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Single molecule fluorescence *vistas* of how lipids regulate membrane proteins

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Abstract

The study of membrane proteins is undergoing a golden era, and we are gaining unprecedented knowledge on how this key group of proteins works. However, we still have only a basic understanding on how the chemical composition and the physical properties of lipid bilayers control the activity of membrane proteins. Single-molecule (SM) fluorescence methods can resolve sample heterogeneity, allowing to discriminate between the different molecular populations that biological systems often adopt. This short review highlights relevant examples of how SM fluorescence methodologies can illuminate the different ways in which lipids regulate the activity of membrane proteins. These studies are not limited to lipid molecules acting as ligands, but also consider how the physical properties of the bilayer can be determining factors on how membrane proteins function.

Cellular membranes are active solvents that contain lipid ligands that interact with membrane proteins.

Membrane and soluble proteins live in different environments. Soluble proteins are surrounded in all directions by an aqueous solvent. This medium is homogeneous, and is believed to have similar properties throughout the cell (1). However, the scenario for membrane proteins is more nuanced. This type of protein contrasts to soluble proteins in multiple ways, the most defining difference being that membrane proteins do not hide their hydrophobic residues in their interior. Instead, they specifically expose lipophilic side chains into a belt-like region, typically at the center of the protein, known as the transmembrane domain (TMD). As a result, membrane proteins are not stable in aqueous solution and require a lipid membrane to solvate the hydrophobic surface of the TMD. This is the underlying principle that anchors membrane proteins to lipid bilayers and defines their cellular localization and function.

Both hydrophobic (insoluble) and hydrophilic (soluble) ligands can bind to membrane proteins. The domains of membrane proteins that lie outside the membrane, generally

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Conflict of Interest

The authors declare that they have no conflict of interest.

referred to as soluble regions, carry out more critical cellular roles than could possibly be described in this short review. Maybe the most emblematic function of membrane proteins is to bind soluble ligands at the extracellular side of the membrane, and then transmit the information of the binding event across the bilayer (2, 3). This process generally results in the recruitment of protein ligands and effectors to the intracellular region that triggers signal amplification into the cytoplasm. The second large class of ligand modulation involves lipid molecules. Lipids typically interact with the TMD of the target protein, although in some cases they can also bind to soluble domains with transient or stable access to the membrane (4). This review will highlight relevant examples of the different ways that the lipid bilayer can modulate the activity of membrane proteins.

Eukaryotic membrane proteins immerse themselves into intricate lipid solvents. This medium consists of hundreds or thousands of different lipid molecules, depending on the cell type (5), which are asymmetrically distributed across the two parallel lipid layers that form the membrane (6). An obvious consequence of such transversal lipid asymmetry is that the amino acids in contact with the cytosolic leaflet interact with different lipids than those facing the extracellular medium (Fig. 1A). For some membrane proteins, the key biological roles that lipid modulation play have been uncovered (7, 8). From the large diversity of lipid ligands, those that most commonly regulate membrane proteins are sterols, like cholesterol (Chol) (9, 10), and phosphorylated phosphoinositides (PI), particularly phosphatidylinositol 4,5-bisphosphate (PIP_2) (11, 12). However, despite decades of work devoted to the study of protein-lipid interactions (13), the general principles that define protein regulation by lipids have yet to emerge. This shortcoming is likely linked to the fact that we still do not understand the function of many lipids or why membranes are so heterogeneous.

A fact that would be useful not to dismiss is that lipid bilayers are liquid fluids. Bilayer fluidity, as well as other lipid properties, are tightly controlled by the cell (14), underscoring their biological importance. Probably the best known example of how a physical characteristic of the lipid bilayer affects membrane proteins is the average bilayer thickness. In fact, it has been found for multiple membrane proteins that optimal function is only achieved when the thickness of the TMD matches that of the lipid molecules surrounding it (7). The mechanical properties of the bilayer are multi-faceted and can only be fully defined using a set of often interdependent physical and material parameters. These include bilayer bending elasticity (15–18), area compressibility modulus (19), membrane spontaneous curvature (20, 21), bilayer inter-leaflet coupling (22), membrane fluidity (23, 24), membrane charge (5), and potentials (25, 26), lateral pressure profile (27), membrane tension (28), as well as the average bilayer thickness (29) and the amplitude and rate of thickness fluctuations (30). We are only beginning to unravel how these bilayer properties modulate membrane proteins (Fig. 1B). It is also important to note that the mechanical properties of membranes need to be optimized for proper membrane protein insertion and stability, as recently reviewed in (31).

