Experimental Manipulation of Phosphoinositide lipids, from cells to organisms

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Abstract

Phosphoinositides (PIs) play critical roles in various cellular, physiological, developmental, pathological and infectious processes. They are signaling phospholipids that can affect every aspect of membrane biology, including protein function (e.g., recruitment, activity), membrane physicochemical properties (e.g., curvature, surface charges, packing), and the generation of second messengers. PIs act at very precise locations within the cell in a dose-dependent manner, and their local concentration can vary drastically during signaling and trafficking. Techniques able to manipulate PI amounts acutely and with subcellular accuracy are thus paramount to understand the role of these lipids *in vivo*. Here, we review these methods and emphasize the approaches recently developed to perturb PI levels in multicellular organisms.

Phosphoinositides (PIs) constitute a dynamic and interlinked network of landmark lipids in cellular membranes

PIs are phosphorylated derivatives from the glycerophospholipid phosphatidylinositol (PtdIns) [1]. The inositol head group can be phosphorylated in position three, four or five, forming up to seven distinct species of phosphorylated PtdIns, collectively known as phosphoinositides (PIs): PtdIns3P, PtdIns4P, PtdIns5P; PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ (**Figure 1**). Of note, the kinases that phosphorylate PtdIns(4,5)P₂ on the 3rd position are only present in animals and as a result PI(3,4)P₂ and PI(3,4,5)P₃ are not detected in fungal and plant cell membranes [2].

Each PI species localizes in specific organelles [3]. PI differential accumulation in membranes is critical for many cellular processes, and requires various cascading enzymatic activities to interconvert PIs into one another (**Figure 1**) [1, 4, 5]. PI kinases and phosphatases or phospholipases have various substrate and catalytic activities, subcellular localizations, spatiotemporal expression patterns and control mechanisms. Thus, **PI-related enzymes** (Glossary Box) constitute an extensive toolkit that precisely define PI concentrations and localization. Most of these enzymes act in a non-redundant manner at the cellular and organismal levels, although they may carry similar catalytic activities [1, 6]. Additionally, long-term PI modifications may lead to indirect and pleiotropic effects that can be difficult to interpret because PIs: (i) regulate many cellular processes; (ii) are present in multiple cellular compartments at the same time; and (iii) are highly interdependent on each other. Furthermore, depleted enzymes might have functions beyond their catalytic activity, making it even more hazardous to conclude that the observed effects originate from the modulation of the PI species

itself. Thus, even though knockout and knockdown strategies for depleting the PI levels are very useful, inducible and targeted methods are essential to untangle the direct and indirect function of PIs. Deciphering the direct impacts of PIs in cells and how their perturbation leads to complex phenotypes and diseases remains one of the most pressing issues in lipid biology and membrane signaling.

Here, we review the diverse methodologies used to manipulate PI levels in cells and organisms and discuss their respective advantages and limitations. We consider the pharmacological approaches developed to inhibit PI-related enzymes. We address how the inhibitions of PI-related enzymes are applied to disturb the flux within the PI network and to assess the function of each PI species. We also examine how lipid analogs, including caged lipids, have recently been harnessed to heighten the concentration of a given PI. Furthermore, we report on recent genetic strategies established to modify PIs in an inducible and targeted manner, featuring **chemogenetics**, **optogenetics** and **voltogenetics** approaches. We discuss their optimal implementation for *in vitro* studies in cell lines and for experiments with animal and plant models. We describe how PI-related enzymes have been repurposed and engineered to build complex inducible systems, with ever-increasing spatiotemporal resolution and tailored specificities. Finally, as key take-home messages, we provide examples on how these arrays of experimental techniques can be used to tackle the complex functions of PIs in cells and organismal development and physiology.

Pharmacological approaches for PI manipulation

It is rather intuitive that, to modulate the respective levels of PIs, drugs preventing their synthesis or their hydrolysis would be tools of choice. Figure 1 recapitulates some of the compounds used to perturb the PI network. Most are chemicals targeting PI kinases (in particular PtdIns 3-kinases and PtdIns 4-kinases which are high-value targets in oncology, immunology and virology). These compounds curb the production of a given PI species while it is still consumed by PI phosphatases and phospholipases [7, 8]. PI levels can also be adjusted through inhibition or activation of phosphatases and phospholipases that hydrolyze them (**Figure 1**). For example, several inhibitors of the Phosphatase and TENsin homolog (PTEN) and Src Homology2-containing 5-phosphatase (SHIP) directly modulate the pool of PtdIns(3,4,5)P₃ (**Figure 1**) [9, 10]. Note that relatively few compounds can affect the levels of PtdIns(4,5)P₂ by either targeting PtdIns4P 5-kinases or PtdIns(4,5)P₂ 5-phosphatases (**Figure 1**), despite the importance of this lipid in cell biology.

PI manipulation via small molecules has been used in isolated cells, in plant and animal models and in clinical set-ups [7, 8, 10-13]. However, as tools to address fundamental questions about PI function, they have some limitations: (i) they are only active within a specific concentration range; (ii) they may have off-target effects; (iii) they may not penetrate easily in complex tissues; and (iv) they impact the whole sample and cannot target precise tissues, cells or organelles. Nonetheless, pharmacological modulation of PI-related enzymes constitutes a relative fast and easy modification of PI levels without the use of complex experimental and genetic systems.

