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Experimental Manipulation of Phosphoinositide lipids, from cells to organisms

Mehdi Doumane, Marie-Cécile Caillaud*, Yvon Jaillais*.

Laboratoire Reproduction et Développement des Plantes (RDP), Université de Lyon, ENS de Lyon, CNRS, INRAE, F-69342 Lyon, France.

* Corresponding authors: yvon.jaillais@ens-lyon.fr , marie-cécile.caillaud@ens-lyon.fr

keywords

Optogenetics, lipid, membrane, targeted manipulation, model organism

Abstract

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

11 **Phosphoinositides (PIs) constitute a dynamic and interlinked network of landmark lipids 12 in cellular membranes**

13 PIs are phosphorylated derivatives from the glycerophospholipid phosphatidylinositol (PtdIns)
14 [1]. The inositol head group can be phosphorylated in position three, four or five, forming up
15 to seven distinct species of phosphorylated PtdIns, collectively known as phosphoinositides
16 (PIs): PtdIns3P, PtdIns4P, PtdIns5P; PtdIns(3,4)P₂, PtdIns(3,5)P₂, PtdIns(4,5)P₂ and
17 PtdIns(3,4,5)P₃ (**Figure 1**). Of note, the kinases that phosphorylate PtdIns(4,5)P₂ on the 3rd
18 position are only present in animals and as a result PI(3,4)P₂ and PI(3,4,5)P₃ are not detected
19 in fungal and plant cell membranes [2].

20 Each PI species localizes in specific organelles [3]. PI differential accumulation in membranes
21 is critical for many cellular processes, and requires various cascading enzymatic activities to
22 interconvert PIs into one another (**Figure 1**) [1, 4, 5]. PI kinases and phosphatases or
23 phospholipases have various substrate and catalytic activities, subcellular localizations,
24 spatiotemporal expression patterns and control mechanisms. Thus, **PI-related enzymes**
25 (Glossary Box) constitute an extensive toolkit that precisely define PI concentrations and
26 localization. Most of these enzymes act in a non-redundant manner at the cellular and
27 organismal levels, although they may carry similar catalytic activities [1, 6]. Additionally, long-
28 term PI modifications may lead to indirect and pleiotropic effects that can be difficult to
29 interpret because PIs: (i) regulate many cellular processes; (ii) are present in multiple cellular
30 compartments at the same time; and (iii) are highly interdependent on each other. Furthermore,
31 depleted enzymes might have functions beyond their catalytic activity, making it even more
32 hazardous to conclude that the observed effects originate from the modulation of the PI species
33

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

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

53

54 **Pharmacological approaches for PI manipulation**

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

67

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

76

77 **Delivery of PIs and PI analogues**

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

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

95
96 When interpreting results from PI delivery assays, it is critical to remember that exogenously
97 added lipids may artificially concentrate in non-endogenous compartments or on the wrong
98 membrane leaflet. They may also be rapidly modified into another lipid species by endogenous
99 enzymes. A solution to these problems is the use of **caged-PI** analogs that can be acutely
100 activated (**Figure 2B**) [24-26]. In that case, the PI head is protected by a photoremovable group,
101 which allows light-regulated uncaging at specific organelles [25, 27]. Membrane permeable,
102 photoactivatable PtdIns(4,5)P₂ can be loaded into cells in an inactive form and subsequently
103 uncaged by light (**Figure 2B**). Such photoactivation is extremely fast (i.e., less than a second)
104 and thus allow manipulating PtdIns(4,5)P₂ levels faster than its metabolism. Caged
105 PtdIns(4,5)P₂ were used to study the role of this lipid in exocytosis in mouse adrenal chromaffin
106 cells [24].

107
108 Taken together, PI analogs are valuable chemicals, that can even be suitable for studies in
109 multicellular organisms. However, lipid analogs remain expensive and their synthesis requires
110 specialized skills, which limits the widespread use of this technique [25, 28]. Genetically
111 encoded strategies for PI perturbation complement the approaches mentioned above. They rely
112 on stable or transitory transfer of genetic constructs that, when expressed by host cells, will
113 perturb PI homeostasis [28, 29]. Among these strategies, we will distinguish chronic from acute
114 modulation techniques.

115 116 **Constitutively expressed genetic systems for PI manipulation**

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

127
128 It is also possible to repurpose PI modifying enzymes to localize their enzymatic activities to
129 specific membrane compartments (**Figure 3B**). For instance, the endogenous PtdIns4P
130 phosphatase called *Sac1* normally resides in the membrane of the Endoplasmic Reticulum (ER).
131 However, by taking the isolated phosphatase domain of *Sac1* and targeting it to the plasma
132 membrane via a membrane anchor (e.g., **Myristoylation And Palmitoylation** – MAP), it is

133 possible to rewire the Sac1 function to specifically erase PtdIns4P at the plasma membrane, not
134 at the ER (**Figure 3B**). Such rewiring approach was used in transiently transformed *Nicotiana*
135 *benthamiana* plants [31]. Upon expression of *MAP-Sac1*, PtdIns4P biosensors are solubilized
136 from the plasma membrane and delocalized to intracellular compartments, indicating a decrease
137 of the PtdIns4P pool at the plasma membrane [31]. Sac1-mediated PtdIns4P depletion in
138 transient assays showed that PtdIns4P is the main anionic lipid responsible for the membrane
139 electrostatic field in plant cells [31, 32] and that it is required for the plasma membrane
140 association of many plant proteins [33-36].
141