The wide range of membrane physical properties allow for proteins to be embedded in solvents with different characteristics. We favor the view that bilayers act on membrane proteins as “active” solvents. In contrast to the aqueous medium, which plays a more passive role, although still certainly important. It seems intuitive that evolution would

have synergistically optimized the lipid environment where proteins operate in the cell. Indeed, the lipid composition of membranes is different between eukaryotic membranes. As a few relevant examples, in the cell there is a gradient of bilayer thickness: the plasma membrane has the thickest bilayer, the Golgi apparatus has an intermediate thickness, and the ER membrane being the thinnest of them (32, 33). This trend agrees with the TM domain thickness of the proteins in these membranes, and indeed proteins do dictate the average membrane thickness (32). Additionally, the cholesterol levels are higher in the plasma membrane than in intracellular organelles (5). Moreover, the membrane of different cellular organelles have characteristic PIP compositions (34). Taken together, such specific differences suggest that the overall composition of the membrane as a solvent contributes to the adequate sorting and activity of membrane proteins, which are likely optimized for the appropriate cellular localization for a given membrane protein.

Activation of membrane proteins often involves a conformational switch between different states (35). As an example, an X-ray solvent contrast modulation study of the Ca^{2+}-ATPase, revealed dynamic coupling with lipid molecules as the protein underwent large conformational changes during the reaction cycle (36). It is likely that this ATPase is not an exception, and that lipids can also modulate the dynamics of other membrane proteins. As a result, studies that aim to determine how lipids regulate membrane proteins should include the ability to study protein dynamics. Single-molecule (SM) fluorescence methods are ideally suited for this endeavor. As we will describe next, several experimental modalities of this family of techniques allow us to identify and investigate different conformational states of membrane proteins, and importantly also the dynamic parameters that define the transitions between them.

Overview of single molecule fluorescence methods

SM fluorescence provides discrete information from multiple copies of a molecule. The analysis of SM data can identify and quantify different molecular states. Total internal reflection fluorescence (TIRF) microscopy provides insight at a SM level by overcoming some limitations of standard fluorescent microscopes. By restricting incident light to a precise focal plane, out-of-focus light is reduced resulting in an enhanced signal-to-noise ratio. This limited excitation plane allows for SM detection and effectively reduces photobleaching of fluorescent probes (37). The constricted focal plane is achieved by generating an evanescent field, of around 100 nm (Fig. 2A), which excites fluorophores at the interface of two materials with different refractive indices. There are two main illumination pathways to generate the TIR phenomenon: prism and objective based systems (38). Prism based TIRF microscopy will be used as an example in this review (Fig. 2A), and is further described in (37). Samples are typically added to a liquid channel that lies between the slide and coverslip. As seen in the magnified area of Fig. 2A, proteins can be isolated and reconstituted using various methods (39–41), which are not limited to those depicted. Compared to traditional approaches, SM methods can more accurately inform on the structure and dynamic changes of membrane proteins at the molecular level. TIRF microscopy can also be applied to study large protein complexes and image fluorescence in live cells (37, 38). This allows tracking of individual molecules in cells, a powerful

technique to study membrane proteins (42–46) that will not be further described here due to space limitations.

A variety of established techniques can be coupled with TIRF microscopy to study protein complexes. One example is probe photobleaching (Fig. 2B, Top). This approach monitors the step-wise fluorescence bleaching that can reveal the oligomeric state of membrane proteins (47). Förster resonance energy transfer (FRET) can provide insights with SM resolution into protein conformational dynamics based on donor-accepter distance changes (48–50) (Fig. 2B, Middle). A third important SM method is protein induced fluorescence enhancement (PIFE), where the fluorescence intensity of the dye informs on local structural changes (Fig. 2B, Bottom) (51). For example, Lamichhane *et al.* reported a ground-breaking example of PIFE used to detect dynamic changes in the activation of the β2 adrenergic receptor (52, 53). Other powerful SM approaches include fluorescence quenching (54, 55) and surface-induced fluorescence attenuation (56). We will describe next recent examples of how SM fluorescence can be used to reveal mechanistic insights on how lipids regulate the activity of membrane proteins.

smFRET allows understanding of KirBac1.1 closure induced by PIP_2.