Delivery of PIs and PI analogues

Modulation of PI levels can also be achieved by directly increasing the number of PI molecules. PIs are highly anionic and, as such, are largely impermeable to membranes when added exogenously. To tackle this issue, membrane permeable PI analogs have been developed (**Figure 2A**, [14-16]). The negative charges of these lipids are neutralized by protective groups, making them membrane permeable. After entering the cell, endogenous cytosolic esterases

hydrolyze the chemical groups, exposing the functional inositol head. Delivery of PIs and PI analogs showed that PtdIns(3,4)P₂ and PtdIns(3,4,5)P₂ stimulate clathrin-mediated endocytosis, tyrosine kinase receptor recycling and cell protrusion in animal cultured cells [17-19]. Injection of PtdIns(4,5)P₂ analogs in *Drosophila* embryos undergoing cellularization, increased the GFP-PH^{PLC} PtdIns(4,5)P₂ **biosensor** signal at the plasma membranes, illustrating that the method efficiently elevated PtdIns(4,5)P₂ levels [20]. In these conditions, myosin-II was more stable at the plasma membrane, suggesting a role of PtdIns(4,5)P₂ in myosin membrane association *in vivo* [20]. Non-modified (as well as fluorescent) PIs were also successfully provided to plant cells, including Arabidopsis seedlings, using polyamine carriers neutralizing the negative charges of PI phosphate groups [21-23]. Delivery of PtdIns(3,5)P₂ led to vacuolar defects mimicking the phenotype observed upon loss-of-function of the putative 5-phosphatase AtSAC2–AtSAC5 [21].

When interpreting results from PI delivery assays, it is critical to remember that exogenously added lipids may artificially concentrate in non-endogenous compartments or on the wrong membrane leaflet. They may also be rapidly modified into another lipid species by endogenous enzymes. A solution to these problems is the use of **caged-PI** analogs that can be acutely activated (**Figure 2B**) [24-26]. In that case, the PI head is protected by a photoremovable group, which allows light-regulated uncaging at specific organelles [25, 27]. Membrane permeable, photoactivatable PtdIns(4,5)P₂ can be loaded into cells in an inactive form and subsequently uncaged by light (**Figure 2B**). Such photoactivation is extremely fast (i.e., less than a second) and thus allow manipulating PtdIns(4,5)P₂ levels faster than its metabolism. Caged PtdIns(4,5)P₂ were used to study the role of this lipid in exocytosis in mouse adrenal chromaffin cells [24].

Taken together, PI analogs are valuable chemicals, that can even be suitable for studies in multicellular organisms. However, lipid analogs remain expensive and their synthesis requires specialized skills, which limits the widespread use of this technique [25, 28]. Genetically encoded strategies for PI perturbation complement the approaches mentioned above. They rely on stable or transitory transfer of genetic constructs that, when expressed by host cells, will perturb PI homeostasis [28, 29]. Among these strategies, we will distinguish chronic from acute modulation techniques.

Constitutively expressed genetic systems for PI manipulation

Overexpression of genetic constructs that encode PI kinases, phosphatases or phospholipases, allows the chronic modification of PI levels (**Figure 3A**). However, the constitutive and ubiquitous perturbation of PIs is rarely viable in multicellular organisms. To by-pass lethality, it is possible to express the enzyme in specific tissues. For example, PtdIns(4,5)P₂ depletion in *Drosophila* spermatocytes was achieved by expressing the *Salmonella* PtdIns(4,5)P₂ phosphatase *SigD* in this cell type [30]. Furthermore, the overexpression of the PtdIns4P 5-kinase *Skittles* specifically in the germ line led to the opposite phenotypes compared to those induced by *SigD* ectopic expression [30]. Constitutive manipulation of PIs in few cell types avoids potential problems due to lethality and therefore allows studying the roles of PIs in cells that are in a physiological context.

It is also possible to repurpose PI modifying enzymes to localize their enzymatic activities to specific membrane compartments (**Figure 3B**). For instance, the endogenous PtdIns4P phosphatase called Sac1 normally resides in the membrane of the Endoplasmic Reticulum (ER). However, by taking the isolated phosphatase domain of Sac1 and targeting it to the plasma membrane via a membrane anchor (e.g., **Myristoylation And Palmitoylation** – MAP), it is

possible to rewire the Sac1 function to specifically erase PtdIns4P at the plasma membrane, not at the ER (**Figure 3B**). Such rewiring approach was used in transiently transformed *Nicotiana benthamiana* plants [31]. Upon expression of *MAP-Sac1*, PtdIns4P biosensors are solubilized from the plasma membrane and delocalized to intracellular compartments, indicating a decrease of the PtdIns4P pool at the plasma membrane [31]. Sac1-mediated PtdIns4P depletion in transient assays showed that PtdIns4P is the main anionic lipid responsible for the membrane electrostatic field in plant cells [31, 32] and that it is required for the plasma membrane association of many plant proteins [33-36].

Inducible expression of PI-related enzymes

As an alternative to the constitutive expression, it is also possible to use **inducible expression systems**. For example, estradiol-inducible expression of Arabidopsis *PtdIns4P 5-Kinases* (*PIP5Ks*) (induction for 24 h) allowed to study the developmental impact of their overexpression and particularly their role in polar auxin transport and root gravitropism [37, 38]. However, by using endogenous enzymes, it is difficult to know whether the phenotypes are caused by the overexpression of the enzyme itself or the accumulation of its product (i.e., PtdIns(4,5)P₂). To circumvent this pitfall, Gujas et al., exploited a genetic approach that enabled them to overexpress the highly processive human *PIP5K* (*HsPIP5K α*) in a tissue-specific inducible manner. GFP-HsPIP5K α could be detected only three hours post induction, and PtdIns4P and PtdIns(4,5)P₂ levels were already affected one hour after induction [39]. This approach revealed that ectopic PtdIns(4,5)P₂ production impacted the differentiation of plant vascular tissues. A similar strategy was also used for the inducible depletion of PtdIns(4,5)P₂ in plant cells (**Figure 3C**) [40]. The system is based on the expression of the catalytic domain of *Drosophila* *OCRL* (encoding a PtdIns(4,5)P₂ 5-phosphatase), which unlike human OCRL is active at 20°C, fused to a membrane targeting sequence (i.e., MAP) and to a red fluorescent protein. Using a suite of genetic control, this tool was shown to specifically targets PtdIns(4,5)P₂ without massively influencing the other anionic lipids present at the plasma membrane. The synthetic enzyme was detected three hours after the gene induction and PtdIns(4,5)P₂ levels already decreased approximately one hour post induction [39, 40]. This strategy was deployed to study the impact of PtdIns(4,5)P₂ depletion on plant root and shoot development and on the localization of plasma membrane proteins involved in endocytosis and cytoskeleton dynamics [40].

