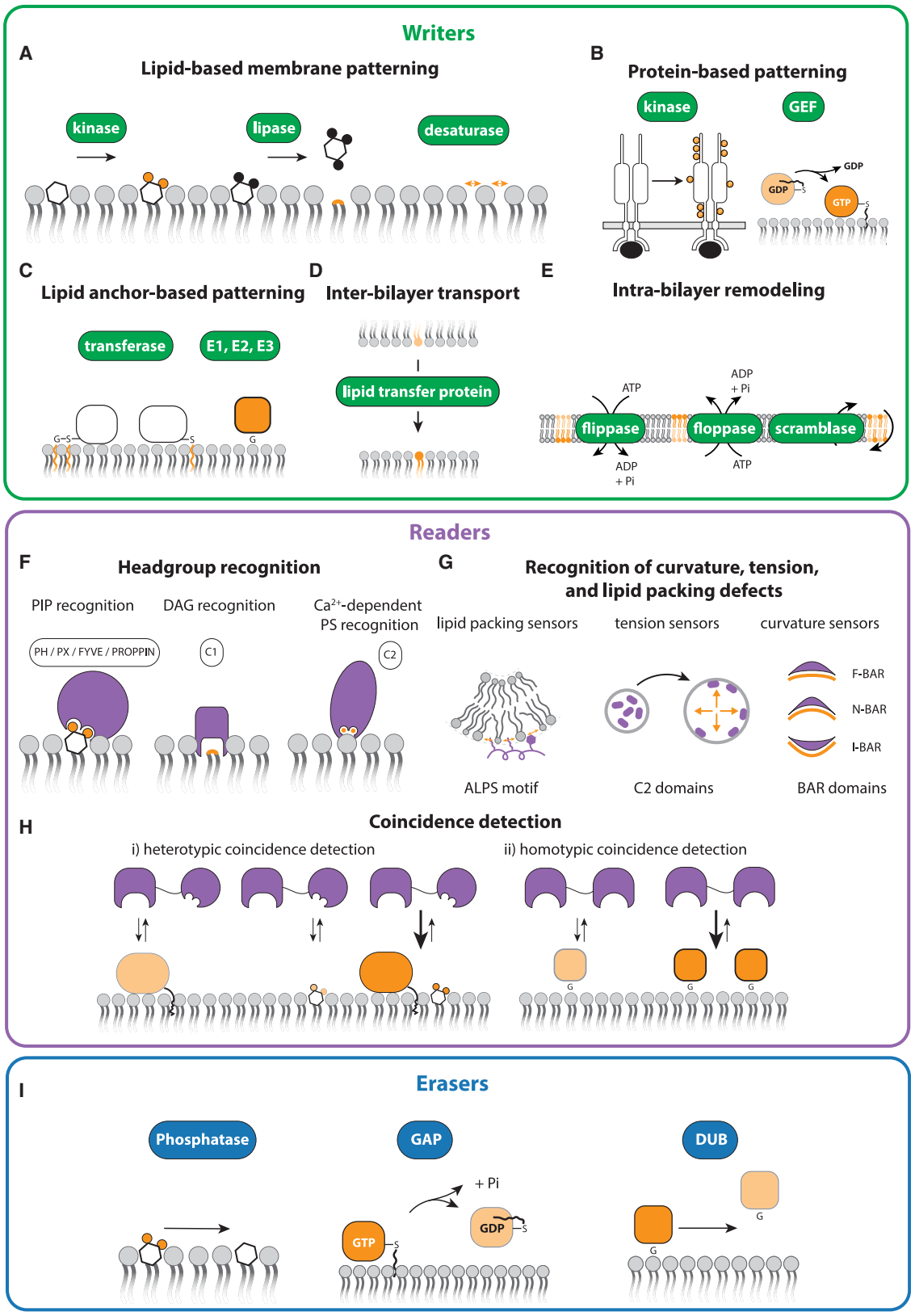
THE READER/WRITER/ERASER PARADIGM IN THE CONTEXT OF MEMBRANE-LOCALIZED PROTEIN INTERACTIONS

Basic cellular processes depend on the precise execution of molecular programs in space and time. The fundamental logic of these reactions can be understood by using the writer/reader/eraser paradigm (Box 1A). Originally introduced to characterize the histone code,[30] this paradigm has been successfully applied to many other cellular signaling networks.[31,32] Accordingly, enzymes that modify the membrane surface by adding marks will be referred to as *writers*, whereas proteins that detect and bind to membrane surface marks will be referred to as *readers*. Finally, enzymes that remove membrane surface marks will be considered as *erasers*. Under certain circumstances, erasers that generate a new mark by removing another mark could also be considered as writers. The counteracting activities of writers and erasers control membrane surface-based marks in a time- and space-dependent manner. Readers then recognize and interpret these time-limited and spatially defined marks on the membrane surface. These functions can be part of the same protein or of a protein complex, allowing for the functional coupling of enzymatic activities (Box 1B).

WRITERS: HOW MEMBRANES ARE PATTERNED IN CELLS

The membrane surfaces of individual intracellular compartments comprise unique combinations of lipids and proteins. These biochemical identities, or 'zip codes,' are generated by writers whose activity in space and time is precisely controlled. With more than 1,000 lipid species and nearly 24,000 proteins, of which approximately 6,500 are integral membrane proteins[33,34] and a further 3,500 predicted to bind membranes,[35] human cells have evolved an enormous arsenal of tools to provide unique biochemical identities.

In the cell, the activities of different writers are often functionally coupled in a precise and highly dynamic manner.[36] For example, phosphoinositide kinases phosphorylate the hydroxyl groups of the inositol ring of phosphatidylinositols (PIs) at specific positions (Figure 2A). The dynamic interconversion of phosphoinositides (PIPs) controls membrane trafficking in the endolysosomal pathway[37] as well as cell polarity.[38] For a comprehensive overview of the roles played by PIPs in scaffolding biological processes at discrete locations in the cell


Writers
A
Lipid-based membrane patterning
kinase
lipase
desaturase
B
Protein-based patterning
kinase
GEF
GDP
GTP
C
Lipid anchor-based patterning
transferase
E1, E2, E3
D
Inter-bilayer transport
lipid transfer protein
E
Intra-bilayer remodeling
ATP
ADP + Pi
flippase
floppase
scramblase
Readers
F
Headgroup recognition
PIP recognition
DAG recognition
Ca2+-dependent PS recognition
PH / PX / FYVE / PROPPIN
C1
C2
G
Recognition of curvature, tension, and lipid packing defects
lipid packing sensors
tension sensors
curvature sensors
F-BAR
N-BAR
I-BAR
ALPS motif
C2 domains
BAR domains
H
Coincidence detection
i) heterotypic coincidence detection
ii) homotypic coincidence detection
Erasers
I
Phosphatase
GAP
DUB
+ Pi
GTP
GDP

with high temporal resolution, we refer readers to an excellent review.[39]

In addition to lipid kinases, lipases can pattern membranes by specifically removing lipid headgroups. For example, phospholipase C (PLC) hydrolyses the headgroup of phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$), generating diacylglycerol (DAG). For an in-depth treatment of signal integration, transduction, and regulation of the six mammalian PLC families, we refer the reader to an excellent review.[40] Furthermore, the membrane can be rendered more prone to binding by proteins that partially insert into the bilayer by the action of lipid desaturases (Figure 2A).