Complementary SM approaches have been used to study KirBac1.1 using smFRET (48, 49). This ion channel is a prokaryotic homologue of eukaryotic inward-rectifier potassium (Kir) channels. Kir channels regulate potassium conductance at the membrane and therefore are critical for membrane homeostasis in pancreatic beta cell hormone secretion, regulation of membrane potential of nerve cells, and scores of other important cellular processes (57). The activity of Kir channels is modulated by PIP_2, which causes marked activation of eukaryotic channels. Paradoxically, this phosphoinositide is instead a strong inhibitor of prokaryotic KirBac channels. While it is known that PIP_2 causes closure of KirBac1.1, the dynamics of this process had only been studied via ensemble methods, which are likely to overlook mechanistic clues of the conformational changes that occur during channel closing.

The two SM studies (48, 49) employed PIP_2 to stabilize the closed state of the channel. One of the papers reconstituted KirBac1.1 in lipid (POPE:POPG) nanodiscs using the MSP1E3D1 membrane scaffold protein (49). Kir channels are homo-tetramers. Homo-oligomers present intrinsic challenges for FRET experiments due to uncertainties in labeling stoichiometry. The solution used by Sadler *et al.* to limit labeling to a single donor and an acceptor fluorophore was to perform protein labeling using sub-stoichiometric amounts of both dyes (lower than 1 in 1,000 in molar equivalents) (49). This strategy prevents the problem presented by the existence of multiple dye labeling sites, which would complicate accurate data interpretation. Data were acquired using confocal-in-solution alternating-laser excitation (ALEX) microscopy. This imaging technique requires no sample attachment to the microscope slide and can identify dynamic equilibrium between different conformations due to its high resolution. Studies of channel inhibition by PIP_2 (by addition to the solution of 20 μM di-C8-PIP_2) and acidic conditions strongly supported a pore closure by the twist-to-shrink model of pore contraction and dilation.

These results agree with the conclusions of the second SM study with KirBac1.1 (48). For this work, the channel was reconstituted in liposomes doped with biotinylated lipid, which allowed the use of neutravidin immobilization for prism-based TIRF imaging. In this case, controlled dye labeling was achieved by using a concatenation strategy, consisting of cloning and expressing the tetramer as a contiguous polypeptide that lacks several cysteine residues, to allow for single maleimide labeling of this residue (Fig. 3A). Multiple conjugations schemes were carried out, allowing robust experimental data (Fig. 3B). The FRET results indicate that the cytoplasmic region fluctuates between two structural states that become less dynamic and separate from the pore upon channel closing. Specifically, when PIP_2 caused channel closure there was an increase of the channel population with FRET efficiency of ~0.15, and a reduction in the population with FRET ~0.25. (Fig. 3B). Their data additionally showed that the extracellular region of the channel is structurally rigid in all cases. Both papers are examples of how different techniques can investigate ion channel gating dynamics in relation to the lipid environment at a SM level.

PIP_2 promotes specific dimerization of receptor tyrosine kinases to stabilize the active conformation.

The receptor tyrosine kinase (RTK) EphA2 is overexpressed in multiple cancers, including breast, prostate, and pancreatic subtypes (58, 59). Loss of EphA2 ligands, like ephrinA1, is often associated with EphA2 overexpression, which can lead to poor cancer prognosis and high rates of metastasis. Therefore, EphA2 is a relevant target candidate for cancer therapeutics. Two different activation mechanisms have been identified for this receptor. EphA2 follows the canonical RTK activation mode, whereby binding of ephrinA ligands promotes receptor dimerization which activates the intracellular kinase domain. In addition to this ligand-dependent activation mechanism, EphA2 can be activated in the absence of ligand through phosphorylation by intracellular kinases (60–62). This ligand-independent activation triggers an oncogenic signal, while the ligand-dependent activation results in the opposite effect (Fig. 4A).