Altogether, these studies highlight that inducible genetic approaches are useful to address the developmental impact of lipid modification in multicellular organisms. They act in the range of a few hours, which is still slow compared to the speed of the endogenous PI metabolism. Alternative assays allow acute PI modification and rely on genetically encoded systems that can be tuned by a chemical (**chemogenetics**), by light (**optogenetics**) or by voltage (**voltogenetics**) [29].

Acute pharmacological induction of endogenous PI metabolic enzymes

PIs are known for their roles as **secondary messengers**. In particular, PtdIns(4,5)P₂ is hydrolyzed by Phospholipase C (PLC) into Diacylglycerols (DAG) and Inositol 1,4,5-trisphosphate Ins(1,4,5)P₃ and both act as secondary messengers, triggering Ca²⁺ influx in the cytoplasm and modulating K⁺ channels conductivity. PLCs also breakdown other PI species to a lesser extent. PLC-mediated hydrolysis of PtdIns(4,5)P₂ occurs in response to (nor)-epinephrine or acetylcholine perception by G-Protein Coupled Receptor (GPCR) (**Figure 4A**, [41]). It is possible to take advantage of these signaling pathways by stimulating them, thus acutely depleting plasma membrane PIs. Many studies used cell types that did not express the

corresponding receptors, and therefore the cells had to be transfected with the signaling components [42, 43]. In vitro mammalian cells transfected with the muscarinic acetylcholine receptor M1 (M₁R) and stimulated with the acetylcholine agonist oxotremorine M (Oxo-M) for 60 s showed a decreased PI level by roughly 70% [44]. PtdIns(4,5)P₂ depletion was observed at 20 s post activation using the PH^{PLC} fluorescent reporter solubilization or **resonant energy transfer biosensors** read-outs [45, 46]. Oxo-M-triggered PtdIns(4,5)P₂ perturbation substantiated the function of OCRL in dephosphorylating PtdIns(4,5)P₂ on endocytic vesicles [47]. Relying on the M₁R receptor has the advantage of being very rapid and of exploiting endogenous components. However, the M₁R signaling pathway itself regulates endogenous cellular processes, which must be considered when interpreting results from PLC-induced depletion of PIs.

Chemogenetics: chemically-induced genetic strategies for acute manipulation of PIs

The rapamycin-inducible method allows to acutely recruit lipid modifying enzymes from the cytosol to a given membrane within few seconds [28]. It is based on the inducible heterodimerization of the FKB domain of the mammalian Target of Rapamycin (mTOR) protein with FKBP12. One of the components (e.g., FRB domain) is anchored to a specific organelle via a membrane targeting sequence, while the other (e.g., FKBP domain) is fused to the catalytic domain of a PI-related enzyme [48-51]. Because the second construct consists only of the isolated catalytic domain, its default localization is in the cytoplasm. When it is not attached to a membrane, the enzymatic module has a low activity, which is likely due to poor access to its lipid substrate. However, a treatment with rapamycin induces the recruitment of the chimeric construct to its target membrane, allowing the enzyme to meet its substrate and thus to almost simultaneously catalyze the reaction. Using a rapamycin-inducible system, a 5-phosphatase domain can associate with the plasma membrane in ~30 s, and PtdIns(4,5)P₂ biosensors are concomitantly solubilized, indicating acute PtdIns(4,5)P₂ depletion (**Figure 4B**, [49-51]). Alternatively, the 3-phosphatase MTM1 can be recruited to Rab5-containing early endosomes to specifically deplete PtdIns3P from the membrane of this compartment [48]. Since the first publications of this lipid modifying approach [48-51], the method was rewired to modify virtually all PI species through the recruitment of phosphatases, phospholipases or kinases at different subcellular localization (see **Table 1**). For example, concomitant rapamycin-inducible depletion of plasma membrane PtdIns(4,5)P₂ and PtdIns4P showed that PtdIns4P, together with PtdIns(4,5)P₂, contributes to the electrostatic field at the surface of animal cell plasma membrane and to the localization of the polarity protein DISCS LARGE (Dlg) in *Drosophila* [52, 53]. This system was also utilized to challenge the presence and the function of various subcellular pools of PIs, including PtdIns, PtdIns4P, PtdIns(3,4)P₂ and PtdIns(3,5)P₂ [54-60]. In addition, rapamycin-inducible dimerization was employed to probe the importance of PIs in membrane contact sites structure-function relationship. In particular, this strategy allowed asking whether the PtdIns4P phosphatase Sac1 acts in *cis* (i.e., at the ER membrane) or in *trans* (i.e., at the plasma membrane or in the trans-Golgi Network) in animal cells [61, 62]. FRB/FKBP-mediated accumulation of PtdIns(3,4,5)P₃ was reported in *Drosophila* larva, proving that the approach was viable for PI manipulation in multicellular organisms [63]. This method was also recently implemented in plants using transient assays in *N. Benthamiana* for the inducible depletion of PtdIns4P at the plasma membrane [64].