142 **Inducible expression of PI-related enzymes**

143 As an alternative to the constitutive expression, it is also possible to use **inducible expression**
144 **systems**. For example, estradiol-inducible expression of Arabidopsis *PtdIns4P 5-Kinases*
145 (*PIP5Ks*) (induction for 24 h) allowed to study the developmental impact of their
146 overexpression and particularly their role in polar auxin transport and root gravitropism [37,
147 38]. However, by using endogenous enzymes, it is difficult to know whether the phenotypes
148 are caused by the overexpression of the enzyme itself or the accumulation of its product (i.e.,
149 PtdIns(4,5)P₂). To circumvent this pitfall, Gujas et al., exploited a genetic approach that enabled
150 them to overexpress the highly processive human *PIP5K (HsPIP5K α)* in a tissue-specific
151 inducible manner. GFP-HsPIP5K α could be detected only three hours post induction, and
152 PtdIns4P and PtdIns(4,5)P₂ levels were already affected one hour after induction [39]. This
153 approach revealed that ectopic PtdIns(4,5)P₂ production impacted the differentiation of plant
154 vascular tissues. A similar strategy was also used for the inducible depletion of PtdIns(4,5)P₂
155 in plant cells (**Figure 3C**) [40]. The system is based on the expression of the catalytic domain
156 of *Drosophila OCRL* (encoding a PtdIns(4,5)P₂ 5-phosphatase), which unlike human OCRL is
157 active at 20°C, fused to a membrane targeting sequence (i.e., MAP) and to a red fluorescent
158 protein. Using a suite of genetic control, this tool was shown to specifically targets
159 PtdIns(4,5)P₂ without massively influencing the other anionic lipids present at the plasma
160 membrane. The synthetic enzyme was detected three hours after the gene induction and
161 PtdIns(4,5)P₂ levels already decreased approximately one hour post induction [39, 40]. This
162 strategy was deployed to study the impact of PtdIns(4,5)P₂ depletion on plant root and shoot
163 development and on the localization of plasma membrane proteins involved in endocytosis and
164 cytoskeleton dynamics [40].
165

166 Altogether, these studies highlight that inducible genetic approaches are useful to address the
167 developmental impact of lipid modification in multicellular organisms. They act in the range of
168 a few hours, which is still slow compared to the speed of the endogenous PI metabolism.
169 Alternative assays allow acute PI modification and rely on genetically encoded systems that
170 can be tuned by a chemical (**chemogenetics**), by light (**optogenetics**) or by voltage
171 (**voltogenetics**) [29].
172

173 **Acute pharmacological induction of endogenous PI metabolic enzymes**

174 PIs are known for their roles as **secondary messengers**. In particular, PtdIns(4,5)P₂ is
175 hydrolyzed by Phospholipase C (PLC) into Diacylglycerols (DAG) and Inositol 1,4,5-
176 trisphosphate Ins(1,4,5)P₃ and both act as secondary messengers, triggering Ca²⁺ influx in the
177 cytoplasm and modulating K⁺ channels conductivity. PLCs also breakdown other PI species to
178 a lesser extent. PLC-mediated hydrolysis of PtdIns(4,5)P₂ occurs in response to (nor)-
179 epinephrine or acetylcholine perception by G-Protein Coupled Receptor (GPCR) (**Figure 4A**,
180 [41]). It is possible to take advantage of these signaling pathways by stimulating them, thus
181 acutely depleting plasma membrane PIs. Many studies used cell types that did not express the

182 corresponding receptors, and therefore the cells had to be transfected with the signaling
183 components [42, 43]. In vitro mammalian cells transfected with the muscarinic acetylcholine
184 receptor M1 (M₁R) and stimulated with the acetylcholine agonist oxotremorine M (Oxo-M) for
185 60 s showed a decreased PI level by roughly 70% [44]. PtdIns(4,5)P₂ depletion was observed
186 at 20 s post activation using the PH^{PLC} fluorescent reporter solubilization or **resonant energy**
187 **transfer biosensors** read-outs [45, 46]. Oxo-M-triggered PtdIns(4,5)P₂ perturbation
188 substantiated the function of OCRL in dephosphorylating PtdIns(4,5)P₂ on endocytic vesicles
189 [47]. Relying on the M₁R receptor has the advantage of being very rapid and of exploiting
190 endogenous components. However, the M₁R signaling pathway itself regulates endogenous
191 cellular processes, which must be considered when interpreting results from PLC-induced
192 depletion of PIs.