Together with PIPs, membranes are also identified by their protein content. For example, protein tyrosine kinases mark receptor tails with phosphotyrosine, creating docking motifs for a plethora of adaptor proteins and effector kinases. The activity of GEFs leads to the membrane association of small GTPases of the Rab family due to their post-translational lipid modifications. These molecular switches can bind reversibly to the membrane and act as important markers of endomembranous cell compartments (Figures 2B and 2C). The compartment specificity of these GTPases is largely governed by their cognate GEFs (Figure 2B).[41] All known GEFs are peripheral membrane proteins themselves[42] with ancillary reader domains that often recognize molecular marks or interaction partners that regulate their nucleotide exchange activity via the allosteric control of autoinhibition.[43] These complex biochemical networks can also include non-linear feedback controls.[19] Indeed, reconstitution experiments with Rab5, its exchange factor Rabex5, and effector Rabaptin5, on supported lipid bilayers showed that this minimal protein system includes a positive feedback loop that gives rise to an ultrasensitive, collective activation and spatial patterning of Rab5 on the membrane.[17,44]

The activity of writers can also be functionally coupled to give rise to signaling cascades. For example, during the maturation of early endosomes along the endolysosomal pathway, the activity of Rabex5:Rabaptin5 is followed by that of the Rab7 GEF complex Mon1-Ccz1.[45] As a result, Rab5 is replaced by Rab7 in a process termed Rab conversion.[45] *In vitro* experiments have demonstrated that Mon1-Ccz1 recognizes a combination of membrane-bound Rab5-GTP and phosphatidylinositol 3-phosphate (PI(3)P), which, together, enhance their activity toward Rab7.[46] The subsequent down-regulation of PI(3)P synthesis by WDR91, a Rab7 effector, is essential for endolysosomal trafficking and neuronal development in mice.[47] During polarized cargo trafficking from the Golgi, the generation of GTP-bound ARF6 drives the recruitment of the lipid kinase phosphatidylinositol 4-phosphate 5-kinase type-1 gamma (PIP5K1C), which mediates the conversion of phosphatidylinositol 4-phosphate (PI(4)P) into $PI(4,5)P_2$ and the consequent tethering of cargo vesicles to the plasma membrane by the exocyst complex.[48]

Protein myristoylation, prenylation, or palmitoylation is yet another way in which the surfaces of membranes can be patterned[49] (Figure 2C). For the GTPases ARF6 and Ras, the differential intracellular distribution of modifying enzymes can establish a spatial organizing system that contributes to a defined distribution of lipidated proteins on different organelles.[50,51] In addition, the ubiquitin-like protein ATG8 is conjugated to phosphatidylethanolamine (PE), a reaction that is referred to as lipidation and is accomplished by a complex of E1-, E2-, and E3-like enzymes, similar to ubiquitylation.[52] The recruitment of the ATG12-ATG5-ATG16L1 E3-like complex to the nascent phagophore during macro-autophagy is mediated by a subset of the PI(3)P-binding β-propeller WIPI proteins of the PROPPIN family,[53] connecting the class III phosphatidylinositide 3-kinase (PI3K)-mediated synthesis of PI(3)P, which itself is dependent on WIPI2 in a positive feedback loop,[54] to the lipidation of ATG8 with PE.[55] ATG8 can also undergo conjugation to phosphatidylserine (PS) in pathways that are different from macro-autophagy.[56] The marking of the nascent phagophore with ATG8 provides a template for the interaction of cargo adapters and receptors and eventual encapsulation of the cargo for targeting to the lysosome. The ATG8-interacting motif, also referred to as the LC3-interacting region (LIR), drives the recruitment of interaction partners to ATG8-positive membranes.[57] Recently, it was shown that ubiquitin as well as the ubiquitin-related proteins NEDD8 and ISG15 can also be directly conjugated to the headgroup of PE.[58]

Although vesicular trafficking inevitably leads to the redistribution of lipids within cells, the majority of lipids reach their destination via lipid transfer proteins (Figure 2D).[59] Patterning of cellular membranes can also be accomplished by integral membrane

Figure 2. Writing, reading, and erasing the membrane code

Writers

(A) Lipid-based membrane patterning can consist of the kinase-mediated phosphorylation of the headgroup of phosphatidylinositol (PI); the lipase-mediated hydrolysis of headgroups, for example, to generate diacylglycerol (DAG); or the desaturation of lipids to increase inter-lipid spacing.

(B) Protein tyrosine kinases catalyze the phosphorylation of specific tyrosine residues in the cytoplasmic tails of receptors, creating docking sites for adaptor and effector proteins. GEFs catalyze the exchange of GDP for GTP in Arf, Ras, and Rab family GTPases, thereby promoting their membrane binding.

(C) Myristoyl-, palmitoyl-, farnesyl-, and geranylgeranyltransferases modify proteins such as Arf, Ras, and Rab family GTPases, allowing them to bind to specific domains in membranes. E1, E2, and E3(-like) enzymes mediate the conjugation of ubiquitin(-like) enzymes to lipid headgroups.

(D) Lipid transfer proteins transport lipids between different membranes and can, thereby, add or remove signaling lipids.

(E) Flippases, floppases, and scramblases facilitate the inter-leaflet transport of lipids and can, therefore, change the chemical nature of the membrane surface.

Readers

(F) Signaling lipids such as phosphorylated forms of PI are recognized by PH, PX, and FYVE domains or the PROPPINs, whereas C1 domains bind to DAG. C2 domains recognize phosphatidylserine (PS)-rich membranes, frequently in a Ca^{2+}-dependent manner (orange dots indicate Ca^{2+} ions).

(G) Amphipathic lipid packing sensors (ALPS) sense hydrophobic defects in the membrane, some C2 domains are able to sense membrane tension, and different degrees and types of membrane curvature can be recognized by F-, N-, or I-BAR domains.

(H) Frequently, marks on the membrane are read out in a combinatorial manner by coincidence detection, where the simultaneous presence of two marks results in more efficient recruitment of a protein to the membrane (i). A variation of this principle is the detection of a single signal by multivalent interactions (ii).

Erasers

(I) Membrane marks can be removed by erasers such as phosphoinositide phosphatases, which dephosphorylate PIPs, by GTPase-activating proteins such as Arf and Rab GAPs, which convert these GTPases to their GDP-bound state and, thereby, facilitate their dissociation from the membrane, as well as by proteins having deubiquitinase(-like) activities, which release ubiquitin family proteins from the membrane.

proteins that facilitate the intra-bilayer reorganization of lipids and their derivatives (Figure 2E). These proteins include the following: flippases, which drive the ATP-dependent movement of specific phospholipids from the extracellular leaflet of the plasma membrane to the cytoplasmic face; floppases, which catalyze phospholipid movement in the opposite direction; and scramblases, which randomize the distribution of lipids in the bilayer in an ATP-independent manner.[60]

READERS: HOW ARE MEMBRANE SURFACES AND PATTERNS RECOGNIZED?

