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## Novel roles of phosphoinositides in signaling, lipid transport, and disease

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### Abstract

Phosphoinositides are lipid signaling molecules that act as master regulators of cellular signaling. Recent studies have revealed novel roles of phosphoinositides in myriad cellular processes, and multiple human diseases mediated by misregulation of phosphoinositide signaling. This review will present a timely summary of recent discoveries in phosphoinositide biology, specifically their role in regulating unexpected signaling pathways, modification of signaling outcomes downstream of integral membrane proteins, and novel roles in lipid transport. This has revealed new roles of phosphoinositides in regulating membrane trafficking, immunity, cell polarity, and response to extracellular signals. A specific focus will be on novel opportunities to target phosphoinositide metabolism for treatment of human diseases, including cancer, pathogen infection, developmental disorders, and immune disorders.

### Introduction

Phosphoinositides (PPIs) are lipid signaling molecules that coordinate numerous aspects of membrane trafficking and cell signaling in eukaryotic cells. Their action is essential in cell growth, metabolism, and cell death [1]. The enzymes that modify phosphoinositides (kinases, phosphatases, and lipases) are critically linked to multiple human diseases, with mutations leading to cancer, immune disorders, developmental disorders, and inflammatory diseases [2–4]. There are 7 different PPI species (Fig. 1A), with all of them acting as regulators of temporal and spatial signaling events (Fig. 1B). The canonical view of

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#### Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Conflict of interest

The authors declare no conflict of interest

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phosphoinositide signaling has focused on their role in both the recruitment (Fig. 2A) and allosteric activation of proteins at specific intracellular locations (Fig. 2B). Phosphoinositide mediated recruitment of proteins to specific organelles can mark their final destination in membrane trafficking [5], modify biophysical properties of membranes [6], and provide spatial regulation of signaling.

However, recent years has seen an explosion in our understanding of other key roles of phosphoinositides, including their role in modulating integral membrane protein signaling (Fig. 2C), and regulating lipid transport (Fig. 2D). This review will highlight recent discoveries in phosphoinositide biology, focusing on four specific areas: novel roles of phosphoinositides in signaling, phosphoinositide regulation of integral membrane proteins, how phosphoinositides and lipid transport proteins mediate lipid exchange against thermodynamic gradients, and finally novel discoveries towards treating human disease by targeting phosphoinositide metabolizing enzymes. For more comprehensive coverage of PPIIn function in e.g. cytoskeletal dynamics and nuclear function, we refer the reader to several recent, comprehensive reviews [7,8].

### **Non-canonical roles of phosphoinositides in recruitment of cellular signaling machinery**

The canonical view of phosphoinositide signaling is the recruitment of specific lipid binding domains to intracellular membranes, with multiple domains putatively identified as specific PPIIn binders (PX, PH, FYVE, etc., for an in-depth review readers are advised to consult [9,10]). The recruitment of these domains to specific intracellular organelles can mediate signaling not only through localization, but also through lipid binding mediated conformational changes [11–13]. Exhaustive analysis of these specific domains is revealing a more complicated picture. Detailed biochemical and biophysical studies of 39 of the 49 PX domains found in the human genome [14] revealed striking differences in PPIIn binding. Four different clusters of PX domains were identified based on their lipid specificity: those that do not specifically bind lipid (i), specific PI3P binders (ii), those that bind other PPIIn species (iii), and those that bind PI3P along with other PPIIn species (iv). These distinct groups are not coupled to their evolutionary history. This highlights the extreme importance in the vigorous biochemical, biophysical and cellular analysis of the lipid binding specificity of putative PPIIn binding domains. Intriguingly, phosphorylation of the lipid binding loop of the PX domain of sorting Nexin 3 (SNX3) disrupts membrane recruitment, and reveals another level of how membrane recruitment can be regulated by PTMs [15].

Many proteins are known to be recruited to phosphoinositides independent of PPIIn binding domains. This can be mediated through multiple different molecular mechanisms, including disordered polybasic motifs. Our focus is on recent discoveries of signaling complexes that are regulated by phosphoinositide recruitment/activation in the absence of PPIIn binding domains. This includes the spatially localized PI(3,4)P<sub>2</sub> plasma membrane recruitment of RasGAP proteins to mediate cell polarity [16], the PI4P-mediated dispersed Trans-Golgi recruitment of the NLRP3 inflammasome to promote inflammatory signaling [17], the PI(4,5)P<sub>2</sub> mediated plasma membrane recruitment of the inflammatory DNA sensing cGAS protein to govern self-nonsel self discrimination [18], and the PI3P mediated pre-autophagosome (PAS) recruitment of the core autophagy protein ATG16L1 to mediate

autophagy [19]. This highlights a nuanced picture of PPIIn recruitment, where recruitment will be dependent not only on specific PPIIn binding domains, but also through non-canonical PPIIn binding sites in proteins. Important to consider is that these sites will likely exhibit less strict dependency on any PPIIn species, and will require extensive testing of other coincidence signals that may mediate recruitment.