193

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

195 The rapamycin-inducible method allows to acutely recruit lipid modifying enzymes from the
196 cytosol to a given membrane within few seconds [28]. It is based on the inducible
197 heterodimerization of the FKB domain of the mammalian Target of Rapamycin (mTOR)
198 protein with FKBP12. One of the components (e.g., FRB domain) is anchored to a specific
199 organelle via a membrane targeting sequence, while the other (e.g., FKBP domain) is fused to
200 the catalytic domain of a PI-related enzyme [48-51]. Because the second construct consists only
201 of the isolated catalytic domain, its default localization is in the cytoplasm. When it is not
202 attached to a membrane, the enzymatic module has a low activity, which is likely due to poor
203 access to its lipid substrate. However, a treatment with rapamycin induces the recruitment of
204 the chimeric construct to its target membrane, allowing the enzyme to meet its substrate and
205 thus to almost simultaneously catalyze the reaction. Using a rapamycin-inducible system, a 5-
206 phosphatase domain can associate with the plasma membrane in ~30 s, and PtdIns(4,5)P₂
207 biosensors are concomitantly solubilized, indicating acute PtdIns(4,5)P₂ depletion (**Figure 4B**,
208 [49-51]). Alternatively, the 3-phosphatase MTM1 can be recruited to Rab5-containing early
209 endosomes to specifically deplete PtdIns3P from the membrane of this compartment [48]. Since
210 the first publications of this lipid modifying approach [48-51], the method was rewired to
211 modify virtually all PI species through the recruitment of phosphatases, phospholipases or
212 kinases at different subcellular localization (see **Table 1**). For example, concomitant
213 rapamycin-inducible depletion of plasma membrane PtdIns(4,5)P₂ and PtdIns4P showed that
214 PtdIns4P, together with PtdIns(4,5)P₂, contributes to the electrostatic field at the surface of
215 animal cell plasma membrane and to the localization of the polarity protein DISCS LARGE
216 (Dlg) in *Drosophila* [52, 53]. This system was also utilized to challenge the presence and the
217 function of various subcellular pools of PIs, including PtdIns, PtdIns4P, PtdIns(3,4)P₂ and
218 PtdIns(3,5)P₂ [54-60]. In addition, rapamycin-inducible dimerization was employed to probe
219 the importance of PIs in membrane contact sites structure-function relationship. In particular,
220 this strategy allowed asking whether the PtdIns4P phosphatase Sac1 acts in *cis* (i.e., at the ER
221 membrane) or in *trans* (i.e., at the plasma membrane or in the trans-Golgi Network) in animal
222 cells [61, 62]. FRB/FKBP-mediated accumulation of PtdIns(3,4,5)P₃ was reported in
223 *Drosophila* larva, proving that the approach was viable for PI manipulation in multicellular
224 organisms [63]. This method was also recently implemented in plants using transient assays in
225 *N. Benthamiana* for the inducible depletion of PtdIns4P at the plasma membrane [64].

226

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

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

243

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

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

258

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

275

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

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

294
295

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

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

311

312 **Concluding Remarks**

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

328

329

330

331 **Glossary box**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

378

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390

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591

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*.

Table and Figure Legends

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

594

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

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

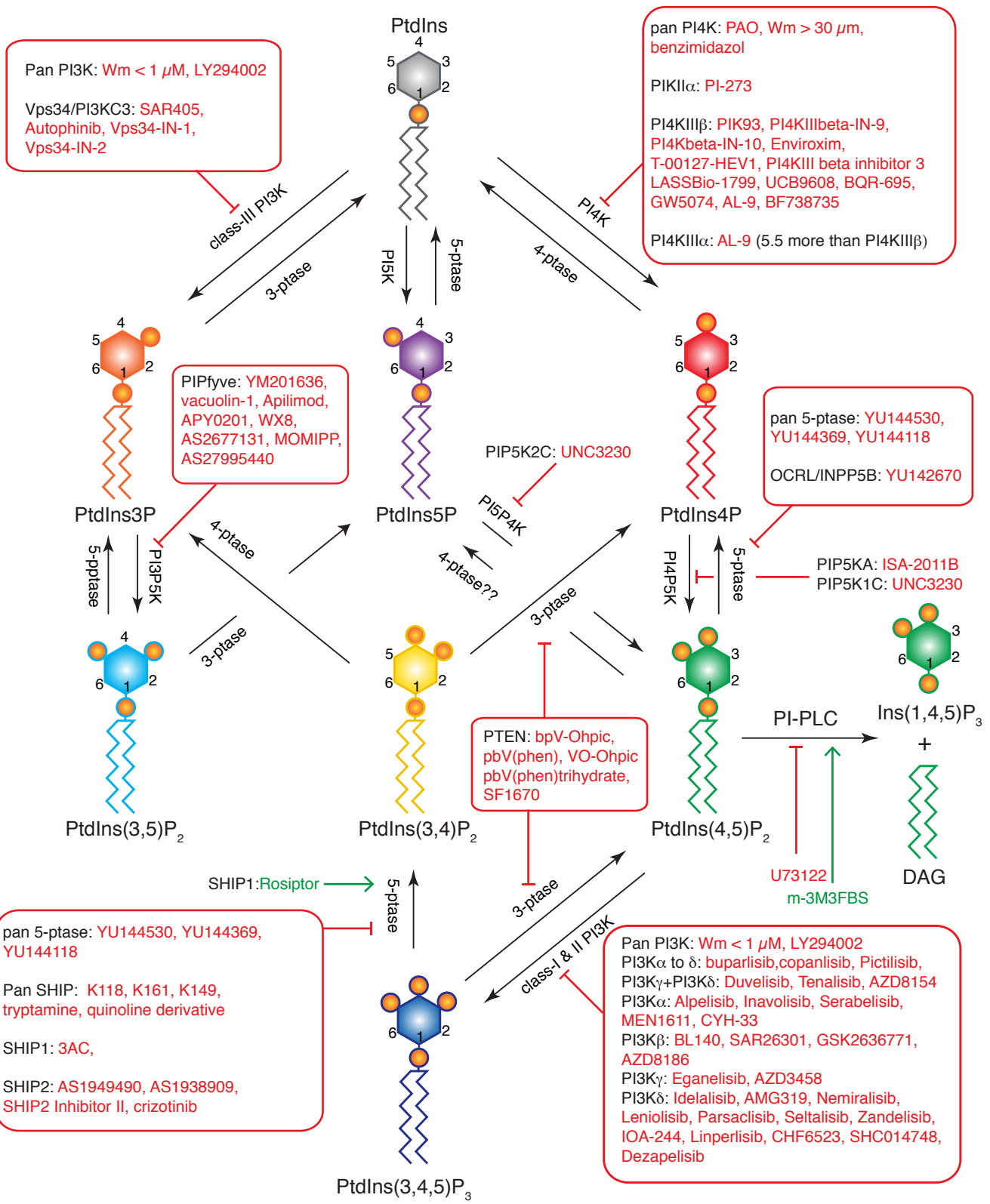
599 ^bControls consisting of catalytically dead enzyme exist for most systems but are not indicated
600 in this table for clarity purpose.