The single TM domain of EphA2 participates in receptor dimerization, and the nearby juxtamembrane (JM) segment modulates the activity of the downstream kinase domain (3). The TM region is believed to dimerize using different conformations in the two activation modes, with different transmembrane crossing angles (Fig. 4A) (63, 64). We have recently used hydrophobic matching to stabilize these two alternate conformations with a peptide that contains the TM domain and a portion of the JM segment (known as TMJM EphA2) (65). We observed that thin bilayers, consisting of the lipid 14:1-PC, stabilize the ligand-dependent conformation while in thicker bilayers (22:1-PC) a lower crossing angle is observed, in agreement with the ligand-independent (oncogenic) conformation (47). Experiments of TMJM EphA2 using lipid vesicles were complemented with SM photobleaching studies. This approach quantified dimer formation in the two alternative conformations. For these studies, TMJM EphA2 was incorporated into lipid nanodiscs formed using the copolymer styrene maleic acid (SMA) to form SMA lipid particles (SMALPs). The SM results indicate that the peptide is in a monomer-dimer equilibrium in both bilayers (Fig. 4C). Interestingly, dimerization was promoted by 3% PIP_2, but

only in the oncogenic conformation stabilized in 22:1 PC bilayers. In contrast, 10% phosphatidylserine (PS), the negatively charged lipid that is most abundant in human cells, caused no significant impact on dimerization (47). These results suggest that PIP_2 specifically modulates the activity of EphA2, probably by electrostatic attraction with basic residues in the JM segment. Stabilization of the dimer by PIP_2 might prevent oncogenic signaling by EphA2, as the monomer is the pro-oncogenic EphA2 species (66).

The Sako group reported that PIP_2 also promotes TM dimerization in a second RTK, the epidermal growth factor receptor (EGFR) (67). EGFR is a driver of cell proliferation and differentiation, and its misregulation can cause tumor malignancy (68). smFRET experiments were performed in MSP nanodiscs for an EGFR TMJM peptide labeled with a FRET donor/acceptor pair. The sensitivity of the technique revealed that the JM region adopts different intermediate conformations *en route* to dimerization. The dynamics of the FRET traces allowed to measure the rate constant for the transition between the different states. The authors observed that ~5% PIP_2 increases the transition rate constant from the intermediate to the dimeric conformation. 30% PS caused a different effect, as it instead stabilized an intermediate state. The paper also investigated the effect of phosphorylation in JM residue Thr654, which reduces kinase activity. The results provide a molecular rationale for this effect, as this post-translational modification reduced dimerization. Since phosphorylation reduces the basic nature of the JM segment, a dynamic interplay between this event and changes in acidic lipid composition is expected to bias the monomer/dimer equilibrium, and thus the activation of EGFR. It is interesting that both the studies for EphA2 and EGFR show clear differences between the effect of binding to the JM of PIP_2 and PS. This agreement suggests that the interaction between the conserved positively charged JM residues (69) and negatively charged lipids is not a simple electrostatic attraction. Indeed, the data point to a more nuanced scenario, where PS acts as “partial agonist”, and full effect is only achieved for PIP_2, probably due to its higher charge density.

The dimerization into the native conformation of a transporter is determined by lipid defects.

The forces that determine membrane protein oligomerization have remained elusive due in part to the lack of robust experimental systems that can accurately quantify membrane protein association. In order to elucidate the reversible equilibrium interaction of proteins in a lipid membrane, the Robertson group used SM photobleaching to study the dimerization of the *Escherichia* coli Cl^-/H^+ antiporter, CLC-ec1 (70). CLC-ec1 homodimerizes through a non-polar dimerization interface composed mainly of isoleucine and leucine residues. To determine the effect of tryptophan substitutions on the dimerization equilibrium in membranes, the authors labeled three CLC-ec1 species. The wild type (WT) subunit was modified so that a partially buried cysteine was more accessible for conjugation with Cy5-maleimide (C85A/H234C), and the interface tryptophan mutations W (I422W) and WW (I201W/I422W) were evaluated. Each construct was reconstituted in 2:1 POPE/POPG (both with 16:0–18:1 acyl chains) membrane vesicles and immobilized at various mole fractions to measure as a function of density the population of monomer, dimer, or larger oligomers. TIRF microscopy coupled with SM bleaching analysis was used to measure the probability

of oligomer populations per vesicle. It was possible to quantify the equilibrium dimerization free energy by diluting the CLC-ec1 subunit and measuring the shift in monomer and dimer populations. The rationale was that if the system exhibits a state of dynamic equilibrium, then the dilution of the transporter will shift the system to the monomeric state. SM analysis of CLC-ec1 WT showed a dependent relationship between the oligomeric state and the subunit/lipid mole fraction. The comparison of the free energy of dimerization for the W and WW mutants confirmed that this substitution at the protein-protein interface destabilizes the dimer, with the WW mutation imposing a greater effect.