### Phosphoinositides as regulators of integral membrane protein signaling

The binding of phosphoinositides to integral membrane proteins can modulate their activity, and can allow for activation of integral membrane proteins to only occur when they are in specific membrane organelles. Phosphoinositides can regulate integral membrane proteins through multiple mechanisms: they can induce allosteric conformational changes and/or mediate coupling to protein binding partners. Phosphoinositides have long been known to be key regulators of ion channels, with PI(4,5)P<sub>2</sub> identified as a key regulator of channel opening [20,21], with the first molecular insight on the role of PI(4,5)P<sub>2</sub> being shown in the structure of the Kir2.2 ion channel [22]. For the Ca<sup>2+</sup> ion channel TRPML1 the binding of PI(4,5)P<sub>2</sub> inhibits channel opening, while binding of PI(3,5)P<sub>2</sub> leads to channel activation [23], providing a lipid mediated switch making sure the channel is only active in PI(3,5)P<sub>2</sub> containing endolysosomal compartments. This reveals the potential multifaceted roles of PPIIn in both positively and negatively regulating integral membrane protein signaling. For example, this could play an important role in coordinating how long-range signals can be propagated physiologically, with modification of PI(4,5)P<sub>2</sub> levels discovered to play an important role in blood flow in the neural vasculature: this could be controlled by the ability of PI(4,5)P<sub>2</sub> to activate Kir2.1 ion channels and inhibit Transient receptor potential cation channel subfamily V member 4 (TRPV4) channels [24,25], which can be further tuned by G<sub>q</sub>PCR receptors activating PLC and depleting PI(4,5)P<sub>2</sub> levels.

Advances in native mass spectrometry [26–28], Cryo electron microscopy [23,29–36], and molecular dynamic simulations [37–40] have been particularly powerful in providing insight into the molecular mechanisms that mediate how PPIIn can bind and regulate integral membrane protein function [41]. A survey of a selection of recent structures of phosphoinositides bound to different membrane proteins (ion channels, flippases) and the molecular basis for how they regulate protein function is shown in Fig. 3. An important note is that for almost all interactions of PPIIn with integral membrane protein there is no distinct polypeptide that mediates binding, which is instead mediated by the complex tertiary and quaternary protein architecture; as such probes for specific PPIIns derived from integral membrane proteins should be approached with caution [42].

The activation of G-protein coupled receptors (GPCRs) can be allosterically modulated by surrounding phospholipids [43], and potentially this may play a role in controlling their oligomeric state [44]. The adenosine A2A GPCR can bind PI(4,5)P<sub>2</sub>, with this interaction potentiating G-protein activation [27]. GPCRs are able to integrate multiple signals, and generate specific downstream outputs, including G-proteins and beta-arrestin signals [45]. The formation of the GPCR beta arrestin complex requires GPCR phosphorylation downstream of GPCR kinases (GRKs). The presence of PI(4,5)P<sub>2</sub> promotes formation of a complex between the beta-2 adrenergic GPCR with the GPCR kinase GRK5 [46]. Beta-

arrestin mediated endocytosis of GPCRs is proposed to require arrestin binding to phosphoinositides [47]. Recent work suggests the possibility of an active beta-arrestin conformation on membranes that is catalytically activated by GPCRs, but independent of a stable GPCR-arrestin complex. The dissociation of signaling competent active beta-arrestin is putatively mediated by phosphoinositides [48]. Biased agonists can specifically favor one pathway over another, with the exact molecular details of this process being unknown. GPCRs primarily generate signals at the plasma membrane, but can also be active in endosomal compartments.

Together these studies bring up an intriguing possibility. They suggest that phosphoinositides may spatially organize multiple aspects of GPCR signaling, both affecting the coupling of GPCRs to G proteins, GPCR kinases and arrestin, and affecting the activation of signaling proteins downstream of GPCRs. This suggests different PPIIn species may in some cases act as spatially localized lipid switches to bifurcate and modulate GPCR signaling.

The disruption of PPIIn regulation of integral membrane signaling can lead to human disease through surprising ways. In Niemann-Pick Type C Disease the disruption of lysosomal transport of cholesterol leads to upregulation of the ATP-binding cassette transporter ABCA1 (ABC1A), which through its PI(4,5)P<sub>2</sub> floppase activity decreases PM PI(4,5)P<sub>2</sub> levels, leading to inactivation of the KCNQ2/3 potassium channel and aberrantly modified neuronal signaling patterns [49].

One of the key implications for the role of phosphoinositides in regulating integral membrane signaling is that unlike in peripheral membrane recruitment this interaction will almost certainly be driven by both interactions between the PPIIn headgroup, and the acyl chains. The majority of PPIIn species contain an 18:0 sn-1 chain, and a 20:4 sn2 acyl chain [50], however, there is extensive variability within different cells and tissues. This has long been known to be critical in the activation of the Kir2.1 channel, with PIP<sub>2</sub> containing saturated acyl chains only weakly activating channel opening [51]. This has also been shown for the ER protein SEIPIN which can bind PA and PI3P and exhibits a strong binding preference towards unsaturated fatty acids [31]. Further biophysical and biochemical analysis will be required to define the full complement of mechanisms by which specific PPIIn species (head group and acyl chain variants) may activate specific integral membrane signaling processes.

### **Phosphoinositides and Lipid Transfer Proteins (LTPs).**

Evidence is emerging that lipid transport proteins (LTPs) can utilize phosphoinositides in a novel way (Figure 4): as a means to couple the energy of ATP hydrolysis to the transport of lipid cargoes against a concentration gradient at membrane contact sites (MCS). So far, evidence implicates this mechanism for enrichment of TGN cholesterol and PM PS [52,53]. In both cases, members of the oxysterol binding protein (OSBP) related protein (ORP) family and the PPIIn PI4P are involved.