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

603

604

605



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

613 **Key legend:** PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdIns3P,
614 phosphatidylinositol-3-phosphate; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns5P,
615 phosphatidylinositol-5-phosphate; PtdIns(3,4)P₂, phosphatidylinositol-(3,4)-bisphosphate;
616 PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate; PtdIns(3,5)P₂, phosphatidylinositol-
617 (3,5)-bisphosphate; PtdIns(3,4,5)P₂, phosphatidylinositol-(3,4,5)-triphosphate; DAG,
618 diacylglycerol; Ins(1,4,5)P₃; inositol-(1,3,5)-triphosphate; PI3K, PtdIns 3-Kinase; VPS34,
619 vacuolar protein sorting34; PI4K, PtdIns 4-Kinase; PI5K, PtdIns 5-Kinase; ptase, phosphatase;
620 PIKfyve, phosphoinositide kinase with a specificity for the five position containing a fyve
621 finger; OCRL, Lowe oculocerebrorenal syndrome protein; INPP5, Inositol polyphosphate 5-
622 phosphatase; PI3P5K; PtdIns3P 5-Kinase; PI5P4K; PtdIns5P 4-Kinase; PI4P5K; PtdIns4P 5-
623 Kinase; PI-PLC; phosphoinositide-specific phospholipase C; SHIP; Src homology 2 (SH2)
624 domain-containing inositol-5-phosphatase; Wm, wortmannin; PAO, phenylarsine oxide.

625

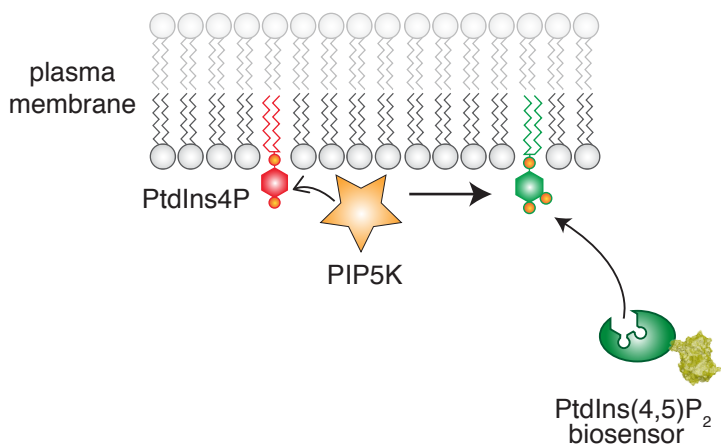
626

627 **Figure 2. Strategy for the delivery of PI molecules into cells.** A) Example showing the
628 delivery of membrane permeable PtdIns(4,5)P₂ through the neutralization of the PI negative
629 charges via protective groups, such as acetoxymethyl (AM) esters. The protective groups are
630 subsequently removed inside the cell and the PI then becomes biologically active. B) Example
631 showing the delivery of caged PtdIns(4,5)P₂ into cells. These molecules are membrane
632 permeable and will become active upon blue light irradiation, which can be highly targeted
633 through the use of a directed light beam (i.e., photomanipulation).
634 **Key legend:** PI, phosphoinositide; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate;
635 AM, acetoxymethyl esters.