The FRB/FKBP system shows acute manipulation of PIs in membranes of *in vitro* animal cell lines but also in plants using transient assays. The FRB/FKBP strategy has, however, some limitations, including the irreversibility of dimerization induced by rapamycin. This drawback can be overcome by the addition of a competitive ligand [65]. Also, rapamycin treatment interferes with endogenous mTOR complexes that have pleiotropic functions. To avoid this

issue, rapamycin analogs that do not target endogenous mTOR proteins [66] and controls consisting of catalytically inactive enzymes must be used. Finally, rapamycin-inducible strategies have two components and thus require the genetic expression of two constructs simultaneously. Expressing proteins from several plasmids is not always easy, especially when working with cells or organisms that are difficult to transform and can lead to heterogeneous accumulation levels. To circumvent these difficulties, it is possible to express both parts on a single construct (e.g. by using self-cleaving peptides) [67]. Alternatively, single-component chemogenetic designs are emerging that are based on the so-called self-localizing ligand-induced protein translocation (SLIPT) strategy [28, 68-70]. In SLIPT, a ligand is engineered to accumulate at a specific subcellular localization and then it recruits a protein of interest (e.g., PI-related enzyme) (**Figure 4C**).

Optogenetics: light-induced genetic systems for acute manipulation of PIs

Optogenetic lipid modification approaches resemble rapamycin-inducible methods in the sense that they often rely on inducible dimerization strategies, aiming at rewiring subcellular localization of PI-related enzymes. However, in the case of optogenetics, specific wavelengths of light trigger heterodimerization between a **photoreceptor** and its downstream partner. Most lipid-modifying optogenetic techniques exploit plant or bacterial blue-light photoreceptors (e.g., **cryptochrome**, **LOV-domain**) (**Figure 5A and Table 1**) [29, 71-75]. It is also possible to use **phytochrome**-based strategies [76]. Phytochromes have the advantage to induce protein dimerization in response to red light, with far-red light reversing the reaction, therefore acting like an active ON-OFF toggle switch (**Figure 5B**). Each photoreceptor has distinct sensitivity and kinetics of dimerization; thus, they can be engineered to achieve various optogenetic properties in terms of speed of action, reversibility and responsiveness [77]. Like the rapamycin-inducible method, it is now possible to manipulate the levels of many PI species using various enzymatic modules at different intracellular membranes (see **Table 1**).

The advantages of the optogenetic strategies include: (i) no treatment with potentially toxic molecules; (ii) no small molecule diffusion into the medium and inside the cell; (iii) their speed of action; (iv) their reversibility; and (v) the ability to pattern light for stimulation of the optogenetic tool in a group of cells or subcellular compartments. Furthermore, using optogenetics, it is possible to obtain subcellular precision by targeting one of the components to specific organelles (see **Table 1**); and through **photomanipulation**. For instance, actuation of CRY2-OCRL by an evanescent blue light wave, generated at the coverslip, can selectively perturb the pool of PtdIns(4,5)P₂ located at the plasma membrane contacting the coverslip [71]. Also, a blue light pulse on a region of the plasma membrane can manipulate PI concentration locally [71, 78]. On the other end, like for chemogenetics, optogenetic approaches often require the genetic expression of two constructs simultaneously. However, a system dubbed optoPB, based on a blue-light-sensitive LOV domain enables inducible translocation to the plasma membrane with a single engineered protein [74]. In optoPB, blue light induces a conformational change that exposes a polybasic tail [74, 79]. The polybasic tail in turn interacts with the negatively charged plasma membrane, thereby triggering cytosol-to-plasma membrane relocalization using a single chimeric construct (**Figure 5C**).

Optogenetics strategies have limitations to consider. First, optogenetic systems are induced by light; thus, they may require dedicated equipment, such as a green room to manipulate the samples under non-inducible lights. Patterning light in a rapid and reproducible manner under the microscope may also require dedicated hardware for photomanipulation [76]. Second, the use of the optogenetic constructs limits the number of channels that are available for imaging. Indeed, if possible, it is best to avoid fluorescent proteins that are excited by the same

wavelength as the photoreceptor present in the optogenetic actuator. Third, photoreceptors require a chromophore for function. Some of these chromophores are produced endogenously in most living organisms (e.g., flavin-based chromophores used by blue-light photoreceptors). In that case, the availability of the chromophore is not an issue. The presence of the chromophore may, however, be limiting for phytochrome-based strategies. Indeed, phytochromes require a light-sensing bilin chromophore, which is not produced by animal cells. The chromophores thus need to be added exogenously before the optogenetic experiments [76]. Bilin chromophores, such as phycocyanobilin, are relatively unstable, and their injection in living metazoans can be inefficient (e.g., poor or no diffusion to the target tissues) and unreliable (e.g., rapid degradation). By contrast, bilin chromophores are endogenously synthesized in plants. However, the development of optogenetics in the green lineage is challenging, because photosynthetic organisms need light to survive.