For ORP function, PI4P has two roles: firstly, as the counter lipid for the exchange cycle; and secondly, in its canonical capacity, to anchor the ORP to the acceptor membrane (See

figure 2). Thus, lipid transfer can only be accomplished when PI4P levels are sufficient to localize the ORP to the MCS. This creates a putative feedback mechanism: PI4P synthesis at the TGN by PI4KB was reported to be inhibited by high sterol concentrations, depleting PI4P and thus de-localizing OSBP and stopping further sterol traffic [54]. The cycle can be re-initiated at distal TGN sites in response to another, cholesterol-activated PI4K, PI4K2A [54].

A similar feedback mechanism regulates ORP5 and -8 proteins at the PM [55]. The ORP5 and ORP8 PH domains have an adjacent polybasic domain that together facilitate concurrent binding of both PI4P and PI(4,5)P<sub>2</sub>, which co-operate to localize the LTP to ER-PM contact sites. This forms the basis of a homeostatic mechanism: when the cardinal regulatory PM lipid PI(4,5)P<sub>2</sub> is depleted (e.g. by phospholipase C), ORP5/8 is not localized to the PM and cannot transfer PI4P to the ER; in this way, all newly synthesized PM PI4P can be directed towards PI(4,5)P<sub>2</sub> synthesis until levels of the latter recover sufficiently [55]. Intriguingly, such a feedback mechanism must be balanced by a component that stops PI4P to PI(4,5)P<sub>2</sub> conversion once levels are restored. Whilst these data imply that PI(4,5)P<sub>2</sub> plays an exclusive regulatory role, PI(4,5)P<sub>2</sub> transfer by ORP5 and 8 has also been reported, with this lipid putatively being transported instead of PI4P [56,57]. However, these data are not consistent with other reports that ORP5 and ORP8 over-expression can deplete PM PI4P, but not PI(4,5)P<sub>2</sub> [52,55]. For now, the exact roles of PI4P and PI(4,5)P<sub>2</sub> as localizing factors versus exchange currency are still subject to debate.

Another lipid that is greatly enriched at the PM is cholesterol, so it has been tempting to speculate this may be fueled by a PPIIn-mediated exchange cycle. The first empirical evidence for such a cycle has just been reported, with the observation that ORP2 can facilitate cholesterol enrichment and PI(4,5)P<sub>2</sub> depletion from the PM [57]. PM cholesterol homeostasis also requires negative feedback, for instance when highdensity lipoproteins (HDLs) deliver exogenous cholesterol to the PM of steroidogenic tissues. This is removed by a novel family of ER proteins, GRAMD1-A, -B and -C (a.k.a. “aster” proteins). Asters localize to PM contact sites in response to elevated cholesterol levels via their GRAM domains, facilitating removal of cholesterol via the VASSt/aster lipid transfer domain [58]. In this case, cholesterol flows down a concentration gradient, so counter-transport is not required, and PPIIn are not implicated in their function or localization [58,59]. On the other hand, PPIIn are needed to localize the related GRAMD2a and -b proteins, which lack the transfer domain but do form ER-PM contact sites that house other lipid and ion exchange reactions [59].

Elegant and fulfilling as these lipid exchange mechanisms are, they are not quite established enough to enter the textbook. Although ORPs and other LTPs can certainly facilitate lipid exchange reactions in the test tube (e.g. [53,56,60]), whether this reflects their true function in cells, or is simply a test tube manifestation of a more subtle activity, is actively debated [61]. It has been proposed instead that several LTPs, including ORPs, can utilize lipid transfer domains to present PPIIn to kinases and phosphatases in membranes replete with their lipid counter-ligand. Thus, rather than exchangers, they are sensors of local lipid composition that charge PPIIn synthesis (or turnover) as a function of membrane composition – a so called “instructive synthesis” model in the context of kinase presentation [61] or

“instructive metabolism” more generally. Indeed, recent work has shown that ORP4 can function to present PI(4,5)P<sub>2</sub> to PLCβ3 for hydrolysis at the PM [62]. Other work has proposed a hybrid of the models, whereby ORP-mediated lipid transfer produces a nanoscale environment enriched with sterol and unsaturated PS that favors PIP5K activation and PI(4,5)P<sub>2</sub> synthesis [63]. However, another group has reported that mammalian PIP5K are in fact most potently activated by PI(4,5)P<sub>2</sub> itself [64]. This produces a feed-forward scenario, with local physical constraints on lipid enrichment and diffusion together with local phosphatase activity constraining PI(4,5)P<sub>2</sub> synthesis [64].

A key problem for the field is that most evidence in intact cells for lipid exchange relies on PPI<sub>n</sub> depletion in response to LTP enrichment, or vice versa (e.g. [52,54–56]): yet precisely the same result can be expected from the instructive synthesis model. How can we differentiate the two models? Certainly, lipid exchange cycles demand segregation of PI kinase and phosphatase activity to distinct membrane compartments, whereas instructive synthesis does not. This has recently been tested for the PI4P phosphatase SAC1, which predominantly localizes to the ER. Treatment of cells with oxidants that block SAC1 activity lead to accumulation of PI4P in the ER, consistent with failure to degrade ORP-transported molecules in this compartment [65]. Likewise, molecular engineering shows that an additional ~6 nm of length is needed between the ER anchor and catalytic domain before the enzyme becomes capable of “reaching” substrate in the plasma membrane, implying substrate must be transferred to it in the ER [65]. That said, it has recently been reported that the versatile TGN protein, FAPP1, is able to complex with SAC1 and VAP proteins to stimulate activity of ER-localized SAC1 at TGN MCSs [66], consistent with an instructive metabolism model.