The same method was recently used to further investigate the driving forces underlying membrane protein association in membranes. Specifically, a follow up study by the same laboratory (71) extended the use of their SM photobleaching approach to elucidate the molecular basis of why CLC-ec1 is found as a dimer in cells. The CLC-ec1 narrow dimerization interface is composed of four short α-helices (Fig. 5 A–B). This interface has a reduced hydrophobic thickness compared to the rest of the protein (Fig. 5B). Molecular dynamic simulations of a CLC-ec1 monomer revealed that the membrane locally deforms at the thin dimerization interface (Fig. 5C). To reduce the resulting hydrophobic mismatch, the lipids in contact with the exposed dimerization interface undergo a massive rearrangement, in what is generally referred to as a membrane defect. Specifically, the lipid acyl chains tilt and reduce their packing, allowing interdigitation between the lipid tails of the two membrane monolayers and increased water penetration into the bilayer core. Such lipid rearrangement minimizes the energetic cost of thinning the hydrophobic belt of the transporter.

The authors reasoned that addition of short-chain lipids to the membrane should destabilize CLC-ec1 dimerization, by means of a reduction in hydrophobic mismatch. Their data show that a short-chain lipid (with C12:0 chains, and of the same lipid headgroup composition) efficiently localized into the membrane defects caused by the protein. As a result of this lipid heterogeneity, the monomer-dimer free energy balance was altered resulting in strong stabilization of the CLC-ec1 monomer. This study highlights how the dimerization of a membrane protein can be driven by the properties of the lipids that solvate it. These results indicate the intriguing possibility that lipids with lower propensity to form bilayers can regulate membrane protein self-assembly by alleviation of hydrophobic mismatch. Therefore, mechanisms might exist by which changes in lipid composition, caused by cellular processes, dietary changes or even infection, could specifically alter the activity of transporters and possibly other membrane proteins. These modulatory effects could extend to the stabilization or destabilization of particular signaling states where even transient conformational changes result in changes in hydrophobic thickness of the protein.

Conclusions and perspectives

- Membrane proteins reside in a complex lipid environment. The composition of this medium plays vital roles in the regulation of membrane proteins, influences cell signaling and can contribute to disease progression. However, the exact mechanisms of how lipids regulate membrane proteins remain unknown.

- SM methods are elucidating key lipid-protein interactions that induce changes in the dynamics, folding, structure and oligomerization of membrane proteins, all of which can lead to altered function.
- Determination of the mechanisms that lipids employ to modulate the activity of model membrane proteins could allow for the generation of models. Identifying such rules might allow to predict how the changes in lipid composition that often result from signaling events will impact the biological processes that membrane proteins control.

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List of Abbreviations

ALEX	Alternating Laser Excitation Microscopy
Chol	Cholesterol
CLC-ec1	Chloride Channel Protein-*Escherichia coli* 1
Cryo-EM	Cryogenic Electron Microscopy
EGFR	Epidermal Growth Factor Receptor
EphA2	Ephrin Type-A Receptor 2
FRET	Förster Resonance Energy Transfer
JM	Juxtamembrane segment
MLVs	Multilamellar Vesicles
MSP	Membrane Scaffolding Proteins
PC	Phosphatidylcholine
PI	Phosphoinositide
PIFE	Protein Induced Fluorescence Enhancement
PIP	Phosphatidylinositol Phosphate
PIP_2	Phosphatidylinositol 4,5-bisphosphate
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylglycerol
PS	Phosphatidylserine

RTK	Receptor Tyrosine Kinase
SM	Single Molecule
SMALPs	Styrene Maleic Acid Lipid Particles
smFRET	Single Molecule Förster Resonance Energy Transfer
TIR	Total Internal Reflection
TIRF	Total Internal Reflection Fluorescence
TM	Transmembrane
TMD	Transmembrane Domain
WT	Wild Type