Voltogenetics: voltage-induced genetic approaches for acute manipulation of PIs

The discovery of voltage-dependent PI phosphatases, the so-called VSPs, led to the development of voltage-controlled and genetically encoded (voltogenetics) tools for PI acute manipulation [80-82]. Wild-type Ci-VSP and Dr-VSP are mainly PtdIns(4,5)P₂ 5-phosphatases and are found in *Ciona intestinalis* (Metazoa Urochordata) and zebrafish (*Danio rerio*, Metazoa Teleostei), respectively. Upon transfection into human cultured cells, a ~1 s depolarization (from -20 mV to +100 mV) transiently erases PtdIns(4,5)P₂ [83]. Interestingly, the initial PtdIns(4,5)P₂ levels are recovered ~15 s after repolarization. Using a voltogenetic approach in *Xenopus* oocytes, it was recently confirmed that the activity of K⁺ channel Slo3 is PtdIns(4,5)P₂-sensitive [84]. VSPs can also be engineered to accommodate additional enzymatic activities. The Ci-VSP voltage-sensitive domain (VSD) was fused to PTEN (Ci-VSPTEN), which specifically modifies the pool of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂ in response to electric stimulation *in vivo* [85]. Electrophysiology approaches require specific expertise, and may be difficult to apply to live organisms, but they are fast and reversible and may be used to assess *in vivo* phosphatase activity upon heterologous expression.

Concluding Remarks

In this review, we highlighted various experimental systems deployed to manipulate PIs in different models. These techniques include pharmacological and genetic strategies as well as acute and chronic modifications. We can distinguish approaches that allow rapid and highly targeted manipulations but that are mainly used in cultured cells, from slower perturbation strategies that are more amenable to multicellular organisms such as animals and plants. Researchers should thus choose the methods according to their questions and biological models. Addressing the role of PIs in morphogenesis may not need ultrafast modifications, because of the dynamics of development by comparison to that of PI metabolism. By opposition, the study of very fast processes, such as vesicular trafficking or membrane contact establishment may require the implementation of rapid perturbation techniques. Optogenetics appears as a particularly promising strategy that should allow bridging the gap between research in isolated cells and in whole organisms (**outstanding question box**). One can envision that light-mediated fast lipid perturbations may soon allow analyzing the role of PIs over a large range of time scales. It may then be possible to address both the direct molecular targets of PIs and their downstream developmental or physiological effects.

Glossary box

PI-related enzyme: Enzyme that impacts PI metabolism or dynamics, and that includes PI kinases, phosphatases or phospholipases.

PI biosensors: Transgenic expression of lipid-binding domains that interact with a given PI species in a stereospecific manner, fused with a fluorescent protein (i.e., genetically encoded sensors). Such probe works as a translocation sensor, because they translocate from the cytosol to a given membrane upon interaction with their cognate lipid and they dissociate from that membrane upon removal of the PI species.

Resonant Energy Transfer biosensors: Intramolecular biosensors that change conformation upon binding to a given PI species. The change of conformation is detected by a change in the fluorescence resonant energy transfer (FRET) or bioluminescence resonant energy transfer (BRET) that happens when the distance between the two fluorescent proteins (a donor and an acceptor) present in the biosensor varies. Resonant energy transfer biosensors can detect dynamic and rapid variations in PI levels and are more quantitative than translocation sensors.

Caged PIs: Chemically modified lipids that renders the PI head group mainly inert until it is removed for example using light.

Chemogenetics: Genetically-encoded system that allows manipulating a biological pathway using small molecules.

Optogenetics: Genetically-encoded system that allows manipulating a biological pathway using light.

Voltogenetics: Genetically-encoded system that allows manipulating a biological pathway using electric stimulation.

Secondary messenger: Signaling molecules released in the cell in response to a primary signal and that trigger signal transduction cascade(s).

Rapamycin: Small molecule inhibitor of mTOR derived from *Streptomyces hygroscopicus*, that has potent antiproliferative and immunosuppressant properties.

Photomanipulation: Describes a range of techniques that allow microscopists to target illumination to specific areas of interests in their field of view, e.g., to photoactivate, photobleach or photoablate a given protein, organelle, cells or group of cells.

Inducible expression system: genetically encoded systems that enable researchers to induce the expression of a gene in response to a given stimulus (e.g., small molecule, light).

Myristoylation: Non-reversible lipid modification used to anchor proteins into biological membranes, via the covalent attachment of a myristoyl group to an N-terminal Glycine.

Palmitoylation: Reversible lipid modification of a protein, which promotes membrane association through S-acylation of cysteine residues (e.g., covalent attachment of palmitic acid or other type of fatty acids).

Photoreceptor: Protein specialized in detecting certain wavelengths of light and to transduce this information into specific signals, often via conformational changes. Most photoreceptors function in conjunction with light-sensitive organic molecules known as chromophores.

Cryptochrome2 (CRY2): blue light photoreceptor from plants that uses Flavin adenine dinucleotide (FAD) as a light-harvesting chromophore. Blue light perception triggers the interaction with the N-terminus of the *Arabidopsis thaliana* transcription factor CIB1 (i.e. CIBN).

Phytochrome b (phyB): Plant protein covalently linked to a light-sensing bilin chromophore, that is sensitive to red and far-red light. Red light triggers the interaction with Phytochrome Interacting Factors (PIF) and far-red light their dissociation.

Light-Oxygen-Voltage-sensing (LOV) domain: Protein domain, present in plants, algae, as well as some bacteria and fungi, that in conjunction with a flavin mononucleotide chromophore acts as a blue-light photoreceptor.

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Highlights:

- Phosphoinositides (PIs) are signaling lipids because they are precursors of second messengers or variations in their local quantity act as a signal in itself.
- PI metabolism is fast and interdependent as they are rapidly converted into each other by kinases and phosphatases.
- Mutations of PI enzymes impacts development, but it is difficult to untangle the direct from indirect phenotypes induced by chronic PI perturbations.
- Experimental systems can acutely manipulate PI levels with subcellular accuracy in cultured cells. These include chemogenetic, voltogenetic and optogenetic strategies and operate in relevant time frames that are faster than the endogenous PI metabolism.
- Systems for the inducible manipulation of PIs are becoming available in multicellular organisms, which enable to address the function of these lipids during development and in their in vivo physiological context.