The membrane surface is recognized by readers, proteins that bind to specific lipids and proteins or their combinations. Readers, therefore, decode the biochemical identity of membranes and localize enzymatic activities in a time- and space-dependent manner.

The arrangement of chemically diverse lipids with dynamically adjusted stoichiometries in a membrane provides innumerable features that can be read out by the protein machinery of the cell. At the simplest level, membrane-binding domains bind to lipid headgroups in a stereospecific manner (Figure 2). The known repertoire of membrane-binding domains that recognize lipid headgroups include the PH, PX, and FYVE domains as well as the PROPPINs that typically bind phosphorylated PIPs and C1 and C2 domains that recognize DAG and PS, respectively[61] (Figure 2F).

The presentation of the lipid headgroup on a crowded surface confers additional binding specificity. As such, membrane-binding domains that bind to the same headgroup can localize to distinct membranes within the cell. This is particularly evident for the C1 domains of protein kinase C (PKC). Conventional PKCs typically recognize DAG in the plasma membrane,[62] whereas the C1 domains of the novel PKCs and the PKDs localize to DAG pools in the *trans*-Golgi network (TGN).[63,64] The specific subcellular localization of DAG-binding C1 domains is likely driven by a combination of protein-protein interactions; coincident headgroup recognition in the interfacial region[65]; and the specific molecular surface of the domain, which penetrates the hydrophobic interior of the bilayer.[66]

Lipid packing impacts the presentation of headgroups and, consequently, membrane surface topology (Figure 2G). So-called packing defects can be recognized by specific protein motifs, including the ALPS (amphiphathic lipid packing sensor) motif. The ~40-amino-acid-long ALPS motif is unstructured in solution but folds into a stable amphipathic helix (AH), which inserts hydrophobic residues into one face of highly curved membranes.[67] Curvature-sensing AH motifs have later been identified in the vesicle-tethering Golgin protein GMPA210, the nucleoporin Nup133, and the Osh4p sterol transport protein.[68] An AH in kinesin-1 has recently been proposed to drive the coincident detection of anionic lipids and cargo adaptors to drive lysosome positioning,[69] whereas an AH in the transcriptional repressor Opi1, which orchestrates lipid metabolism, recognizes phosphatidic acid (PA) in the ER.[70] Recent work has also implicated the role of membrane tension in modulating the membrane binding of individual C2 domains,[71] adding yet another layer of control (Figure 2G).

The encapsulation of biochemical processes requires membranes of varying degrees of curvature, from enveloped viruses and endocytic vesicles with very high degrees of curvature to Golgi membranes that are composed of flattened stacks of membranes with highly curved tips. Curvature is, therefore, a common topological feature of membranes that can be recognized or induced by proteins (Figure 2G). The first curvature sensors identified were those of the F-, N- and I-BAR family of proteins,[72] which recognize different degrees and types of membrane curvature.[61] The fusion of synaptic vesicles with the plasma membrane during the exocytosis of neurotransmitter is driven by C2 domains in synaptotagmin-1, which induce a high degree of membrane curvature and, thereby, lower the energy barrier to membrane fusion.[73,74] The $PI(4,5)P_2$-mediated adsorption of the caveolae coat protein, Cavin1, and subsequent membrane penetration of its trimeric helical region 1 (HR1) domain has also been proposed to potentiate membrane curvature generation and drive the recruitment of Caveolin1.[75]

Membrane marks are often read out in a combinatorial manner, and the coincident detection of two or more marks increases the specificity and affinity of interactions (Figure 2H). For example, if a binding partner interacts with two membrane-localized signals with a K_D of 10 μM each, then the overall K_D of its interaction with the membrane is, in theory, 0.1 nM[76] (in practice this value is usually higher due to steric and entropic constraints). The binding of homo-oligomers to homotypic surface patterns is a special case of coincident detection in which a single signal is detected with high affinity by multivalent interactions. For example, the early endosomal autoantigen 1 (EEA1) uses its homodimeric FYVE domains to tether PI(3)P-positive early endosomes to Rab5-positive endocytic vesicles.[77] Multivalent homotypic and heterotypic binding interactions can also exhibit cooperativity in their binding.[78] Importantly, the requirement of coincident signals for productive interactions is akin to logical AND gates. In electronic circuits, an AND gate generates an output only when all inputs are detected. In the biological context, the protein will only be weakly activated by individual inputs, whereas the integration of multiple inputs leads to a strong response.[78,79]

ERASERS: HOW MARKS ARE REMOVED FROM THE MEMBRANE

In order to terminate biochemical signals on the membrane surface, cells must be able to erase the marks generated by the writers (Figure 2I). Eraser proteins, therefore, allow membrane-based processes to be specific, reversible, and processive. In combination with writers and readers, the counteracting biochemical activities of eraser proteins also allow for a rapid response of the biochemical systems upon the removal of a signal.[80]

In endocytosis, phosphatases erase the signals of various PIPs, including $PI(4,5)P_2$, $PI(3,4)P_2$, and PI(4)P, which is required to allow for membrane budding, scission, uncoating, and fusion with endosomes.[39] In the attenuation of growth factor signaling, the phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$) signal is reversed to $PI(4,5)P_2$ by the phosphatase and tensin homolog (PTEN).[81] Different Rab proteins are sequentially activated and inactivated to allow the regulated maturation of organelles.[19] For instance, Gyp1, a GAP for the ER-localized Rab Ypt1, is an

effector of the downstream GTPase Ypt32.[82] A similar principle may be applied to the conversion of Rab5 to Rab7.[19,83,84] Another example is provided by Arf GTPases during COPI vesicle formation. Here, Arf1 has to be inactivated and removed from the membrane to allow the vesicle's uncoating, transport, and subsequent fusion with its target compartment.[85] The targeting of ArfGAP is coupled with vesicle biogenesis via its curvature-sensitive binding to the membrane.[85] The dynamic localization of the neuronal protein ADAP1 is controlled by $PI(3,4,5)P_3$ in concert with the motor protein KIF13B. $PI(3,4,5)P_3$ binding to ADAP1 in the tip of the growing axon both dissociates ADAP1 from KIF13B and activates its GTPase activity against ARF6, thereby locally inactivating it and promoting dendrite branching.[86]

On a more fundamental level, the activity of erasers is needed to counteract the activities of writers to prevent the spread of membrane marks throughout the cell, which would render the signal non-specific. This is illustrated in cells in which Atg4, the DUB that removes lipidated Atg8 from the membrane, is deleted. In these cells, Atg8 proteins eventually mark multiple membrane compartments in addition to dedicated autophagic membranes.[87] The DUB Doa4 removes the ubiquitin conjugated to PE in late endosomes.[58] Similarly, for the reconstituted Rab5-Rabex5:Rabaptin5 activation network, it was found that the GAP RabGAP-5 suppresses homogeneous Rab5:GTP accumulation on the membrane surface. Instead, accelerated GTP hydrolysis limits Rab5 spreading and activation waves.[17] In a negative feedback reaction, phospholipase C beta (PLCβ) limits its own activity at the plasma membrane by stimulating GTP hydrolysis in the heterotrimeric G-protein subunit $G\alpha_q$, thereby triggering its own dissociation from its membrane anchor.[88]

HOW DO MEMBRANES INFLUENCE THE BIOCHEMICAL ACTIVITY OF PROTEINS?