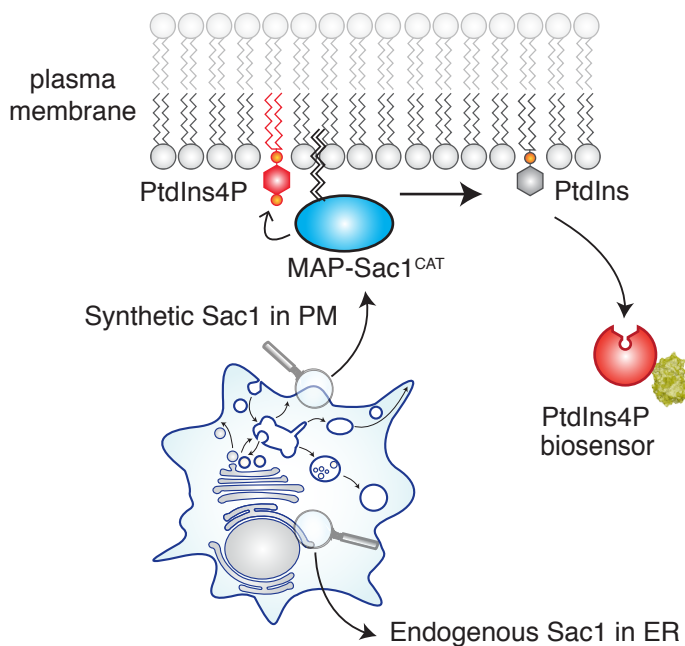
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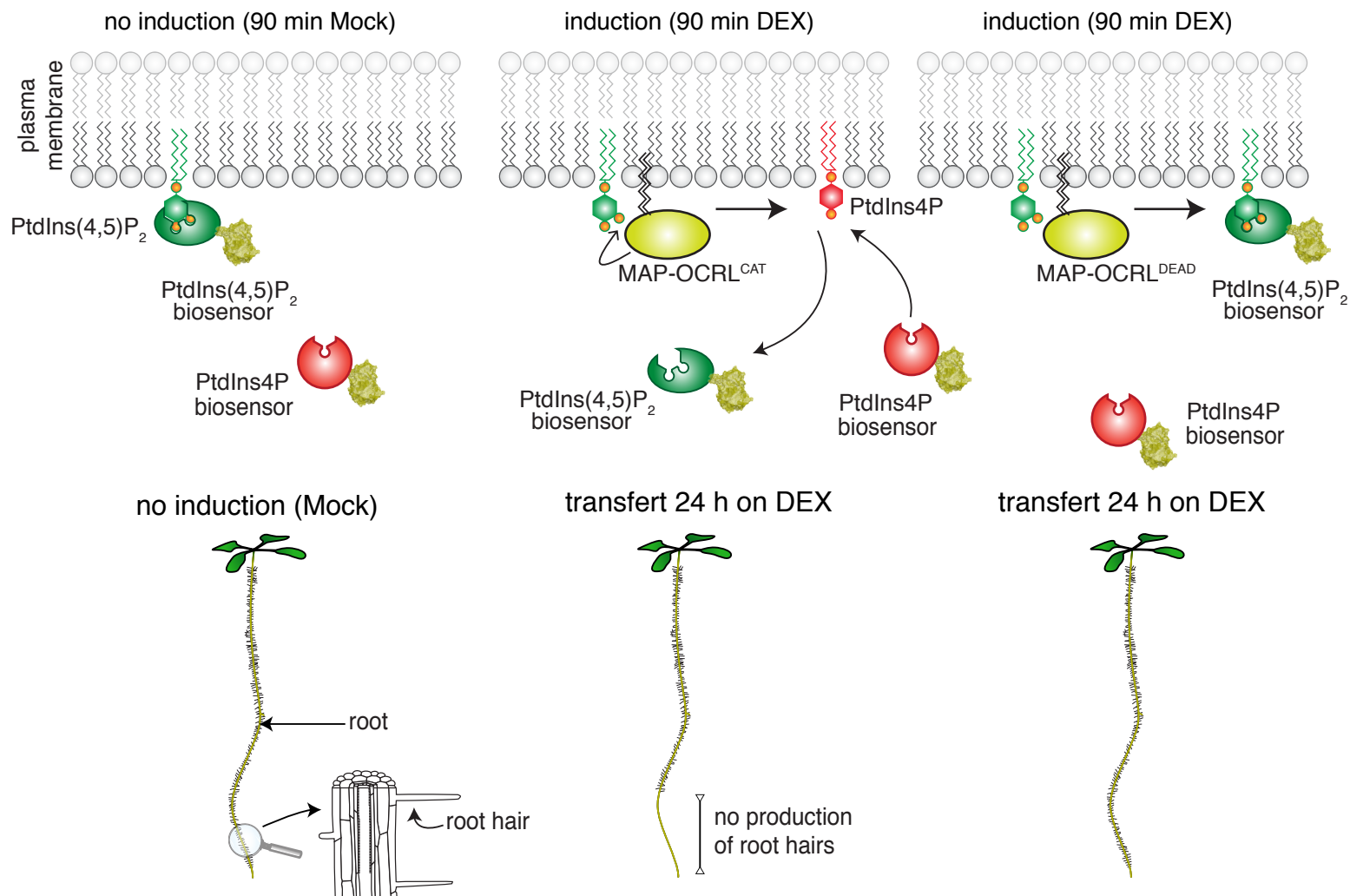
(A) Modulation of PI level via chronic over-expression of endogenous enzyme (e.g. PIP5K)



(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)