Outstanding question box

- Will it possible to manipulate PI levels on demand by complementing the palette of available PI-related enzyme inhibitors? In particular, we urgently need to uncover pan-PIP5K drugs to prevent PtdIns(4,5)P₂ synthesis and to characterize additional PI phosphatase inhibitors, notably to target the SAC and INPP protein families.
- To what extent is it still feasible to improve the diversity of PIs that can be labeled, caged and delivered into cells? We should also strive to make these approaches more widely available to the research community, perhaps by using *in cellulo* tagging strategies.
- Minor pools of lipids can be difficult to visualize because of their tiny concentrations, but they may nonetheless be highly relevant in terms of function and physiology. Can acute perturbation methods be leveraged to uncover the roles of minor pools of specific PI species?
- Can we engineer synthetic manipulation systems to experimentally perturb lipid homeostasis at the nanoscale? Such assay may allow uncoupling the function of different lipid pools that are present on the same membrane and could help to probe PI dynamics and diffusion.
- Most optogenetic lipid modification strategies are based on inducible dimerization approaches. Future research should aim at engineering new light-gated modules to directly manipulate the activity of PI-related enzymes.
- What are the technical limitations in designing acute perturbation systems that are compatible with studies in multicellular organisms and to tackle their roles in development, normal physiology, and disease progression?
- How important is the fatty acid chain composition in PI function *in vivo*? This has remained mainly unanswered and we should design experimental methods to probe and perturb PI fatty acid composition *in vivo*.

Type of inducible system ^a	Construct ^b	Reaction/ targeting ^c	Refs	Addgene #
Rapamycin (or rapalog)-inducible dimerization	FKBP-PI4KA ^{ΔN}	PtdIns → PtdIns4P	[57]	139311
	FKBP-PI4KB	PtdIns → PtdIns4P	[54]	139310
	FKBP-PIP5K	PtdIns4P → PtdIns(4,5)P ₂	[50]	20157
	FKBP-iSH2	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃	[50]	20159
	FKBP-PTEN	PtdIns(3,4,5)P ₃ → PtdIns(4,5)P ₂	[55]	116866
	FKBP-PLCδ1 ^{Δ44-ΔPH}	PtdIns(4,5)P ₂ → Ins(1,4,5)P ₃ and DAG	[58]	-
	FKBP-PJ	PtdIns(4,5)P ₂ → PtdIns4P → PtdIns	[53]	37999
	FKBP-Inp54p	PtdIns(4,5)P ₂ → PtdIns4P	[50, 51]	20155
	FKBP-INPP5E	PtdIns(4,5)P ₂ → PtdIns4P	[49]	67516
	FKBP-PJ ^{INPP5E}	PtdIns(4,5)P ₂ → PtdIns4P	[53]	38001
	FKBP-PJ ^{SAC}	PtdIns4P → PtdIns	[53]	38000
	FKBP-Sac1 ^{ΔTDM}	PtdIns4P → PtdIns	[61]	108132
	Sac1 ^{ΔTDM} -FKBP	PtdIns4P → PtdIns	[61]	108123
	FKBP-BcPI-PLC ^{AA}	PtdIns → Ins1P and DAG	[58]	-
	FKBP-INPP4B	PtdIns(3,4)P ₂ → PtdIns3P	[55]	116864
	FKBP-MTM1	PtdIns3P → PtdIns	[57]	51614
	Lyn ^{N11} -FRB	Plasma membrane	[49-51, 53, 57, 86, 87]	20147, 67517, 38003, 38004, 155228
	GAP43 ^{N19} -FRB	Plasma membrane	[49]	67518
	Lck ^{N10} -FRB	Plasma membrane	[88, 89]	67902
	FRB-Sac1 ^{C67}	Endoplasmic reticulum	[54]	139317
	FRB-PMP ^{C10}	Peroxisome	[54]	13916
	FRB-Giantin	Golgi	[54, 89, 90]	139313, 67903
	FRB-Rab5	Early endosome	[57]	51612, 64209
	FRB-Rab7	Late endosome	[57]	51613, 64210
	Akap1 ^{N31} -FRB	Mitochondria	[54]	139315
	Tom70p ^{N33} -FRB	Mitochondria	[91, 92]	46942, 59352
	FRB-MoA ^{N37}	Mitochondria	[89, 90]	67904
	LAMP-FRB	Lysosome	[55, 90]	-
	TGN38-FRB	Trans-Golgi Network	[59]	-
CRY2-based blue light dimerization system	CRY2-INPP5E	PtdIns(4,5)P ₂ → PtdIns4P	[71]	79561
	CRY2-OCRL	PtdIns(4,5)P ₂ → PtdIns4P	[71]	79566, 79565
	CRY2-iSH2	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃	[71]	66839
	CRY2-PIP5K1	PtdIns4P → PtdIns(4,5)P ₂	[93]	79569, 79570
	CIBN-Ras4B ^{C14}	Plasma membrane	[71, 94]	26867, 79574
	Lyn ^{N11} -CIBN	Plasma membrane	[71]	79572
	ER-P450 C1 ^{N37}	Endoplasmic reticulum	[73]	-
	CIBN-OMP25 ^{C44}	Mitochondria	[73]	-
	CIBN-mitoNEET	Mitochondria	[95]	89480
	Rab3-CIBN	Insulin granule	[96]	-
Magnet blue light dimerization system	pMagFast(3x)-MTMR1	PtdIns3P → PtdIns	[73]	-