Every biochemical reaction is fundamentally limited by the intrinsic rate of the reaction under physiological conditions at saturating substrate concentration. The turnover numbers of enzymes can vary greatly, from 10^4 to 10^6 molecules min^{-1} for the fastest known enzymes to just 30 molecules min^{-1} for some of the slowest.[89] Signaling enzymes, such as kinases and small GTPases, typically exhibit turnover rates orders of magnitude lower than diffusion-limited enzymes and are often regulated allosterically. The acquisition of the correct geometry for phosphorylation[90] and the local concentration of reaction partners are, therefore, essential for catalysis.

The activity of many protein kinases is acutely regulated on membranes (Figure 3). At the simplest level, the membrane provides a platform upon which biochemical reactions are restricted to a fixed distance from the membrane. This is perhaps best exemplified by the myotonic dystrophy protein kinases (DMPKs) that regulate actomyosin contraction in the cell cortex, in which the catalytic kinase domains are separated from the membrane-binding domains by a coiled-coil domain evolutionarily conserved in length (Figure 3A).[91,92]

The generation and turnover of lipid second messengers in the membrane can offer an additional, allosteric layer of regulation. The PI3K pathway is directly coupled with the activation of growth factor receptors in the plasma membrane. Class I PI3Ks, recruited to activated receptors, phosphorylate $PI(4,5)P_2$ to $PI(3,4,5)P_3$, thereby writing a new mark on the membrane. At the apex of the PI3K-elicited signaling cascade is activation of the 'master' kinase, phosphoinositide-dependent kinase 1 (PDK1), by $PI(3,4,5)P_3$. PDK1 is maintained in an inactive conformation in the cytosol in which its $PI(3,4,5)P_3$-binding (reader) PH domain is sequestered in an autoinhibitory interaction with its kinase domain.[93] $PI(3,4,5)P_3$-binding elicits not only the displacement of the PH domain from the kinase domain but also licenses the dimerization and trans-autophosphorylation of its kinase domains (Figure 3B). Sequestration of the dimerization interface of PDK1 ensures that promiscuous autoactivation is unlikely to occur in the absence of $PI(3,4,5)P_3$. Simultaneous sequestration of the lipid-binding pocket also establishes a competition between the membrane and the kinase domain for binding, thereby setting a threshold $PI(3,4,5)P_3$ concentration required for activation. Full-length PDK1, therefore, exhibits strong positive cooperativity in binding to $PI(3,4,5)P_3$, leading to switch-like activation.[93] Trans-autophosphorylation alone, however, is insufficient to convert PDK1 into an active conformation,[93] which depends explicitly on $PI(3,4,5)P_3$ or $PI(3,4)P_2$. In this sense, the membrane and signaling lipid are the ultimate regulators of activity.

Together with PDK1, the pro-growth and survival kinases Akt and Sgk3 are the primary effectors of PI3K signaling. Similar to PDK1, Akt and Sgk3 are characterized by a membrane-binding reader domain followed by a writer kinase domain. Although Akt is allosterically activated by both $PI(3,4,5)P_3$ and $PI(3,4)P_2$[94–96] (Figure 3C), serum- and glucocorticoid-regulated kinase 3 (Sgk3) is activated exclusively by the endosomal lipid PI(3)P in an analogous manner.[97] A large proportion of endosomal PI(3)P originates from the membrane-localized lipid phosphatases SH2-domain-containing 5-phosphatase 2 (SHIP2) and inositol polyphosphate 4-phosphatase (INPP4), erasers that double as writers, sequentially converting class-I-PI3K-derived $PI(3,4,5)P_3$ into the new marks $PI(3,4)P_2$ and PI(3)P.[98–100]

Other regulatory principles are exemplified by the Tec kinases, critical regulators of immune cell signaling. Bruton's tyrosine kinase (Btk) serves to illustrate the concept of coincidence detection. In addition to a $PI(3,4,5)P_3$-sensitive PH domain, Btk contains a conserved module of Src homology 3 (SH3), Src homology 2 (SH2), and kinase domain (for a recent review of the ancient Src-like module, we refer readers to Shah et al.[101]). In the example of Btk, kinase activation depends not only on $PI(3,4,5)P_3$ but also on the sequential recognition of phosphotyrosine in the cytoplasmic tail of the receptor by its SH2 domain and a polyproline motif by its SH3 domain (Figure 3D). Similar to PDK1, lipid-mediated Btk dimerization on the membrane surface has been proposed to facilitate trans-autophosphorylation.[102] Perturbations of the Btk gene, which is located on the X chromosome, are the cause of X-linked agammaglobulinemia (XLA), a disease characterized by the absence of circulating antibodies and a susceptibility to infection.[103,104] Not surprisingly, all of the regulatory mechanisms described above have been observed to be perturbed by XLA-associated mutations.[105]

Specific, lipid-mediated allosteric activation of protein kinases can also be accompanied by their recruitment by small GTPases. Son-of-sevenless (SOS)-dependent activation of Ras, for example, creates a membrane-anchored mark for the recruitment of the effector kinase Raf. In the absence of activated