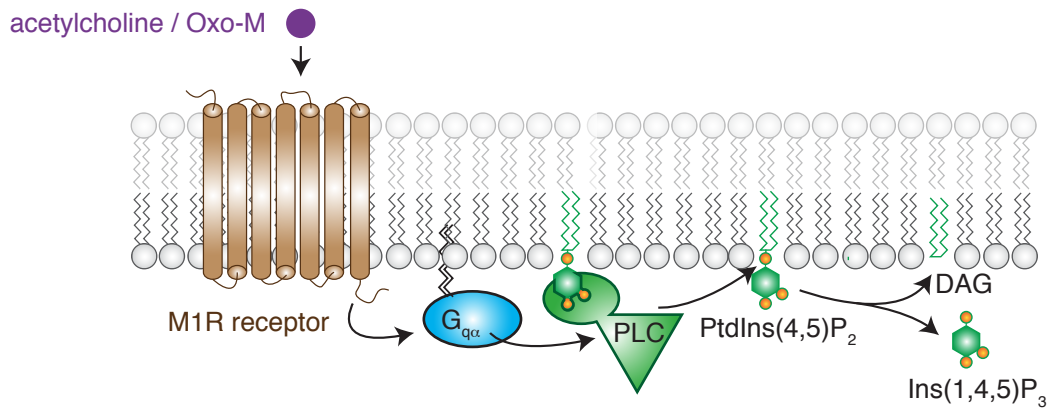


638 **Figure 3. Manipulation of PIs via expression of wild type or engineered PI-related**
639 **enzymes.** A) Example showing PI modification, here increased PtdIns(4,5)P₂ synthesis, via the
640 chronic overexpression of a gene encoding a PI-related enzyme (e.g. PIP5K). B) Example
641 showing the engineering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

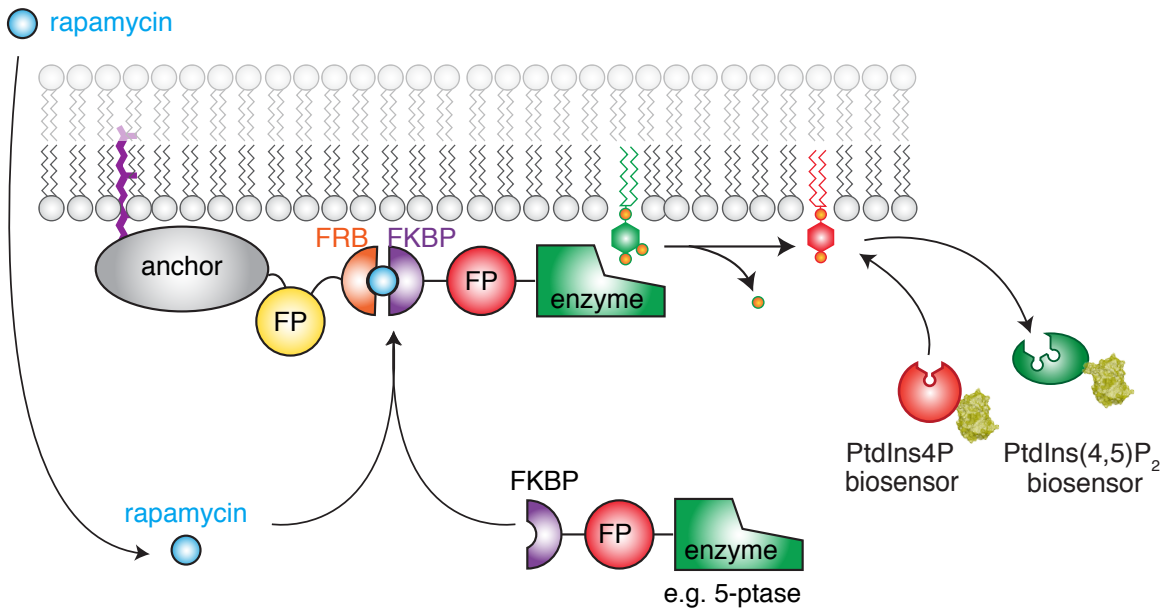
652 **Key legend:** PI, phosphoinositide; PtdIns, phosphatidylinositol; PtdIns4P,
653 phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-(4,5)-bisphosphate;
654 PIP5K; PtdIns4P 5-Kinase; MAP, myristoylation and palmitoylation; SAC, suppressor of actin;
655 PM, plasma membrane; ER,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

657

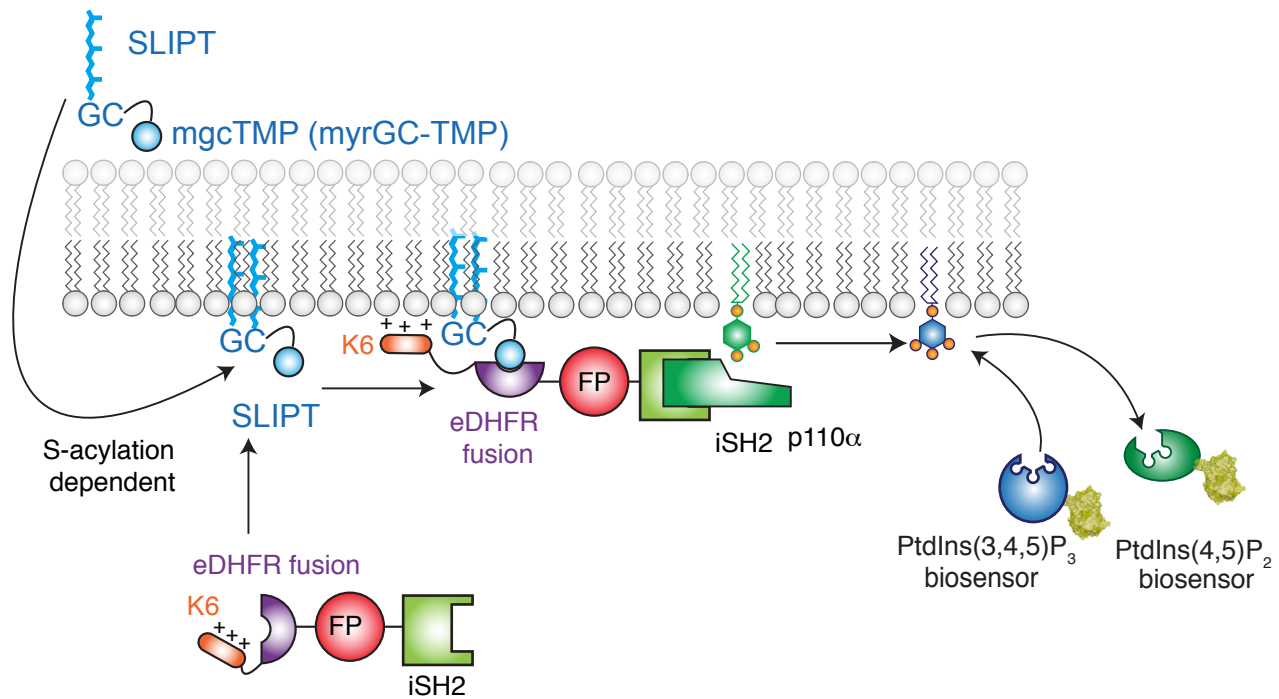
(A) Acetylcholine/Oxo-M induced PtdIns(4,5)P₂ hydrolysis