(Neurospora crassa LOV ^{VIVID} -based)	pMagFast2(3x)-OCRL	PtdIns(4,5)P ₂ → PtdIns4P	[73]	-
	iSH2-pMagFast2(3x)	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃	[75]	67298
	iSH2-pMag(3x)	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃	[75]	67304
	nMag(1x)-KRas4B ^{C14}	Plasma membrane	[73]	-
	nMag(3x)-KRas4B ^{C14}	Plasma membrane	[73]	-
	P450 2C1 ^{N37} -nMag	Endoplasmic reticulum	[73]	-
	nMag(1x)-OMP25 ^{C44}	Mitochondria	[73]	-
	nMag(2x)-OMP25 ^{C44}	Mitochondria	[73]	-
	Rab5-nMag	Early endosome	[73]	-
	LAMTOR ^{N40} -nMag	Lysosome	[73]	-
Enhanced Magnet dimerization system (thermostable)	eMagB ^F -OCRL	PtdIns(4,5)P ₂ → PtdIns4P	[72]	162254
	eMagA ^F -N-Ras ^C	Plasma membrane	[72]	162247
improved Light Dimerization System — iLID (Avena sativa LOV2-based blue light receptor)	SspB R73Q-OCRL	PtdIns(4,5)P ₂ → PtdIns4P	[73]	-
	iLID-KRas4B ^{C14}	Plasma membrane	[87, 97-99]	85680, 1600999, 604411, 155229
	Lyn ^{N11} -iLID	Plasma membrane	[98]	161001
	ADRB2-iLID	Plasma membrane (slow diffusion)	[98]	161002
	Stargazin-iLID	Plasma membrane (slow diffusion)	[98]	161000
	P450 2C1 ^{N37} -iLID	Endoplasmic reticulum	[73]	
	iLID-ActA ^{C46}	Mitochondria	[73]	60413 60413
Single component LOV-based blue light relocalization system	iSH2-optoPB	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃ at Plasma membrane	[74]	-
Phytochrome-based Red/Far Red dimerization switch	iSH2-PIF	PtdIns(4,5)P ₂ → PtdIns(3,4,5)P ₃	[76]	50841
	PhyB- K-Ras4B ^{C14}	Plasma membrane	[76]	50839
Voltage-based system (VSP)	CiDr-VSP L223F	Plasma membrane PtdIns(4,5)P ₂ → PtdIns4P	[81]	140892

Table 1. List of constructs used in chemogenetic, optogenetics and voltogenetic systems to acutely perturb PI levels at precise subcellular localization.

^aTwo-component systems are grouped together with light color-shade indicating the enzyme and dark color-shade indicating the membrane anchor.

^bControls consisting of catalytically dead enzyme exist for most systems but are not indicated in this table for clarity purpose.

^cFor targeting sequences, when a fraction of the protein is used, the number of residues is indicated and their location (^N for N-terminus, ^C for C-terminus)

Figure 1. Small molecule perturbation of the PI network. Schematic representation of PI molecules and their interconversion via kinases/phosphatases mediated by phosphorylation/dephosphorylation cycles. Small molecules described to inhibit (in red) or activate (in green) phosphorylation of dephosphorylation reaction. The enzyme (in human) targeted by each compound are indicated in black, when known. Note that the list of small molecule inhibitors may not be exhaustive and they may not always be fully specific of the indicated enzyme and only show preferential activity toward them.

Key legend: PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdIns3P, phosphatidylinositol-3-phosphate; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns5P, phosphatidylinositol-5-phosphate; PtdIns(3,4)P₂, phosphatidylinositol-(3,4)-bisphosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PtdIns(3,5)P₂, phosphatidylinositol-(3,5)-bisphosphate; PtdIns(3,4,5)P₃, phosphatidylinositol-(3,4,5)-triphosphate; DAG, diacylglycerol; Ins(1,4,5)P₃, inositol-(1,3,5)-triphosphate; PI3K, PtdIns 3-Kinase; VPS34, vacuolar protein sorting34; PI4K, PtdIns 4-Kinase; PI5K, PtdIns 5-Kinase; ptase, phosphatase; PIKfyve, phosphoinositide kinase with a specificity for the five position containing a fyve finger; OCRL, Lowe oculocerebrorenal syndrome protein; INPP5, Inositol polyphosphate 5-phosphatase; PI3P5K; PtdIns3P 5-Kinase; PI5P4K; PtdIns5P 4-Kinase; PI4P5K; PtdIns4P 5-Kinase; PI-PLC; phosphoinositide-specific phospholipase C; SHIP; Src homology 2 (SH2) domain-containing inositol-5-phosphatase; Wm, wortmannin; PAO, phenylarsine oxide.

(A) exogenous treatment

(B)

Figure 2. Strategy for the delivery of PI molecules into cells. A) Example showing the delivery of membrane permeable PtdIns(4,5)P₂ through the neutralization of the PI negative charges via protective groups, such as acetoxymethyl (AM) esters. The protective groups are subsequently removed inside the cell and the PI then becomes biologically active. B) Example showing the delivery of caged PtdIns(4,5)P₂ into cells. These molecules are membrane permeable and will become active upon blue light irradiation, which can be highly targeted through the use of a directed light beam (i.e., photomanipulation).

Key legend: PI, phosphoinositide; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; AM, acetoxymethyl esters.