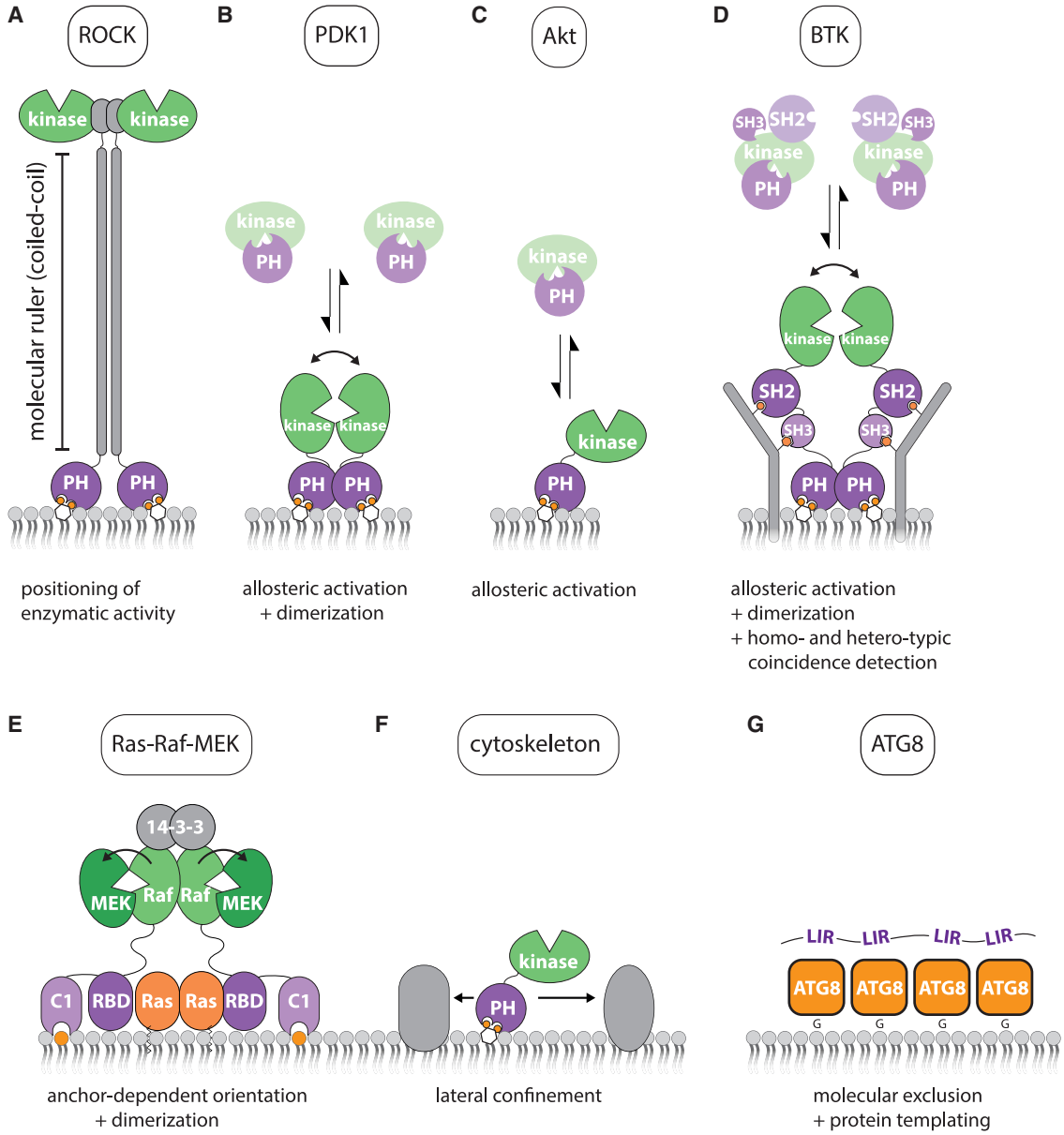


Figure 3. How do membranes influence the biochemical activity of proteins?

(A) Recruitment to the membrane surface can position a catalytic activity of an enzyme, here shown for the ROCK kinase, at a fixed distance. This distance is controlled by the length of a semi-rigid coiled-coil domain connecting the kinase (green) and membrane-binding domains.

(B) Membrane binding can release intramolecular inhibitory interactions by reorienting the membrane-binding domain such as in Akt.

(C and D) (C) Allosteric activation by the release of autoinhibition can be coupled with the promotion of dimerization and transactivation by the clustering of signaling lipids on the surface, as exemplified by the PDK1 kinase. (D) An extension of this principle is the additional coincidence detection of a signaling lipid and the phosphorylated cytoplasmic tails of a transmembrane receptor shown here for the kinase BTK.

(E) The membrane surface can also serve to orient reaction partners relative to each other as shown for the Raf kinase, recruited by Ras (orange), and its substrate kinase MEK.

(F) Membrane-localized reactions can be laterally confined by diffusion barriers, as for example by the actin cytoskeleton.

(G) At high densities, membrane marks such as ATG8 family proteins can exclude other molecules and template macromolecular assemblies.

Ras, Raf exists as an inactive monomer.[106,107] Displacement of its membrane-binding cysteine-rich domain (CRD) upon Ras binding to the Ras-binding domain (RBD) leads to the assembly of an active, back-to-back dimer of the Raf kinase domain,[106] which then propagates the signal by phosphorylating the next kinase in the pathway, MEK (Figure 3E). Accumulating evidence suggests that Ras itself may dimerize on the membrane,[108–112] although this is still actively debated in the field.[113] Presumably, Ras dimerization or clustering would stabilize Raf binding to the membrane through avidity effects. In summary, the active conformation of Ras is intimately tied to its membrane localization and, thereby, its capacity to engage downstream effectors.

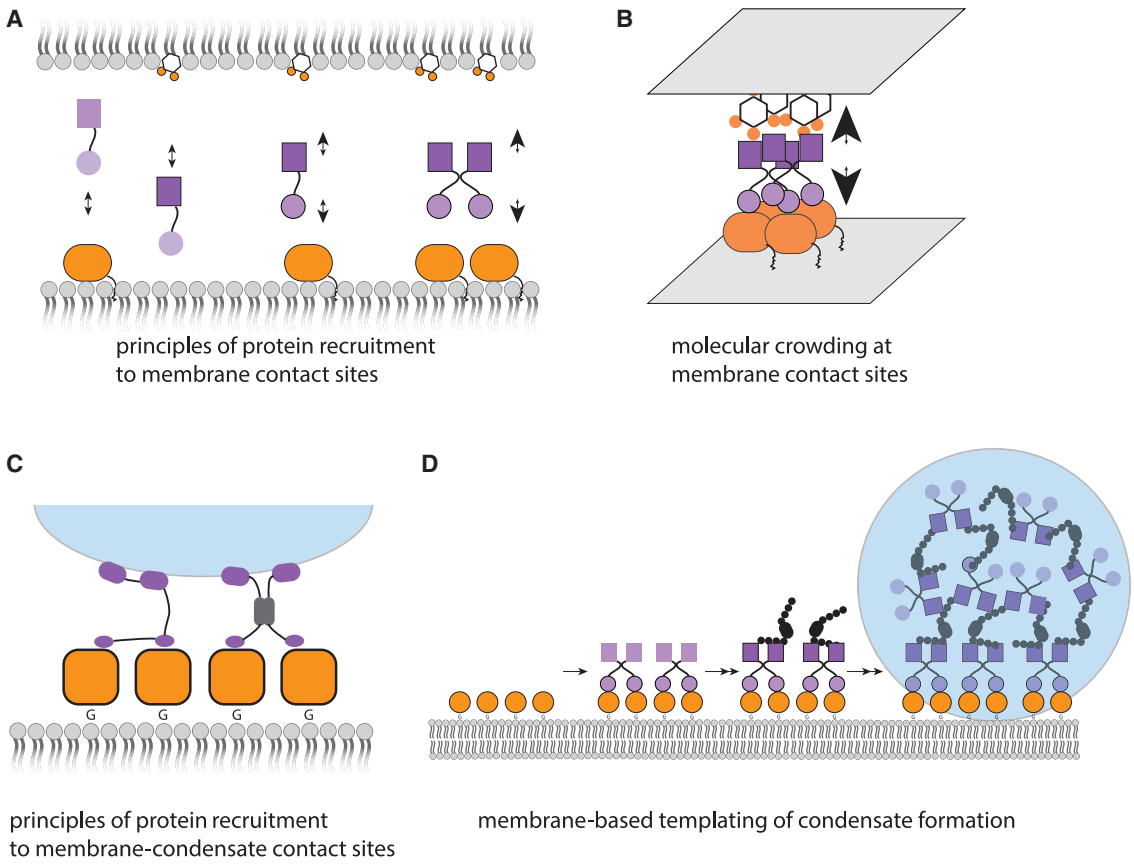


Figure 4. Membrane-membrane and membrane-condensate contact sites

(A) For proteins having two different binding sites for membrane marks, their recruitment to membrane contact sites is facilitated when the two marks are present on the two different membranes. In case multiple membrane-binding domains are present, or upon multimerization, these interactions can be further stabilized.