(B) Rapamycin-inducible relocalization system



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

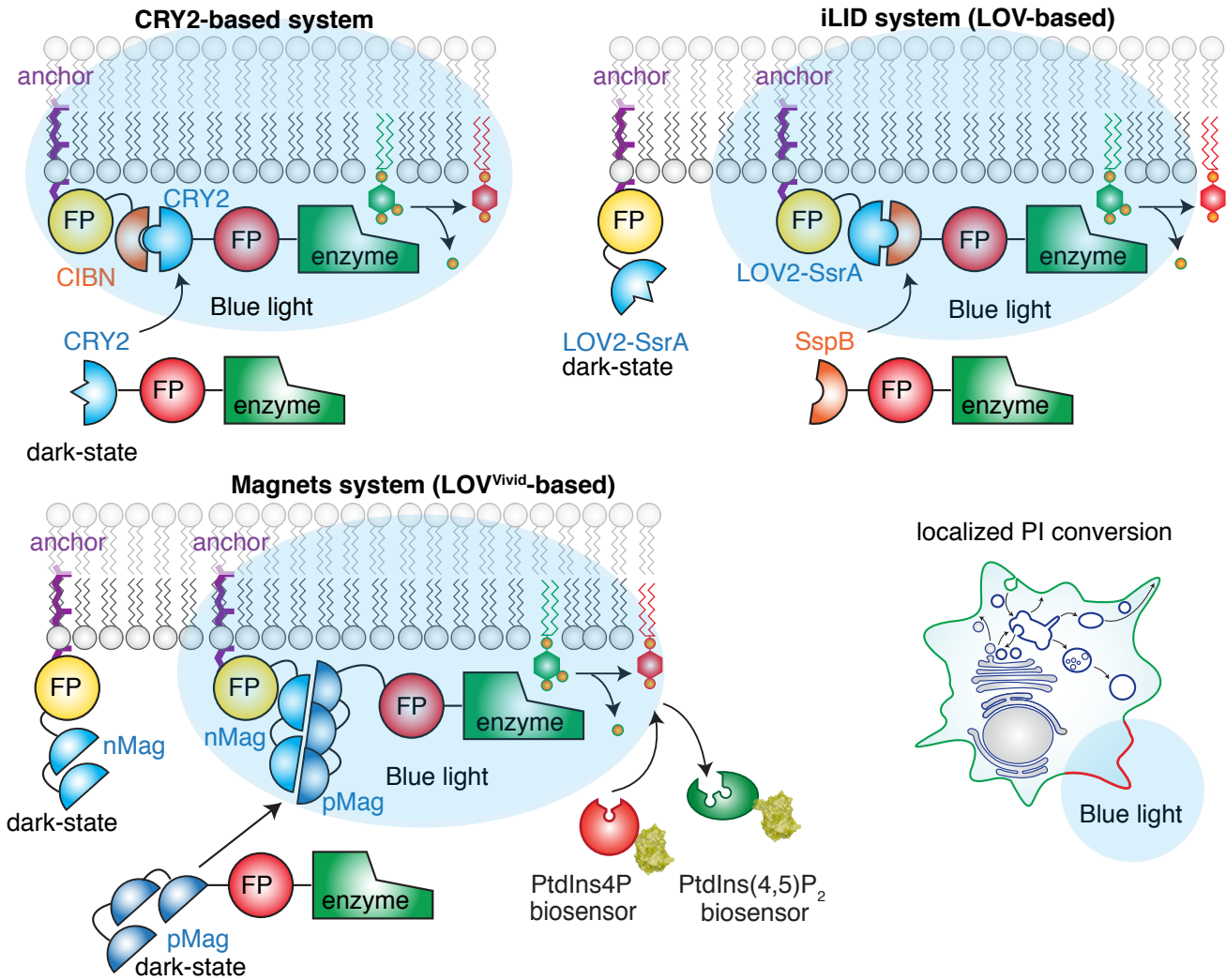


658 **Figure 4. Chemogenetic perturbation of PI levels.** A) Example showing the fast consumption
659 of PtdIns(4,5)P₂ levels via G receptor-coupled signaling and activation of PLCs. Some cells
660 naturally respond to acetylcholine or oxotremorine M (Oxo-M), but other do not. It is possible
661 in that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