(A) Modulation of PI level via chronic over-expression of endogenous enzyme (e.g. PIP5K)

(B) Modulation of PI level via over-expression of synthetic enzyme (e.g. MAP-Sac1)

(C) Modulation of PI level via inducible over-expression of synthetic enzyme (e.g. MAP-OCRL)

Figure 3. Manipulation of PIs via expression of wild type or engineered PI-related enzymes. A) Example showing PI modification, here increased PtdIns(4,5)P₂ synthesis, via the chronic overexpression of a gene encoding a PI-related enzyme (e.g. PIP5K). B) Example showing the engineering of PI-related enzyme to synthetically target a specific enzymatic activity to a given subcellular localization. In this example, the endogenous Sac1 protein acts at the membrane of the ER, but a synthetic Sac1 enzyme, consisting of only its catalytic domain (Sac1^{CAT}) and a plasma membrane targeting sequence (e.g. myristoylation and palmitoylation, MAP), specifically targets the PtdIns4P pool at the cell surface. C) By coupling a synthetic enzyme (as in B) and an inducible expression system (here using dexamethasone, DEX), it is possible to obtain inducible PI modification to study their function during development. In this example, a synthetic PtdIns(4,5)P₂ phosphatase, consisting of the plasma membrane targeting motif MAP and the 5-phosphatase OCRL from *Drosophila* efficiently perturb PtdIns(4,5)P₂ levels and can be used to study root development and morphogenesis in *Arabidopsis* (e.g. formation of root hairs).

Key legend: PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PIP5K; PtdIns4P 5-Kinase; MAP, myristoylation and palmitoylation; SAC, suppressor of actin; PM, plasma membrane; ER, endoplasmic reticulum; DEX, dexamethasone; OCRL, Lowe oculocerebrorenal syndrome protein; CAT, catalytic domain; DEAD, catalytically inactive.

(A) Acetylcholine/Oxo-M induced PtdIns(4,5)P₂ hydrolysis

(B) Rapamycin-inducible relocalization system

(C) Self-localizing ligand-induced protein translocation (SLIPT) system

Figure 4. Chemogenetic perturbation of PI levels. A) Example showing the fast consumption of PtdIns(4,5)P₂ levels via G receptor-coupled signaling and activation of PLCs. Some cells naturally respond to acetylcholine or oxotremorine M (Oxo-M), but other do not. It is possible in that case to express the gene encoding the receptor and G protein in order to render cells responsive to these signals. B) Example showing the principle of rapamycin-induced translocation system based on the conditional FRB-FKBP dimerization. The key to this system is that the catalytic domain of most PI-related enzymes is active at very low levels when they are in the cytosol (i.e. no close contact between the enzyme and its substrate) but they become quickly activated once associated with membranes. C) Example of SLIPT system based on Nakamura et al., (2020). The ligand consists of TMP, a ligand of eDHFR from *Escherichia coli*, connected to the membrane permeable lipopeptide myristoyl-Gly-Cys (myrGC). Once inside the cell, the ligand is inserted in the Golgi membrane, where the cysteine is S-acylated (e.g. palmitoylation) and is then exported to the plasma membrane. However, a fraction remains in the Golgi, likely as unpalmitoylated peptides. In membranes, TMP recruits the eDHFR-containing protein. Because the eDHFR-fusion protein also contains a polybasic hexalysine peptide (K6), it is stabilized at the plasma membrane owing to its high electronegative properties compared to the Golgi. In this example, the authors fused K6-eDHFR with the SH2 domain of p85 (iSH2), which recruits the catalytically active PI3K subunit p110 α and produces PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂.

Key legend: PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PtdIns(3,4,5)P₂, phosphatidylinositol-(3,4,5)-triphosphate; DAG, diacylglycerol; Ins(1,4,5)P₃; inositol-(1,3,5)-triphosphate; M1R, muscarinic acetylcholine receptor M1; Oxo-M, oxotremorine M; G, guanine nucleotide-binding protein; PLC, phospholipase C; FP, fluorescent protein; FKBP, FK506 binding protein; FRB, FKBP-rapamycin-binding; 5-ptase, inositol 5-phosphatase; SLIPT, self-localizing ligand-induced protein translocation; TMP, trimethoprim; eDHFR, *Escherichia coli* dihydrofolate reductase; K6, hexalysine peptide; SH2, Src Homology domain2; iSH2, SH2 domain of p85.

(B) Blue-light inducible two-components relocation system (e.g. CRY2, iLID, Magnets)

(C) Red/Fad Red-light switch relocation system (e.g. phytochrome B)

(A) Blue light-inducible single-component relocation system (e.g. optoPB)

Figure 5. Optogenetics perturbation of PI levels. A) Examples showing three different blue-light inducible translocation strategy based on the CRY2/CIBN, iLID and Magnet systems. B) Example based on Toettcher et al., (2011), showing the phyB-based toggle switch, in which red light triggers the recruitment of a PIF-containing chimeric protein (here PIF is fused with iSH2) to the plasma membrane and far red light induces the release of the PIF-containing protein. C) optoPB is a single component optogenetic tool that allows with a single protein to recruit a domain of interest to the plasma membrane upon blue light illumination. In this example, based on He et al., (2017), the authors fused optoPB with the SH2 domain of p85 (iSH2), which recruits the catalytically active PI3K subunit p110 α and produces PtdIns(3,4,5)P₃ from PtdIns(4,5)P₂.

Key legend: PI, phosphoinositide; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PtdIns(3,4,5)P₂, phosphatidylinositol-(3,4,5)-triphosphate; FP, fluorescent protein; CRY, cryptochrome; LOV, Light-oxygen-voltage-sensing; iLID, improved light-induced dimerization; CIBN, N-terminal region of CIB1; CIB1, Cryptochrome-interacting basic-helix-loop-helix1; SspB, 13-kD adaptor protein from *E. coli*; SsrA, 7-residue peptide that binds SspB; Magnet, engineered *Neurospora crassa* Vivid photoreceptor, which comprises an N-terminal Ncap domain responsible for homodimerization and a C-terminal light-oxygen-voltage-sensing (LOV) domain, nMag, negative Magnet; pMag, positive Magnet; phyB, phytochromeB; PIF, phytochrome interacting factor; SH2, Src Homology domain2; iSH2, SH2 domain of p85.