2016

Escherichia Coli as a New Platform for the Study of Phosphoinositides

Sergio Botero

ESCHERICHIA COLI AS A NEW PLATFORM FOR THE STUDY OF PHOSPHOINOSITIDES

A Thesis Presented to the Faculty of

The Rockefeller University

in Partial Fulfillment of the Requirements for

the degree of Doctor of Philosophy

by

Sergio Botero

June 2016

ESCHERICHIA COLI AS A NEW PLATFORM FOR THE STUDY OF PHOSPHOINOSITIDES

Sergio Botero, Ph.D.

The Rockefeller University 2016

Phosphoinositides are membrane phospholipids involved in a wide variety of processes across the tree of life. In eukaryotic cells they function though their role as integral membrane components, anchors for proteins, membrane identity markers, and signaling molecules. Phosphoinositides are regulated through the phosphorylation and dephosphorylation of the inositol head-group at the 3rd, 4th, and 5th positions, creating a complex and very dynamic interconversion network. They can also be hydrolyzed into an inositide head-group and diacylglycerol which are in turn signaling molecules. The wide variety of functions, and redundancy in their synthesis pathways, makes the in-vivo study of phosphoinositides complex since any experimental alterations can have undesired effects throughout the cell. In this work I engineered the metabolic network required to synthesize the most abundant eukaryotic phosphoinositides in the bacterium *Escherichia coli,* which normally lacks any of these phospholipids. This engineered bacterium is a new tool for the in-vivo study of cell biology models that involve phosphoinositides, allowing for a precise control of the system and avoiding any undesired interactions. To achieve this I built and optimized the expression of the required enzymes into a single plasmid such that it can be used in any strain of *E. coli*.

My system can produce phosphatidyl inositol, phosphatidyl inositol (4) phosphate (PI4P), and phosphatidyl inositol (4,5) diphosphate (PIP2), and is easily controlled trough the addition of inositol to the growth media of the bacterium. As an example application of my system, I use it to confirm the role of PIP2 binding in the non-conventional protein export of human basic fibroblast growth factor (FGF2).

Acknowledgments

I wish to thank my thesis advisor, Dr. Sanford M. Simon, the chair of my thesis committee, Dr. Brian Chait, my other committee member, Dr. Agata Smogorzewska, and Dr. Anant K. Menon, my external examiner for their help with this work. Additionally I'd like to thank the members of the Simon Laboratory, the Chait Laboratory and their collaborators from the Rout Laboratory, who gave me very important insights during lab meetings regarding the project. I also want to thank Savanna Honerkamp-Smith for her help with the editing and design aspects of this thesis, Nick Takacs for his help structuring the initial draft, and Rachel Chiaroni-Clarke for her help with some of the optimization experiments. Finally, I want to thank the Rockefeller University Staff for their help and support, especially the Deans Office, and in particular Cristian Rosario, Marta Delgado, and Kristen E. Cullen.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