(B) At extended membrane contact sites, the crowding of membrane marks and membrane tethers can result in stable assemblies with low diffusion rates.

(C) Similar principles of protein targeting to membrane contact sites are also applicable at membrane-condensate contact sites where multivalent interactions can closely align the membrane and condensate surfaces.

(D) The membrane can additionally induce the assembly of condensates by the recruitment and clustering of proteins, which harbor binding sites for other multivalent macromolecules (schematically exemplified here with the recruitment of cargo receptors and ubiquitinated proteins in autophagy).

Reactions on the membrane can be further controlled by barriers that laterally confine the diffusion of lipids and proteins (Figure 3F). The meshwork of the actin cortex, for example, limits not only 2D diffusion in the plane of the membrane but also three-dimensional (3D) diffusion of components within reaction compartments just beneath it.[114] Actin polymerization dynamics and the activity of motor proteins can generate cortical flows on the membrane surface, which strongly affects the steady-state distribution of lipids and membrane-anchored proteins.[115] The restricted organization of receptors in the membrane that arises from intercellular communication and the consequent physical barriers to receptor transport has also been shown to govern the output of cellular signaling pathways.[116]

The marking of membranes with proteins can template macromolecular assemblies such as the ATG8-labeled nascent autophagosome, which recruits proteins containing the LIR motif, via a direct interaction with ATG8 in a polyvalent manner (Figure 3G). Finally, recent technological advances, particularly in mass spectrometry, have also revealed the roles played by membrane lipids in modulating the structure, conformation, and function of many membrane-embedded proteins.[117]

These examples demonstrate how specific, membrane-licensed interactions serve to template the assembly of supramolecular complexes at discrete locations. The membrane locally concentrates, activates, spatially confines, and organizes reaction partners to drive efficient catalysis. In doing so, the membrane ensures high fidelity and low noise.

MEMBRANE-MEMBRANE AND MEMBRANE-CONDENSATE CONTACT SITES

So far, we have outlined how the membrane can act as an active scaffold to template the assembly of macromolecular complexes and control their catalytic activities. In this section, we discuss special cases in which two membranes come into contact with each other or in which membranes come into contact with membrane-less biomolecular condensates.

Membrane contact sites are defined by the tethered proximity of two organelles.[118] The mechanisms by which reader proteins are specifically targeted to membrane contact sites are manifold (Figures 4A and 4B). It is becoming clear that the unique properties of two membrane surfaces can be exploited to recruit

proteins to their contact site. For example, the lipid transfer protein OSBP is a parallel dimer containing a PI(4)P- and ARF1-GTP-binding PH domain in its N terminus and a central FFAT motif, which binds to VAP proteins.[119–121] The PI(4)P and ARF1-GTP are localized in membranes of the TGN, whereas the VAP proteins reside in the ER membrane.[120,121] The dimeric OSBPs, therefore, prefer to bind to membranes where PI(4)P/ARF1-GTP and VAP proteins are concentrated, respectively. At TGN-ER contact sites, these two membranes are available for binding at the same time; therefore, in total, six binding sites are present for the OSBP readers. Another example is the Num1 protein, which anchors mitochondria to the plasma membrane within a larger structure called the MECA (mitochondria-ER cortex anchor). Num1 harbors a C-terminal PI(4,5)P_2-binding PH domain. The N terminus contains a dimeric coiled-coil domain, which also interacts with the outer mitochondrial membrane.[122] Since the plasma membrane is enriched in the PI(4,5)P_2 mark, the Num1 reader is preferentially recruited to mitochondrial sites close to the plasma membrane where four binding sites for Num1 are available. Here, homotypic and heterotypic coincidence detections are combined to drive the localization of Num1.

In recent years, the presence of membrane-less organelles, also referred to as biomolecular condensates, has attracted considerable attention. These structures are macroscopically stable, but individual components frequently show high mobility, both within the condensates and in exchange with a cytosolic pool. There is extensive crosstalk between membrane-bound and membrane-less organelles (Figures 4C and 4D). Some insights regarding the principles of these interactions have been obtained from the study of cargo-driven selective autophagy in yeast and human cells. Here, the prApe1- (yeast) and p62- (mammals) containing cargo material forms condensates, which initiate the formation of membrane contact sites that connect the ER and ATG9 scramblase-positive vesicles via the lipid transfer protein ATG2.[123–130] This results in the induction of autophagosome biogenesis where the PI(3)P mark on the growing phagophore membrane generated by the writer class III PI3K is detected by readers of the PROPPIN family, which, in turn, recruit the ATG12-ATG5-ATG16L1 writer complex to mark the membrane with ATG8 proteins.[55] During engulfment, cargo receptors in the condensates, such as p62, interact with ATG8 family proteins on the growing phagophore and, thereby, closely align the membrane to the cargo, a process also referred to as wetting.[131] The individual interactions between the receptors in the condensates and the membrane can be very weak because both binding partners are present at high local concentration on the surface of the two structures. At least *in vitro*, even low-affinity, low-specificity interactions between phase-separating polymers with a naked membrane can result in membrane alignment and bending around the condensate.[131] Vice versa, during starvation-induced autophagy, the nascent ATG8-protein-marked phagophore may be read out by polymeric cargo receptors recruiting it to the membrane. The cargo receptors, in turn, recruit poly-ubiquitinated proteins (Figure 4D). Upon the completion of autophagosome biogenesis, the PI(3)P and ATG8 marks are erased by the ATG4 proteins[132,133] and PI(3)P-specific phosphatases,[134] respectively.