ORP2-mediated PI(4,5)P<sub>2</sub> exchange to drive sterol enrichment is supported by the fact that sterol enrichment (and PI(4,5)P<sub>2</sub> depletion) is inhibited by knock-down of endosomal PI(4,5)P<sub>2</sub> phosphatases [57]. Likewise, PI(4,5)P<sub>2</sub> exchange at ER-PM contact sites [56] requires the localization of an ER phosphatase to degrade this lipid; INPP5K seems like an enzyme to fit this role, though its catalytic activity is not yet explicitly linked to PM PS and PI(4,5)P<sub>2</sub> homeostasis; instead, it is required for ER tubule morphogenesis at the cellular level [67], with loss of function in humans causing a form of muscular dystrophy [68,69]. Clearly, delineating the functional distribution of PPI<sub>n</sub> phosphatase activities at the subcellular level, and how this couple to LTP function, requires further work. This will be a key goal to resolve lipid transfer and instructive synthesis.

Finally, although both instructive synthesis and lipid exchange predict the same change in PPI<sub>n</sub> levels in target compartments, the fate of the lipid differs. Exchange specifically predicts that the degraded PPI<sub>n</sub> molecules will be delivered to a recipient compartment for degradation. It is this specific transfer step that has yet to be demonstrated in living cells. The failure to observe it can be explained if lipid exchange reactions simply don't happen in living cells; but they are also explained by the necessarily close coupling of PPI<sub>n</sub> degradation to transport. It therefore seems that if lipid transfer to a destination membrane can be demonstrated in intact cells, the case for lipid exchange cycles will be greatly strengthened. This will require acute control of LTP activity, and/or careful, precise and acute decoupling from the degradative process. These experiments will prove tricky, though



with increasingly sophisticated molecular, optical and chemical genetic approaches (e.g. [54,65,66]) the requisite experimental tool kit may be available. Whichever model ends up favored by the evidence and enters the realm of textbook knowledge (and it could be both, depending on the specific molecular circumstance), it is clear that PPI signaling is central to lipid as well as protein organization.

**Novel insight into targeting pathological phosphoinositide metabolism in disease**—The most clinically advanced strategy targeting pathologies in phosphoinositide signaling has been the targeting the PI3K pathway in cancer, immunodeficiencies, and growth disorders. Currently there are four clinically approved PI3K inhibitors [2,70–73], however, there has been little success in targeting solid tumors with this approach, with various deleterious side effects. Recent discoveries suggest there may be unique additional opportunities to target this pathway in disease. Inhibitors specific to the PIK3CA PI3K isoform lead to a large increase in insulin signaling, which can limit their efficacy.

Suppressing this insulin feedback through either dietary or pharmacological approaches shows promise for increased efficacy of PI3K inhibitors as cancer therapeutics [74]. It has also been found that in breast cancers there are frequent occurrences of two mutations within the same allele of PIK3CA, leading to increased PI3K activity and tumor growth [75]. This is consistent with unique mutations in PIK3CA leading to activation by different molecular mechanisms [76]. Patients expressing the doubly mutated PIK3CA showed a more positive clinical response to PI3K inhibitors, which suggests that cancers driven by these mutations are excellent targets for antiPI3K therapeutics.

In addition to cancer, mutation of PIK3CA is a driver of overgrowth disorders (PIK3CA-related overgrowth syndromes [PROS]), with treatment using PIK3CA specific inhibitors dramatically decreasing symptoms in patients [77]. Activating mutations in the PIK3CD isoform of PI3K is a causative agent of primary immunodeficiencies [78], and PIK3CD specific inhibitors are showing promise as a clinical treatment for these patients [79]. The lipid phosphatase PTEN that antagonizes the PI3K pathway has been discovered to be inactivated by poly-ubiquitination mediated by the E3 ligase WWP1, with small molecule WWP1 inhibitors representing a novel mechanism to reactivate anti-tumorigenic PTEN activity [80].

While PI3K inhibitors are the most clinically advanced molecules for modulation of pathological PPI metabolism, exciting pre-clinical experiments are revealing potential therapeutic strategies. Myotubular myopathy (MTM) is a childhood muscle disease mediated by inactivating mutations in the MTM lipid phosphatases, which dephosphorylate PPI at the 3' position [81]. The development of PIK3C2B inhibitors may be useful as MTM therapeutics [82], but surprisingly it has been found in mouse models that the clinically approved estrogen receptor inhibitor tamoxifen can ameliorate some of the symptoms of MTM [83]. The full mechanistic details of how tamoxifen improves the MTM phenotype is unknown, however, it is proposed to function mainly through the estrogen receptor pathway by extra-nuclear estrogen receptor alpha signaling. Finally, inhibitors towards the parasitic variants of the lipid kinase PI4KB show promise as single-dose anti-malarial [84,85] and anti-cryptosporidium [86] agents, with clinical trials currently ongoing in malaria [87].