General abbreviations

CDP-DAG Cytidine diphosphate diacylglycerol

DAG Diacylglycerol

ER Endoplasmic reticulum

FGF2 Human basic fibroblast growth factor

NanoLuc Nano luciferase

GPI Glycophosphoinositides

PH-domain Pleckstrin homology domain

OSBP-PH domain Oxysterol-binding protein

PLCδ-PH domain phospholipase Cδ1 pleckstrin homology domain

TLC Thin Layer Chromatography

Inositides

IP_3 Inositol tri-phosphate

IP_4 Inositol tetra-phosphate

IP_5 Inositol penta-phosphate

IP_6 Inositol hexa-phosphate

IP_7 5-diphosphoinositol-pentakisphosphate

IP_8 Bisdiphosphoinositol-tetrakisphosphate

Phosphoinositides

PI Phosphatidylinositol

PIP Phosphatidylinositol phosphate

PI3P Phosphatidylinositol-3-phosphate

PI4P Phosphatidylinositol-4-phosphate

PI5P Phosphatidylinositol-5-phosphate

PI(3,4)P_2 Phosphatidylinositol-3,4-diphosphate

PI(3,5)P_2 Phosphatidylinositol-3,5-diphosphate

PI(4,5)P_2 Phosphatidylinositol-4,5-diphosphate

PI(3,4,5)P3 Phosphatidylinositol-3,4,5-triphosphate

PIP2 Phosphatidylinositol-4,5-diphosphate

PIP3 Phosphatidylinositol-3,4,5-triphosphate

Phosphoinositide metabolizing enzymes

PIS Phosphatidylinositol-synthase

PI4K Phosphatidylinositol-4-kinase

PI4P5K Phosphatidylinositol-4-phosphat3-5-kinase

PLC phospholipase C

PTEN Phosphatase and tensin homolog deleted on chromosome 10

1. Introduction

1.1. An evolutionary outlook

As the main components of all cellular membranes, phospholipids are essential components of all living organisms. Their basic structure consists of a hydrophobic lipid component, two fatty acid tails, and a hydrophilic head which includes a phosphate group, hence the name phospholipid. The head-group can be modified further with the addition of other chemical moieties. Their amphipathic character is essential for the formation of biological membranes because their hydrophobic tails can align towards the inside of a bilayer, leaving the hydrophilic head towards the exterior. These membranes create the semipermeable barrier that is essential to separate the interior of cells from the environment, but also to separate the different organelles and vesicles from the rest of the cytoplasm in eukaryotes.

Phosphoinositides are a class of phospholipids that have a 6 carbon-ring *myo*-inositol (commonly referred to simply as inositol) head. This head-group is linked via a phosphate to a cytidine diphosphate diacylglycerol (CDP-DAG) in Bacteria and Eukarya, or to a CDP-archaeol in Archaea [1], and it presents a hydrogen and a hydroxyl in each of the other 5 carbons. The carbon linked to the phosphate is carbon 1 in the *myo*-inositol ring. These hydroxyls can be substituted by phosphate groups in positions 3, 4, and 5, in any combination, creating a code that can be used by cells to differentiate among membranes or sections of them[2]. Figure 1 shows a schematic representation of all

phosphoinositide variants and their known interconversion pathways in mammalian cells. Phosphoinositides are present in all eukaryotes, most archaea, and some groups of bacteria[3]. While the actual lipids are different in Bacteria and Archaea due to the different linkers of each domain (ether linked in Archaea and Ester linked in Bacteria and Eukarya), their synthesis is thought to have a common origin giving the similarity in their biosynthetic pathways[4].

Bacteria and Eukarya phosphoinositides are the same in terms of their overall structure, with differences only in the length and saturation of the lipid tails, but their biosynthetic pathways differ. In Eukarya the first molecule to be synthesized is phosphatidyl inositol (PI), the only phosphoinositide that is un-phosphorylated. In Bacteria, as well as Archaea, PI is not the first molecule to be synthesized; in the prokaryotes a phosphorylated version is the first phosphoinositide to be synthesized, starting from CDP-DAG in Bacteria, or CDP-archaeol in Archaea, and adding a phosphorylated *myo*-inositol to create a phosphatidylinositol phosphate (PIP) in Bacteria or an archaetidylinositol phosphate in Archaea. This PIP or archaetidylinositol phosphate is then dephosphorylated to synthesize PI or archaetidylinositol. The PI-synthase (PIS) and PIP-synthase enzymes for this first step in the biosynthetic pathway can only use their specific substrates but are closely related evolutionarily in all three domains of life despite this difference[3].