The process of membrane-induced condensate formation is even better understood for plasma-membrane-localized receptor signaling.[135] For example, upon TCR activation, the writer kinase Lck phosphorylates the cytoplasmic tail of CD3ζ, which is read out by the ZAP70 kinase. ZAP70, in turn, writes a phosphorylation code on the adaptor protein LAT, which is subsequently read by various proteins, resulting in condensate formation and downstream signaling at the membrane. Apart from activating Ras via Grb2 and SOS, the condensates include N-WASP and ARP2/3, triggering actin polymerization.[136–138] Similarly, the phosphorylated form of the cytoplasmic tail of the Nephrin cell adhesion protein is able to localize 2D condensates of NCK and N-WASP to the membrane.[139,140] In TCR signaling, the condensates exclude the CD45 protein phosphatase, thus spatially separating the eraser from the writers and readers in the signaling condensate on the membrane.[136] At the same time, the actin-containing condensates feed back to the initial writers by clustering the receptors on the membrane.[139]

INTEGRATION OF WRITER, READER, AND ERASER ACTIVITIES

The dynamic writing, reading, and erasing of the membrane code permits the assembly of complex biochemical circuits. A crucial property of such circuits, which are based on a network of many low-affinity interactions, is that the overall assembly can be very stable while individual interaction partners exhibit high exchange rates. This allows other factors to rapidly outcompete specific interactions to modify the entire assembly dynamically over time. For example, in clathrin-mediated endocytosis, a network of low-affinity interactions changes over time, culminating in the assembly of a clathrin cage, which is actively disassembled only at the very end of the process.[141] During bacterial cell division, the tubulin homolog FtsZ and its membrane anchor and actin homolog FtsA form treadmilling filaments on the inner leaflet of the cytoplasmic membrane. These filaments organize into the so-called Z-ring that rotates within the cell circumference, whereas the dynamic recruitment of transmembrane proteins assembles the cell division machinery.[142] Such transient protein assemblies with a high turnover of components are generally favorable for dynamic cellular processes as they do not require a dedicated protein machinery to disassemble stable complexes.[143]

In this section, we focus on the signaling downstream of the epidermal growth factor receptor (EGFR) to illustrate the integration of writers, readers, and erasers to drive signaling in the cell via two alternate pathways (Figure 5). The membrane serves to pre-organize and locally concentrate inactive EGFR. EGF binding to the extracellular domain of the EGFR relieves autoinhibition and induces receptor-mediated dimerization.[144,145] These conformational changes are transmitted to the intracellular tyrosine kinase domain, thereby promoting autophosphorylation.[146] Autophosphorylation of the C-terminal, cytoplasmic tail of EGFR, as well as trans-phosphorylation by other kinases in the cell, creates a scaffold for the assembly of a number of supramolecular complexes at the plasma membrane. Two major intracellular pathways that are activated by EGFR are the Ras-Raf-MEK-ERK pathway and the Ras-PI3K-Akt pathway, which promote cell growth, proliferation, and survival.[147,148]

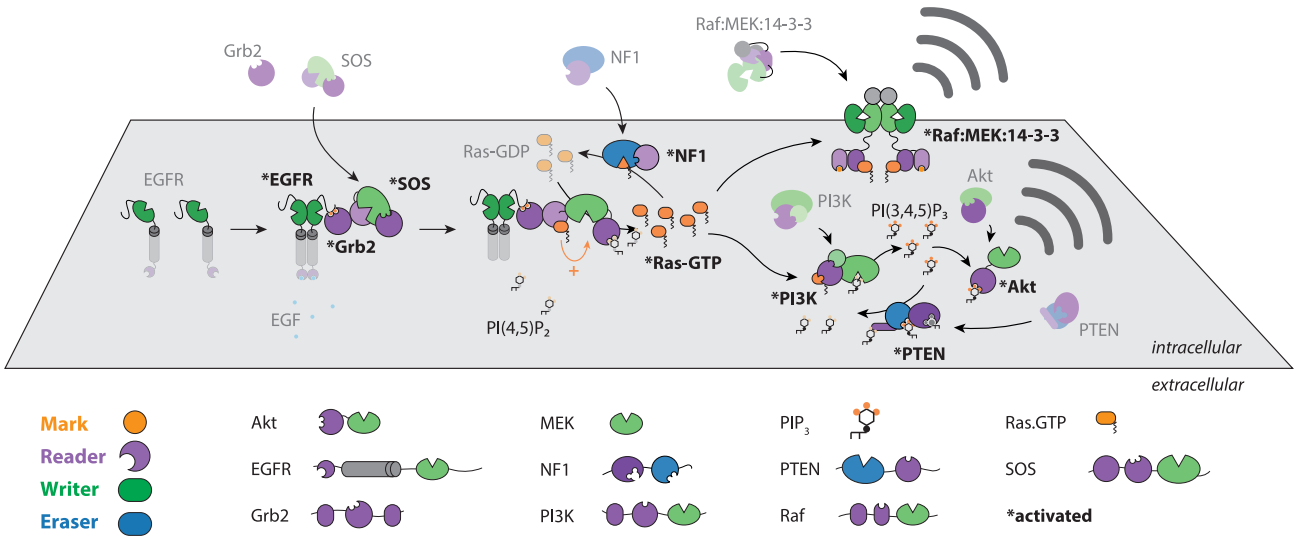


Figure 5. Integration of writer, reader, and eraser activities

Schematic of EGFR signaling to illustrate how the activities of writers, readers, and erasers are integrated at the membrane surface. Upon the activation and subsequent phosphorylation of the cytoplasmic tails of EGFR, the Ras GEF SOS is recruited to EGFR by Grb2. SOS coincidentally binds to PIP_2 via its PH domain, which, in turn, releases its autoinhibition, allowing it to activate Ras. In a positive feedback loop, SOS binds activated, membrane-localized Ras, thereby further increasing its GEF activity. The simultaneous binding to phosphorylated EGFR via Grb2, $PI(4,5)P_2$, and active Ras increases the residence time and catalytic activity of SOS, resulting in a feedforward loop for Ras activation. Active Ras recruits Raf, which additionally binds lipids in the membrane. These two interactions release Raf autoinhibition, allowing it to dimerize and activate the downstream kinase MEK. Upon activation, Ras also recruits and activates the lipid kinase PI3K, which, in turn, produces $PI(3,4,5)P_3$ to recruit Akt to the membrane, whereupon the PH-domain-mediated autoinhibition of Akt is released. The eraser NF1 removes the Ras signal from the membrane by activating its GTPase activity. The activity of NF1 may be sterically excluded from activated EGFR because SOS is stably bound to the receptor via Grb2. The eraser for PI3K signaling, PTEN, is recruited to the membrane via its own product, $PI(4,5)P_2$, resulting in a positive feedback loop. In the scheme, the marks are depicted in orange, the readers in purple, the writers in green and the erasers in blue. Note that this is a simplification, as many writers and erasers frequently have reader domains, and the eraser of one signal can at the same time be the writer of another signal.