## Conclusions and Future Directions

Phosphoinositides are master regulators of signaling in almost every intracellular membrane compartment. The development of novel tools to interrogate PPI metabolism have revealed exciting new insight into their roles in controlling membrane trafficking, metabolism, autophagy, and signaling. The classical understanding of phosphoinositide signaling was as messengers that could recruit and/or activate soluble effector proteins. As we have seen, our knowledge of the repertoire of such proteins and their associated functions is still expanding. Moreover, driven especially by the revolution in structural biology, we now know that these lipids are also crucial to the regulation of integral membrane proteins. We have also discussed evidence that PPI may in fact fulfil a novel role as an energy currency to drive non-vesicular lipid transport. In the meantime, knowledge of the classical PPI-driven pathways is now maturing into a state where pharmacologic inactivation of their metabolism can alleviate a variety of malignant, infectious and inflammatory diseases. As our basic mechanistic knowledge of PPI signaling expands, we expect therapeutic opportunities derived from this knowledge to expand even further.

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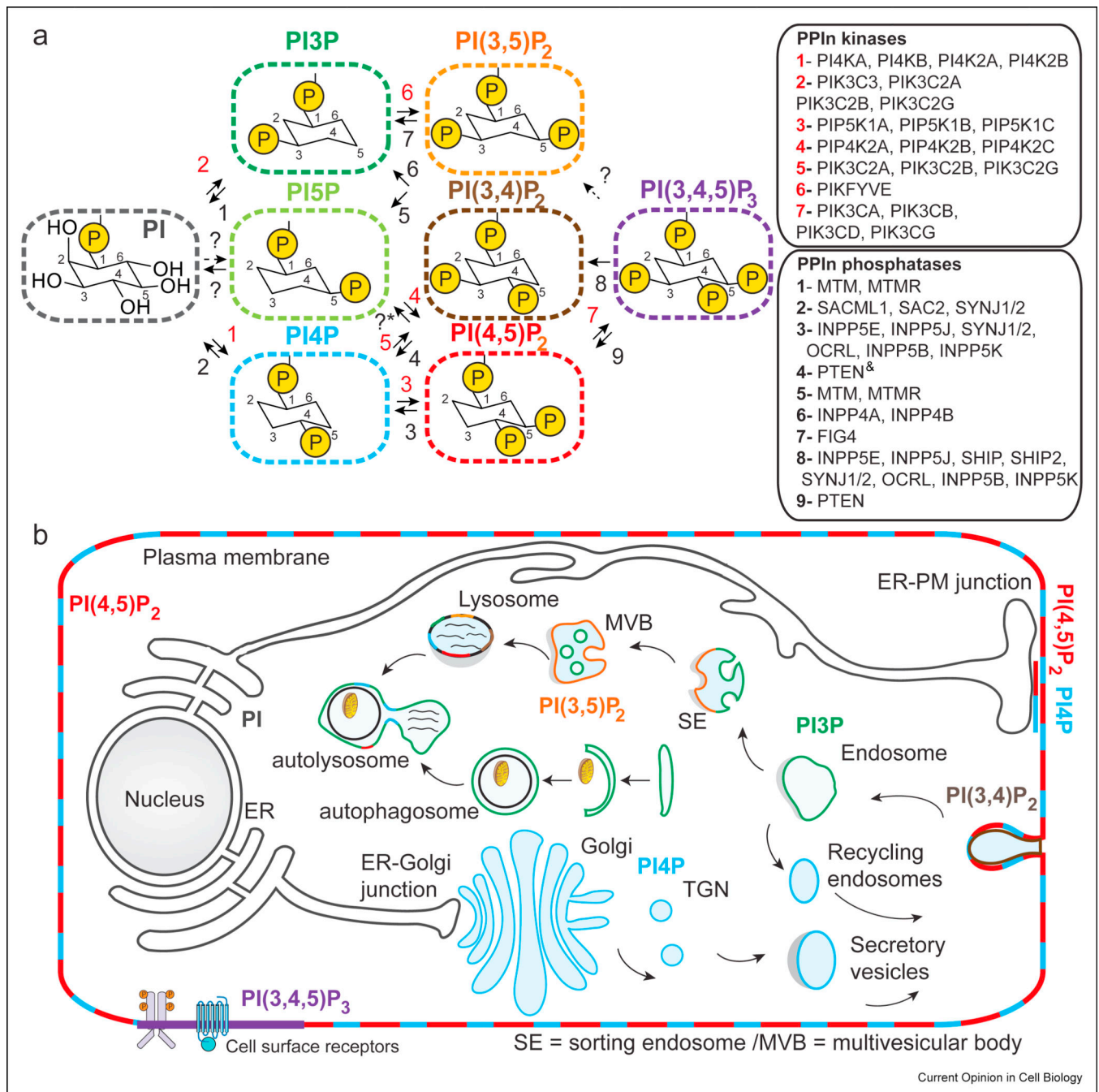
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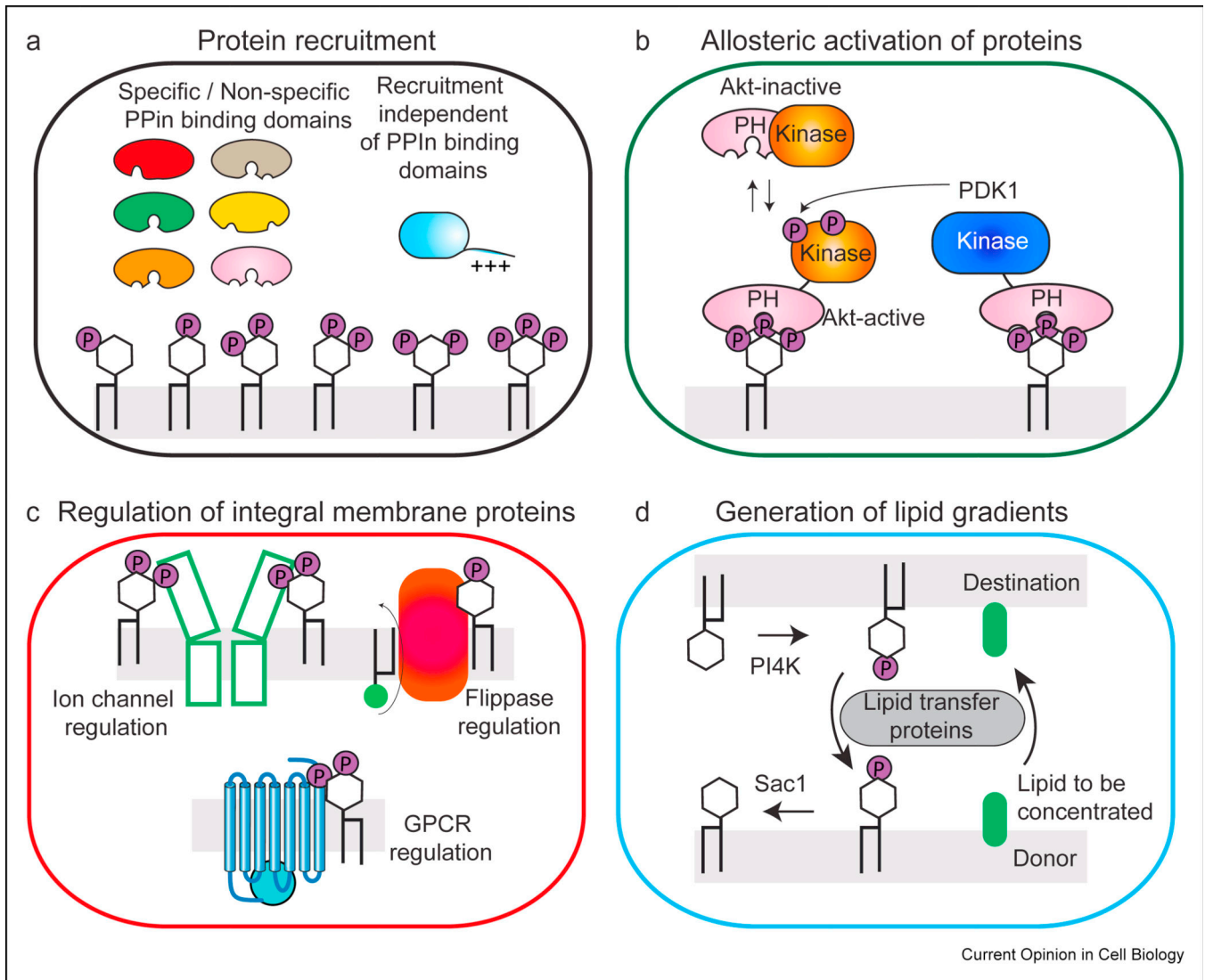