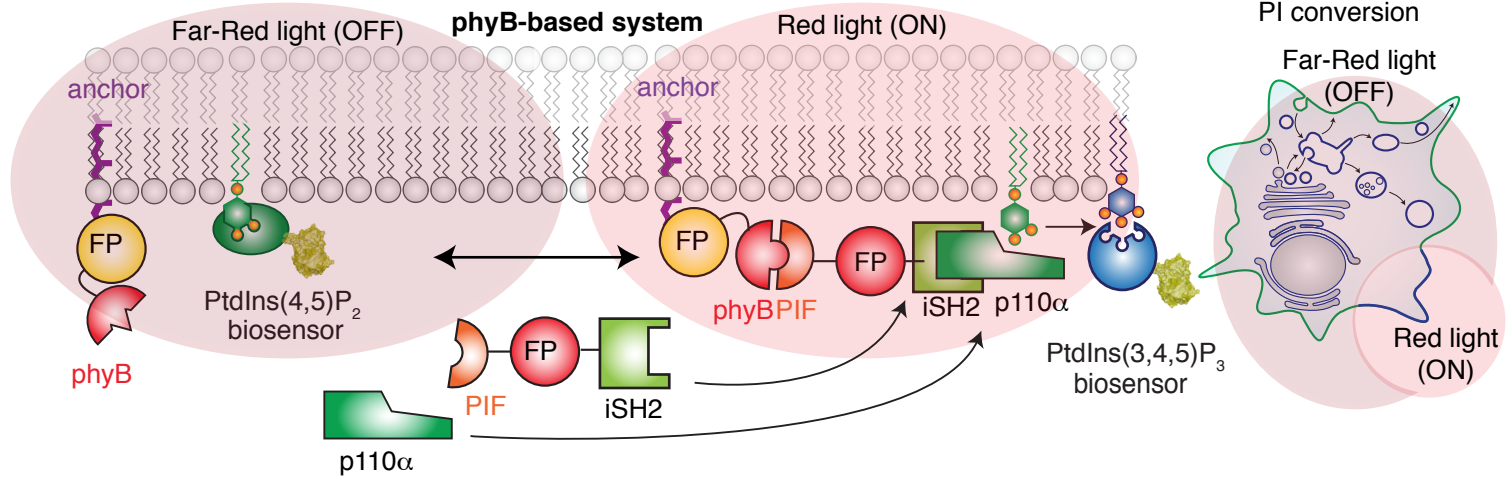
677 **Key legend:** PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P₂, phosphatidylinositol-
678 (4,5)-bisphosphate; PtdIns(3,4,5)P₂, phosphatidylinositol-(3,4,5)-triphosphate; DAG,
679 diacylglycerol; Ins(1,4,5)P₃; inositol-(1,3,5)-triphosphate; M1R, muscarinic acetylcholine
680 receptor M1; Oxo-M, oxotremorine M; G, guanine nucleotide-binding protein; PLC,
681 phospholipase C; FP, fluorescent protein; FKBP, FK506 binding protein; FRB, FKBP-
682 rapamycin-binding; 5-ptase, inositol 5-phosphatase; SLIPT, self-localizing ligand-induced
683 protein translocation; TMP, trimethoprim; eDHFR, *Escherichia coli* dihydrofolate reductase;
684 K6, hexalysine peptide; SH2, Src Homology domain2; iSH2, SH2 domain of p85.

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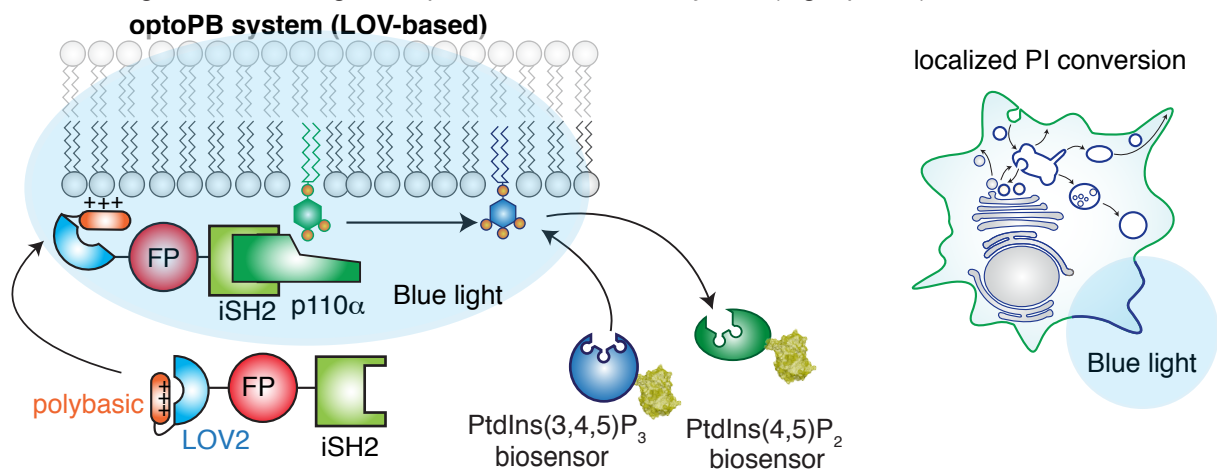
(B) Blue-light inducible two-components relocation system (e.g. CRY2, iLID, Magnets)



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



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



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

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

707