EGF-mediated trans-autophosphorylation leads to the recruitment of the reader protein Grb2 via its SH2 domain.[149] Grb2, in turn, recruits the Ras GEF SOS with its SH3 domain.[150] In the absence of activated receptors, SOS is autoinhibited by its membrane-binding PH and histone domains, which block the allosteric binding site for Ras-GTP,[151] thereby preventing promiscuous Ras activation. Recruitment of SOS by Grb2 results in an increase in the local concentration of SOS at the membrane. Lipid marks such as $PI(4,5)P_2$ and PA promote the relief of SOS autoinhibition by disengaging its regulatory domains.[152,153] Reconstitution of phosphorylated fibroblast growth factor receptors (FGFRs) tails, Grb2, and SOS on SLBs has recently revealed that activated FGFRs undergo phase separation, which enhances the catalytic efficiency of SOS toward Ras.[154] Similar phase transitions of receptors and cytosolic adaptor proteins have been reported in the context of TCR and FGFR2 signaling.[136,155,156] Interestingly, the multivalent recruitment and activation of SOS appears to be sufficient to retain it on the membrane in an active conformation for as long as it takes to inactivate it by endocytosis.[156] Binding of activated Ras to an allosteric site in SOS further enhances its GEF activity[16] in a feedforward loop that can drive the processive activation of thousands of Ras molecules at a time.[156] SOS activation has been proposed to involve condensates containing receptors, Grb2, and SOS.[11] These condensates would permit the iterative sampling of the membrane in which the probability of SOS autoinhibition being released is increased. The relatively slow release of autoinhibition acts as a kinetic "proof reading" such that transient, monovalent recruitment of SOS to activated receptors by Grb2 is unlikely to lead to SOS activation.[11,138] Interestingly, activated Ras is barely found in endocytic vesicles containing EGFR and Grb2 following receptor recycling at the plasma membrane,[157,158] suggesting that endocytosis may terminate growth-factor-dependent Ras signaling but not the plasma membrane-resident Ras-Raf-MEK-ERK pathway.

The activated Ras mark on the membrane also stimulates the PI3K-Akt pathway. Downstream of the EGFR, the class I PI3K alpha (PI3Kα) plays the predominant role in signaling.[159] The structure of p110γ bound to H-Ras has revealed the molecular basis of Ras binding,[160] whereas single molecule studies show how synergistic binding to both the receptor and Ras marks activates the writer function of p110α to drive $PI(3,4,5)P_3$ synthesis.[161,162]

EGFR signaling is antagonized by the erasers for $PI(3,4,5)P_3$ and Ras signaling, PTEN, and neurofibromin (NF1), respectively. Both PTEN and NF1 are tumor suppressors, and their loss or mutation is also common in cancer and overgrowth disorders.[81,163–167] The PTEN eraser is a lipid phosphatase with specificity for the 3′ phosphate of the inositol ring. PTEN is mainly associated with the dephosphorylation of the $PI(3,4,5)P_3$ mark on the plasma membrane, although recent studies have also implicated it in the termination of $PI(3,4)P_2$ signaling on endosomes.[168,169] A $PI(4,5)P_2$-binding motif in the disordered N terminus promotes membrane binding and increased phosphatase activity,[170] demonstrating that the product of the reaction drives a positive feedback loop, similar to the effect activated Ras exerts on SOS.[16,151] The C2 domain of PTEN is tightly associated with the phosphatase domain and is critical for membrane

binding and the orientation of the catalytic site for substrate engagement.[171] Membrane binding, however, is acutely regulated by the multi-site phosphorylation of the C-terminal 47 amino acids of PTEN, which drives specific interactions of the tail with the C2 and phosphatase domains that maintain PTEN in an autoinhibited conformation and weaken its membrane association.[171–174]

Recently determined structures of the eraser NF1, a RasGAP, have revealed a putative autoinhibited conformation in which the catalytic GAP domain is sequestered, along with the membrane-binding surface of the SEC-PH domain, in intramolecular interactions with the central scaffold domains of the molecule.[175,176] These findings imply that significant conformational changes must occur upon membrane recruitment to permit the engagement of GTP-bound Ras. The obligate homodimer of NF1 also implies that Ras dimers[108–111,177] could be the cellular substrates of NF1. Ras interaction with NF1 is antagonized by A-Raf binding,[178] illustrating the mutual exclusivity of the interaction between the activated Ras mark and its reader (Raf) or eraser (NF1). Finally, proximity labeling approaches have been employed to map the time-resolved proximity proteome of EGFR from ligand-mediated activation through receptor internalization and recycling, revealing the changing landscape of signaling protein interactions in both space and time.[179]

In summary, the example of EGFR signaling illustrates how the generation of signaling lipids and proteins by writers, the coincident detection of multiple signals by readers, and constrained diffusion in the plane of the membrane pattern both the plasma membrane and endomembranous compartments. In combination with erasers, which counteract these events, the membrane can scaffold biochemical pathways to drive signal transduction with high fidelity and spatiotemporal resolution.

OUTLOOK

The compartmentalization of biochemical reactions is essential in the crowded environment of the cell. By analogy to the histone code, the dynamic patterning and remodeling of membrane surfaces by writers and erasers facilitate the acute spatial and temporal control of molecular interactions (readers). Local concentration and membrane-licensed interactions of reaction partners as well as the coincident detection of multiple signals can be exploited to drive cooperative, switch-like behavior and high-fidelity transmission of information. The individual interactions, however, that drive the assembly of supramolecular complexes on the membrane are necessarily weak. This presents obvious problems for experimental investigations. *In vitro*, reductionist approaches can suffer from missing components; lacking, incomplete, or incorrect post-translational modifications, or inappropriate boundary conditions. In cells, genetic ablation or ectopic over-expression can lead to improper stoichiometries and cellular concentrations, circumvent physiological expression patterns, or elicit pleiotropic effects beyond the subject of investigation.

It is clear, then, that advances in structural biology, including *in silico* structure prediction,[180–182] cryoelectron microscopy[183,184] and tomography,[185,186] artificial intelligence-driven segmentation and structure template matching,[187] correlative light and electron microscopy,[188,189] and super-resolution microscopy,[190,191] must be applied to image cellular processes *in situ*. In particular, cryoelectron tomography is beginning to yield unprecedented snapshots of membrane-based processes at near atomic resolution.[192] Combining structural information from various sources with mass-spectrometry-based, label-free interactomics is necessary to annotate 3D snapshots of near-native cellular assemblies.[193,194] Machine learning is improving the sensitivity and dynamic range of proteomics applications.[195] Endogenous tagging of proteins using genome editing technology is already proving to be a powerful tool in our arsenal, as it allows the dynamics of proteins and protein complexes to be visualized by live-cell microscopy while maintaining the correct stoichiometries.[196] In addition, when combined with proximity labeling techniques coupled with mass spectrometry,[197,198] endogenous tagging permits the detection of transient interactions at biologically relevant expression levels. Along the same lines, the quantification of the abundance of molecules in the cell is providing essential numbers for concentration and stoichiometry,[196,199] which are ultimately needed for both reconstitution experiments and mathematical modeling. *In vitro*, biochemical reconstitutions of increasing complexity that recapitulate the boundary conditions present in cells are needed to test what is necessary and sufficient for the assembly of large macromolecular machines, signal propagation, or the biogenesis of subcellular organelles. New tools for the detection of single molecules on surfaces can, in principle, be used to rebuild complex membrane-scaffolded assemblies and monitor their dynamics.[200]

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.devcel.2023.06.001.

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DECLARATION OF INTERESTS

S.M. is a member of the scientific advisory board of Casma Therapeutics.

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