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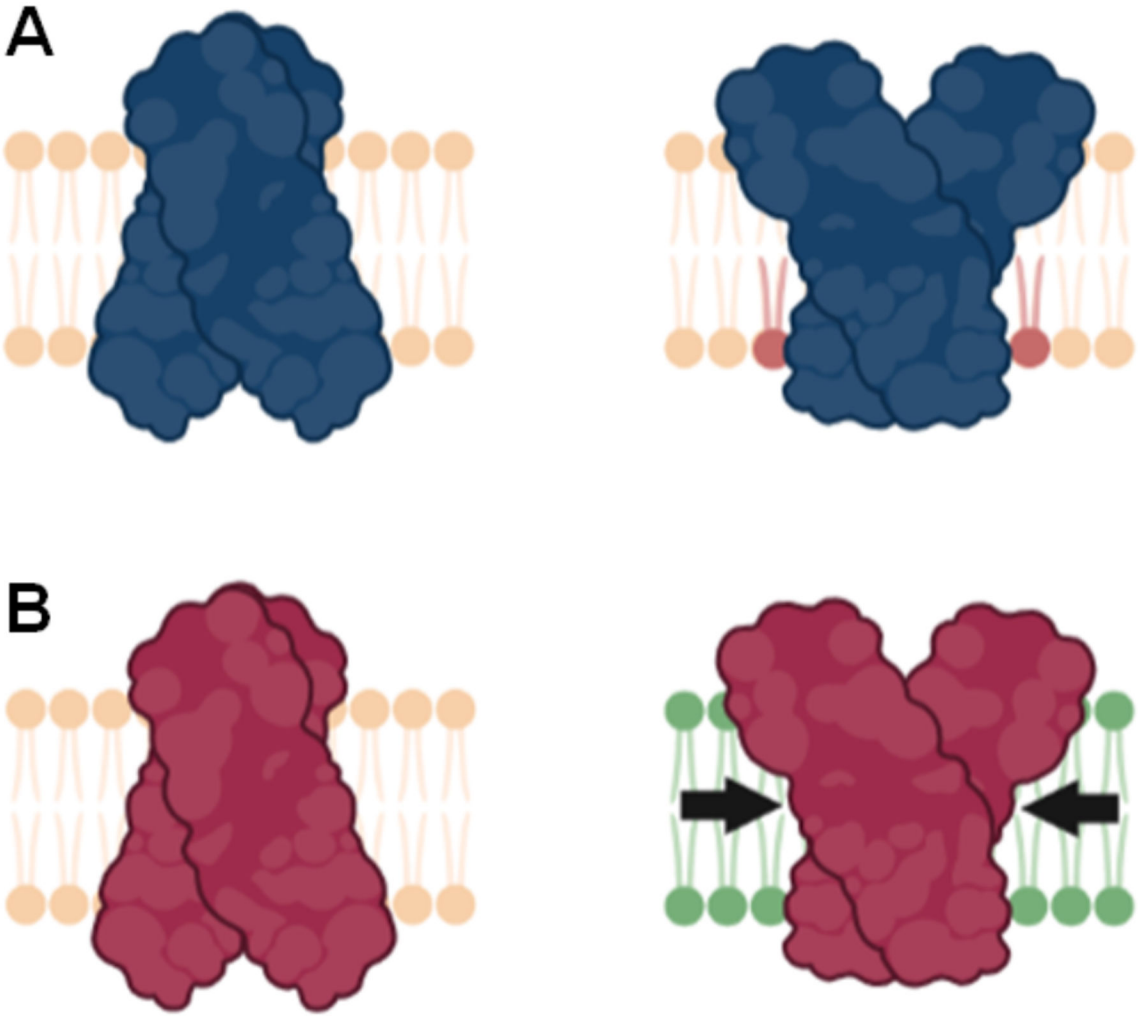

Figure 1. Lipids use different mechanisms to activate membrane proteins.

A, The presence of the lipid ligand PIP_2 (red), which is distributed asymmetrically across the lipid bilayer, can induce a conformational change in a membrane protein (blue) that alters function. **B,** A change in protein conformation, activity, and/or dynamics can also occur when specific lipids (green) surround the TMD causing changes in the physical properties of the protein solvent (marked as arrows).