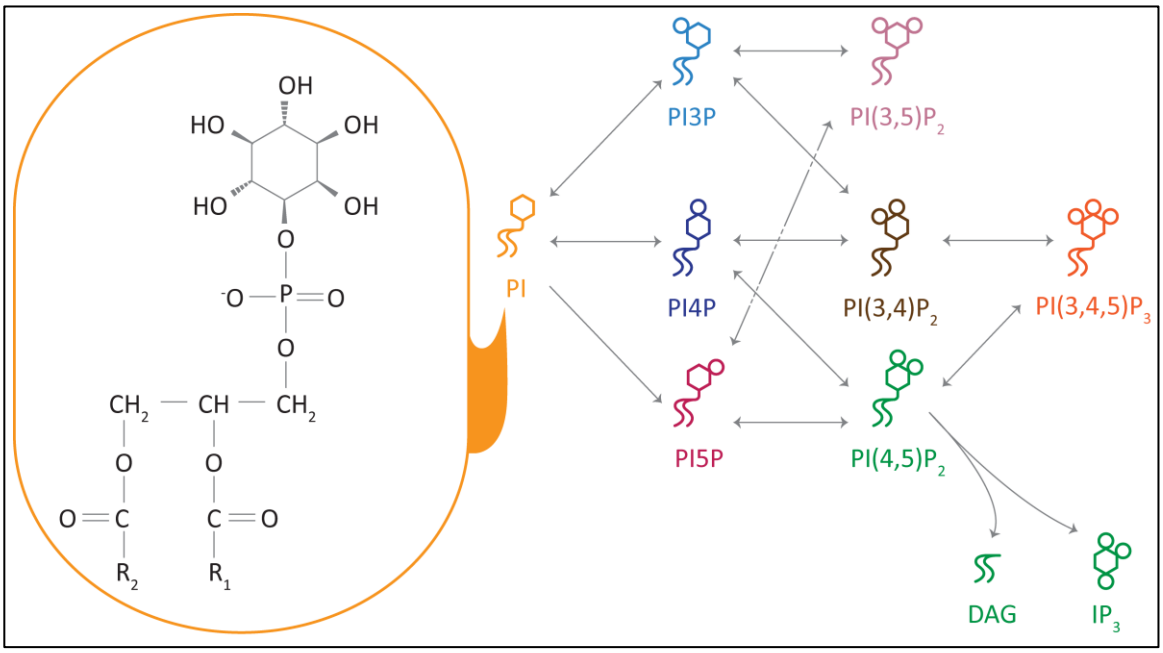


Figure 1. Phosphoinositide chemical structure and interconversion pathways

The pathways shown are present in mammalian cells and represent a eukaryotic generalization although there is variation between the major groups of eukaryotes. Bacteria and Archaea have much simplified pathways involving only PI and monophosphorylated phosphoinositides or no phosphoinositides at all. Only the hydrolysis pathway of PIP2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3) is shown, since this is the best understood, but hydrolysis of phosphoinositide releasing DAG and the corresponding inositide occurs for other phosphoinositides (see text). Figure adapted from the works of Shah and coworkers[5] and Viaud and coworkers[6].

There is little known about prokaryotic phosphoinositides and their functions remain poorly described, especially since until recently it was thought that it was an uncommon occurrence in bacteria[3]. It is however assumed, given their presence in all eukaryotes, that phosphoinositides were present in the last common eukaryotic ancestor and it has been proposed that eukaryotes acquired their phosphoinositides from Archaea [7].

Phosphoinositide functions vary widely across the domains of life. The best understood functions encompass being integral membrane phospholipids[8], membrane anchors for proteins covalently bound to them[9], and anchor sites for proteins with domains specific for their recognition[2]. They can also act as signaling molecules directly[10], or through the products of their hydrolysis into DAG and inositides (the head-group)[11]. Inositides can also act as compatible solutes[12], as cofactors for several processes in the nucleus involving both RNA and DNA regulation[13,14], and as protein modification moieties[15].