### Figure 1: Identity and cellular localization of phosphoinositides in cells

**A.** Phosphoinositides are composed of two acyl chains attached to a glycerol backbone, with a myo-inositol headgroup. There are a total of seven different phosphoinositide species that can be generated downstream of the precursor phosphatidylinositol through phosphorylation of the hydroxyls on the inositol headgroup. These include phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol 4-phosphate (PI4P), phosphatidylinositol 5-phosphate (PI5P), phosphatidylinositol 3,4 bis-phosphate (PI(3,4)P<sub>2</sub>), phosphatidylinositol 3,5 bis-phosphate (PI(3,5)P<sub>2</sub>), phosphatidylinositol 3,4 bis-phosphate (PI(4,5)P<sub>2</sub>), and phosphatidylinositol 3,4,5

tris-phosphate (PI(3,4,5)P<sub>2</sub> (referred to as PIP<sub>3</sub>). The different human lipid kinases and phosphatases that generate them are indicated in the legend according to the numbers. Phosphoinositide conversion reactions that are not fully established are marked with a ? sign. \*The conversion of PI4,5P<sub>2</sub> to PI5P has been implicated to involve the *PIP4P1/PIP4P2* genes (TMEM55A/TMTM55B proteins), however, recent work indicates the biological activity of these proteins is not driven through PI4,5P<sub>2</sub> phosphatase activity [88]. The & sign indicates reactions that have only recently been identified [89].

**B.** The generation of phosphoinositides are master regulators of temporal and spatial localization of cellular signaling and membrane trafficking events, with their location tightly restricted through the action of the lipid kinases and phosphatases that generate them. They play key roles in secretion from the Golgi, endocytosis, and endo-lysosomal trafficking of membranes [90].



**Figure 2: Phosphoinositides roles in protein recruitment/allosteric activation, modulation of integral membrane proteins, and lipid transport**

**A.** Role of Phosphoinositides in the recruitment of proteins to specific intracellular locations.

Many PPI binding domains have been identified, although many of these domains have varying levels of specificity, and also frequently require coincidence detection of other signals (including both additional lipid and protein binding partners). Phosphoinositides can also regulate protein recruitment outside of lipid binding domains, including polybasic stretches, and non-canonical lipid binding sites.