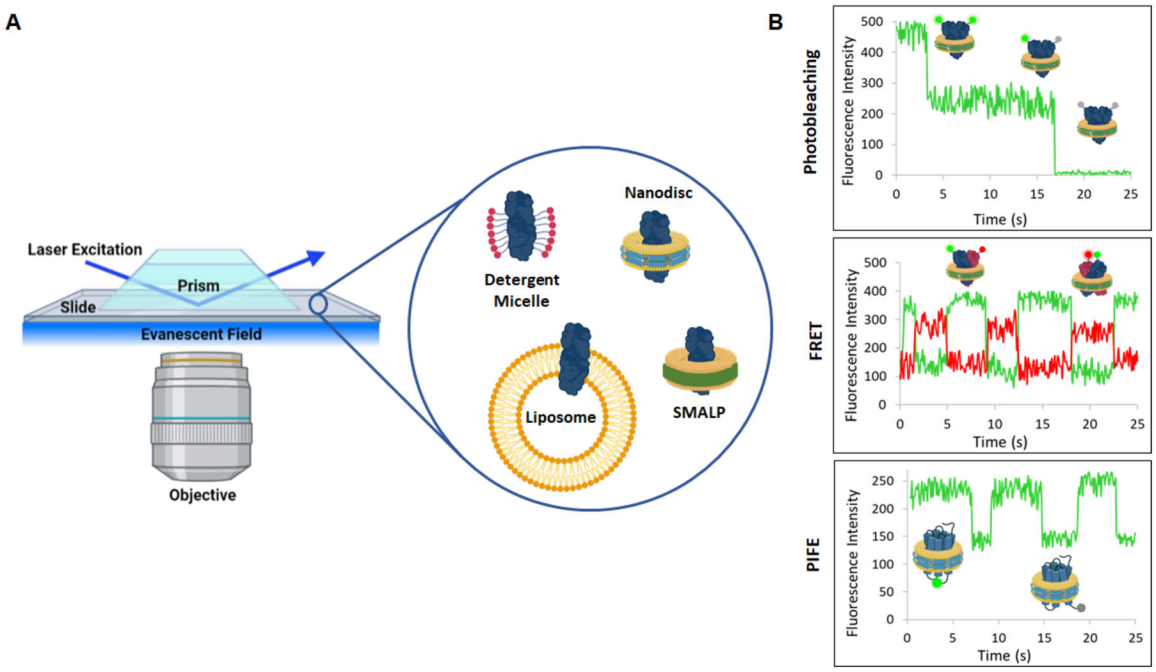

Figure 2. Experimental diagram of TIRF single molecule microscopy.

A, Generalized TIRF microscope schematic. The magnified area shows a membrane protein isolated by detergent solubilization or reconstitution using membrane-scaffolding proteins into nanodiscs (blue cylinders), styrene maleic-acid for SMALPs (green band), and liposomes. **B**, Example fluorescent traces are shown for the main SM modalities: photobleaching, Förster resonance energy transfer (FRET), and protein induced fluorescence enhancement (PIFE). In FRET experiments, green lines denote the donor fluorescence and red traces correspond to the acceptor.

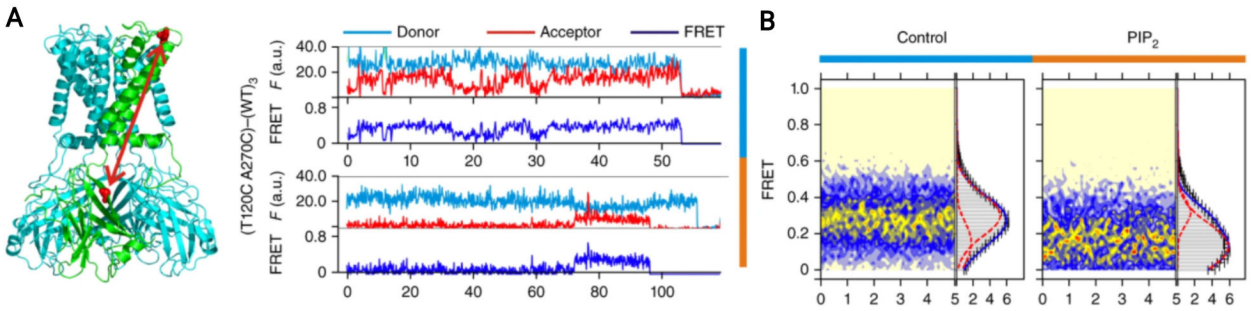

Figure 3: KirBac1.1 closure is induced by PIP_2.

A, The red arrow marks the distance between the cysteine residues where the donor and acceptor dyes are located (T120C and A270C, shown as red dots). The graphs show raw fluorescence and FRET (dark blue). Data are shown for control conditions (top), and in the presence of PIP_2 (bottom). **B,** FRET contour plots show that when the channel is closed by PIP_2, there is an increase in the distance between the TMD and C-terminus domain, revealed as in increase of the channel population with a FRET efficiency of ~0.15. Experiments were performed in liposomes of POPE:POPG (3:1) in the presence of 1% PIP_2. Figure modified from (48), with permission.

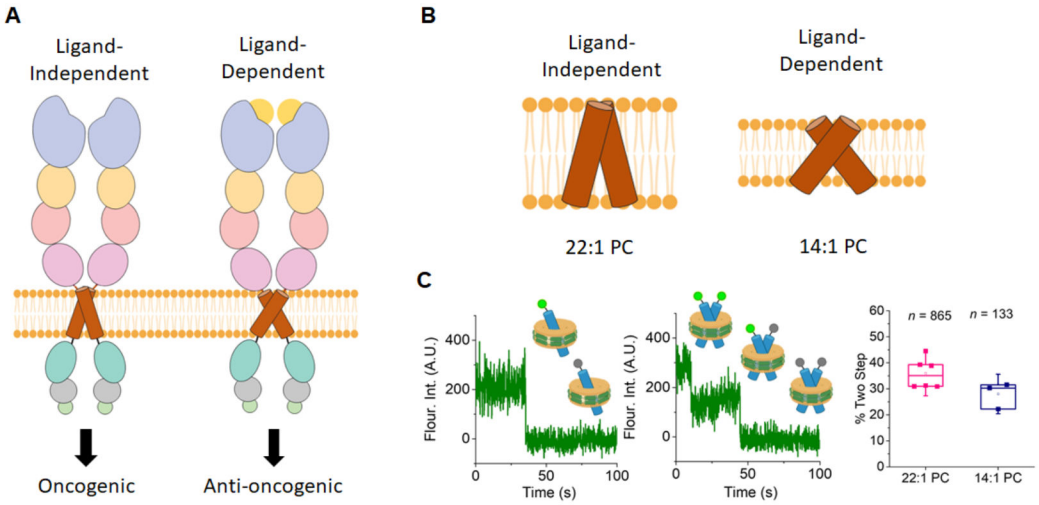

Figure 4. EphA2 signaling is determined by ligand and lipid interactions.

A, Two alternate EphA2 activation mechanisms. **B,** Transmembrane domain crossing angles of EphA2 in thin (14:1 PC) and thick (22:1 PC) bilayers. **C,** Single molecule photobleaching of fluorescently conjugated TMJM EphA2 in SMALPs. Quantification of TMD monomers (left) and dimers (middle) indicate a monomer-dimer equilibrium (right). Adapted from (35) with permission.

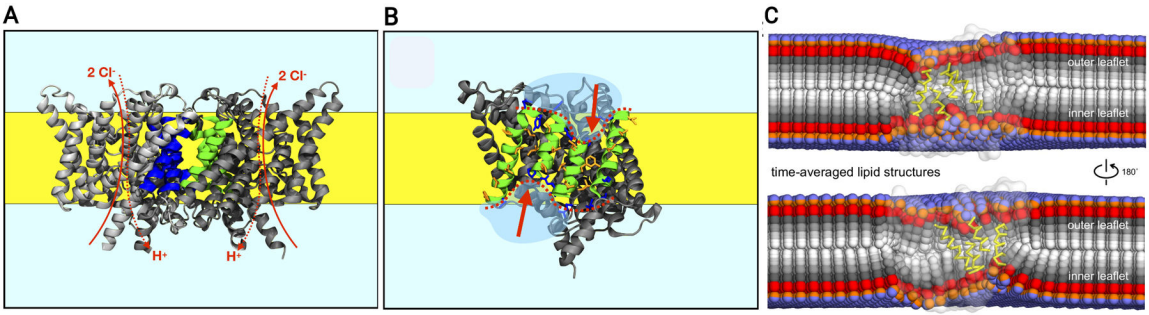

Figure 5. Lipid solvation controls the dimerization of the CLC-ec1 transporter.

A, Dimerization of CLC-ec1 is mediated by a small protein interface, showed in green and blue for each monomer. Arrows indicate the pathways for Cl^- and H^+ transport. The hydrophobic core of the membrane is shown in yellow, and the hydrated regions in cyan. **B,** side view of the interface can be observed when the figure in A is rotated, and a monomer removed. The dashed red lines mark the hydrophobic surface, which show two polar pockets (red arrows) that cause membrane defects. **C,** Molecular dynamics simulation of CLC-ec1 shows acyl chain deformation around the transporter dimerization interface, resulting in local bilayer thinning. Data are shown for a monomer, with interface helices in yellow. Figure modified from (71), with permission.