**B.** Roles of phosphoinositides in allosteric activation of signaling enzymes. Example of the allosteric activation of the pro-growth kinase Akt (PKB) downstream of  $PIP_3$ , where  $PIP_3$  binding to the PH domain disrupts an inhibitory PH-kinase interface, followed by  $PIP_3$  activated phosphorylation of Akt by phosphoinositide dependent kinase 1 (PDK1).

**C.** Phosphoinositides are key regulators of integral membrane proteins, including ion channels, G-protein coupled receptors, and lipid scramblases, flippases and floppases. Phosphoinositides can regulate integral membrane function through allosteric

conformational changes and/or through modulating their coupling to protein binding partners.

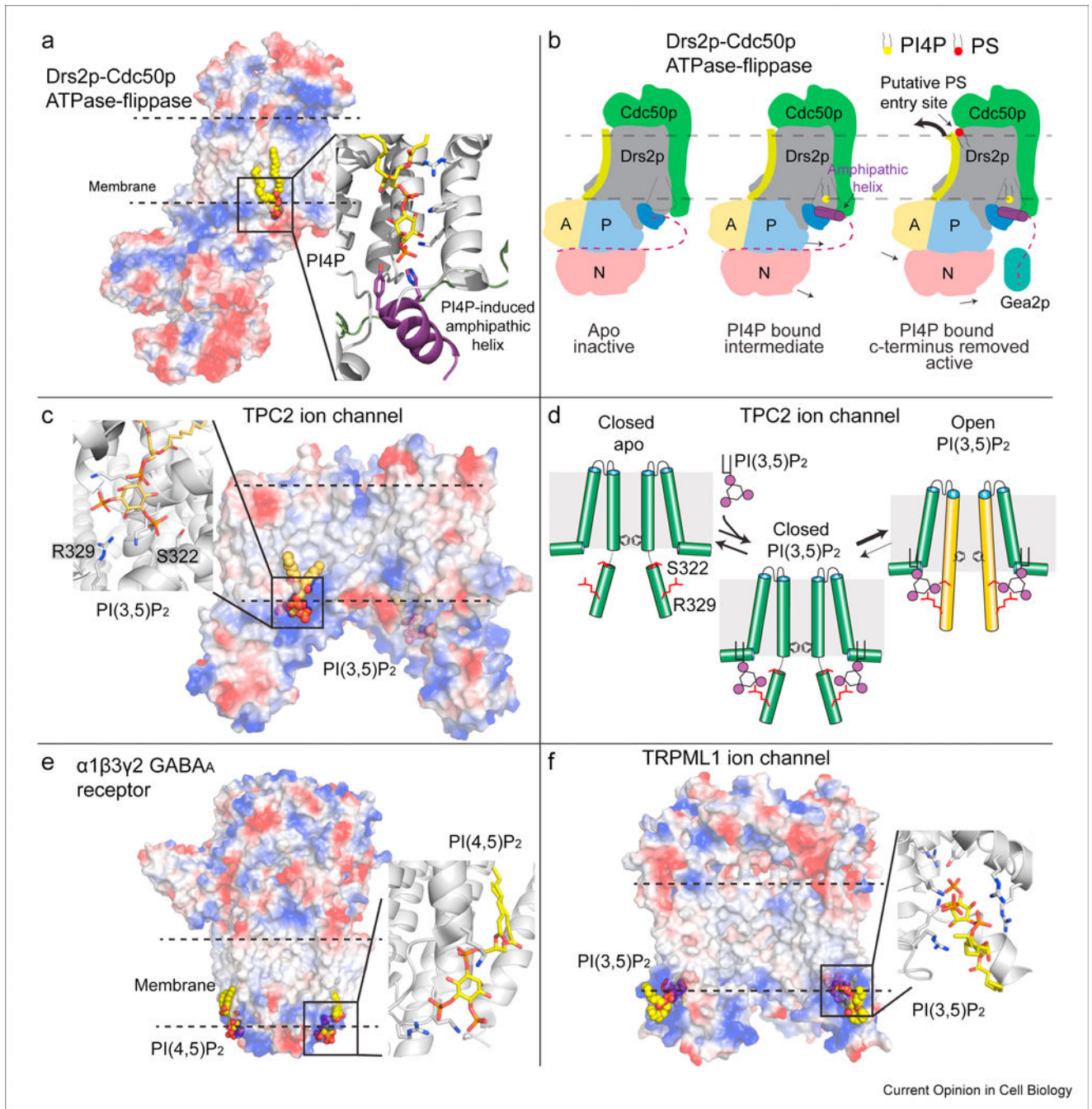
**D.** Phosphoinositides can mediate the transport of lipids against their concentration gradient through the coordinated action of lipid kinases, phosphatases, and lipid transport proteins (LTPs).

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### Figure 3: Structural basis for the regulation of integral membrane proteins by PPIs

**A.** Cryo-EM structure of the yeast dimeric complex of the ATPase flippase Drs2pCdc50p [29]. The protein complex is shown as a surface representation, highlighting charged pockets that mediate lipid binding. The amphipathic helix that binds PI4P is colored purple, with the residues that bind specifically to PI4P shown as sticks.

**B.** A cartoon schematic of the conformational changes that occur during PI4P binding in the flippase catalytic cycle [29,91]. The Cdc50p protein is shown in green, with the Drs2p protein colored according to its domains, with the A, P, and N domains colored in yellow,



blue and red respectively. The inhibitory c-terminus of Drs2p is shown as a dotted line, which is attached to the amphipathic helix that forms upon PI4P binding. The coordinated binding of PI4P and disruption of the c-terminal inhibitory interaction through binding to the Arf-GEF Gea2p leads to an allosteric conformational change in TM2 (colored lime) that opens a putative PS lipid binding pocket, allowing for lipid transfer.

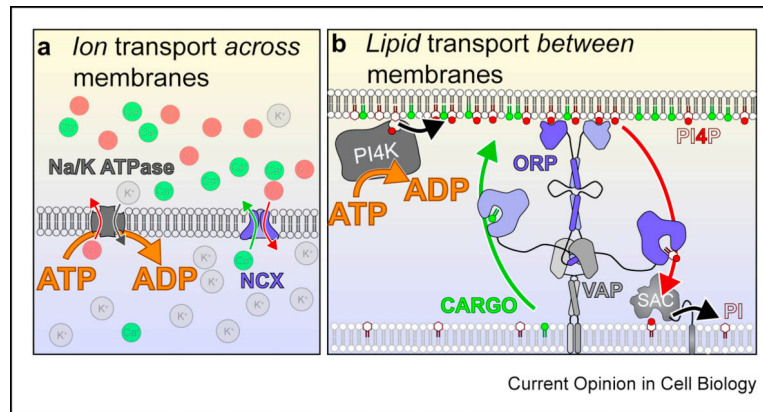
**C.** Cryo-EM structure of the open active form of the human  $\text{Na}^{2+}$  selective two pore channel (TPC2) bound to  $\text{PI3,5P}_2$  [35]. The protein complex is shown as a surface representation, highlighting charged pockets that mediate lipid binding. The specific residues that mediate phosphoinositide binding are shown as sticks. The Arg and Ser residues that interact with  $\text{PI3,5P}_2$  are labeled.

**D.** Cartoon schematic of the molecular mechanism of how  $\text{PI3,5P}_2$  mediates ion channel opening (only the 6 TM-I domain is shown for simplicity). Binding of  $\text{PI3,5P}_2$  biases the equilibrium to the open conformation through allosteric conformational changes in the IS6 helix (colored in orange in the open conformation). This helix contains Ser-322 and Arg-329 which interact with  $\text{PI3,5P}_2$ , and putatively bias the channel towards an open conformation.

**E.** Cryo-EM structure of the type A  $\gamma$ -aminobutyric acid ( $\text{GABA}_A$ ) pentameric ligand gated ion channel bound to  $\text{PI(4,5)P}_2$  [30]. The protein complex is shown as a surface representation, highlighting charged pockets that mediate lipid binding. The specific residues that mediate phosphoinositide binding are shown as sticks.

**F.** Cryo-EM structure of the Transient receptor potential mucolipin 1 (TRPML1) ion channel bound to  $\text{PI(3,5)P}_2$  [23]. The protein complex is shown as a surface representation, highlighting charged pockets that mediate lipid binding. The specific residues that mediate phosphoinositide binding are shown as sticks.





**Figure 4: PPI<sub>n</sub> synthesis powers lipid transfer**

Principles of counter ion (A) and counter-lipid (B) transport. In both cases, transport of a cargo (green) is powered by flow of the counter-molecule (red) down its concentration gradient. Ultimately, the chemical gradient of counter-molecule is established via ATP hydrolysis. This concept mirrors the textbook example of exchangers in ion homeostasis. For example, sodium and calcium ions are both maintained at low cytosolic concentrations relative to the extracellular milieu. The sodium calcium exchanger (NCX) helps maintain low cytosolic calcium concentrations in the absence of ATP hydrolysis, by exchanging cytosolic calcium ions for extracellular sodium ions. Thus calcium moves against its electrochemical gradient (out of cells), but powered by the flow of sodium down its own gradient (into cells). Ultimately, ATP powers this cycle through the sodium-potassium ATPases that actively pump sodium out of cells and maintains the gradient. The same has been proposed for lipid transfer proteins of the ORP family: These can exchange a sterol or phospholipid that is synthesized in the ER for PI4P in another membrane [10]. This membrane is anchored to the ER by the ORP protein itself, usually by PI4P through a PH domain at one end and through an ER anchor (or interaction with the ER receptor VAPa/b) at the other. The ORD domain then exchanges PI4P and cargo lipid. Hydrolysis of the PI4P in the ER by the SAC1 lipid phosphatase ensures vectorial transfer of PI4P, which is unavailable for the return step. Thus the cargo lipid is moved instead, against its own concentration gradient. Ultimately, ATP hydrolysis powers this cycle through the PI 4-kinases that maintains high PI4P levels in the non-ER membrane.

Although this is an apparently novel concept relative to the protein recruitment and activation mechanisms traditionally associated with PPI<sub>n</sub> signaling, there is a unifying theme: In both cases, PPI<sub>n</sub> synthesis couples the energy of ATP hydrolysis to the nonequilibrium acquisition of lipid or protein molecules (or their activity) in restricted membrane compartments. This is an essential function for multi-organelle eukaryotic cells.