In Archaea phosphoinositides have functions as integral membrane components[8] and inositides (just the head-group without a lipid tail) are thermo-protective solutes for some hyperthermophilic Archaea[12]. In Bacteria phosphoinositides have been associated mostly with the Actinobacteria[16], which includes a large number of environmental bacteria but also the *Mycobacterium* genus and *Corynebacterium diphtheria*, the etiological agent causing diphtheria, which use inositides (lacking the lipid tails) as an intracellular redox-buffer[17] and as intermediates in the production of antibiotics in some environmental actinomycetes[18]. Recently it was shown that phosphoinositides are

present in a significantly larger proportion of bacteria than previously thought but their function in these other bacteria remains mostly unexplored[3]. Photosynthesizing cyanobacteria and α-proteobacteria have phosphoinositides so, as expected being their descendants, chloroplast have and synthesize PI[19] but its function remains poorly characterized[7]. Mitochondria have a small phosphoinositide component they do not synthesize and presumably get through their contact with the endoplasmic reticulum. There are no known functions of mitochondrial phosphoinositides but there is an observation that aged mitochondria can recover some of their functionality with the addition of PI[20].

Since all eukaryotes have phosphoinositides in their membranes, their presence is considered to be an ancestral character of the group derived from an archaeal origin, and it is in eukaryotes that their functions are more diverse[21]. Phosphoinositides are used as protein anchors in the form of glycophosphoinositides (GPI) using a complex protein machinery for their synthesis in the ER[9]. Inositol polyphosphates (the non-lipid-bound version of the head-group) are used as osmoregulators by some eukaryotes just as in some prokaryotes[22].

PI is synthesized at the endoplasmic reticulum (ER) by a PI-synthase (PIS) and its concentration is at the highest in this organelle[23]. As mentioned earlier, this PIS is closely related to that of prokaryotes, but it synthesizes PI, in contrast to PIP, in prokaryotes[3]. From PI synthesized in the ER, all other forms of phosphoinositides are formed by the

phosphorylation or dephosphorylation of the 3, 4 and 5 positions, giving seven possible phosphorylated variants. These variants can be localized in different membranes and used as a code for membrane identity, a use that is considered a eukaryotic innovation. The use of inositide-phosphate derivatives, missing lipid tails, gives even a larger number of possibilities as a code since they can also be phosphorylated at the 2nd and 6th position. Using of both phosphoinositides and their inositide derivatives in signaling is considered the most evolutionarily novel adaptation of inositide functionality[21].

In eukaryotes, the basic biosynthetic pathway of phosphoinositides starts from the ER, being rich in PI, with the next step in their biosynthesis being the phosphorylation to create phosphatidylinositol-4-phosphate (PI4P) in the Golgi apparatus, and especially the trans-Golgi system. In this way, ER and Golgi apparatus already have a characteristic membrane phospholipid composition characterized by PI and PI4P respectively. Some of this PI4P is carried to the plasma membrane where it is phosphorylated again in its 5th position to create phosphatidylinositol-4,5-diphosphate (PI(4,5)P_2 or simply PIP2), which is characteristic of this membrane[21]. However, there are variations that make the system more complex. There is evidence, for example, of PI4P synthesis also at the plasma membrane and nucleus[24], but conceptually the ER-Golgi-Plasma membrane respectively matching PI-PI4P-PIP2 in their localization and synthesis is a valid simplification. Through mass spectrometry it was measured that PI constituted 5-10% of the total phospholipids of a mammalian cell while PI4P and PIP2 represent only 0.5-1%[25]. Those three phosphoinositides, PI, PI4P and PIP2 represent the majority of the

cells phosphoinositides, with PI being the bulk of them and PI4P and PIP2 each comprising about 5% with all others being a very small fraction of the phosphoinositides in a "resting mammalian cell"[26]. This is not by any means static, as an example $PI(3,5)P_2$ increases twenty fold upon osmotic stress in yeast[27].

Not surprisingly, the phosphoinositide code eukaryotes develop as a membrane identity system is an essential cellular function since different phosphorylations mark different membranes[2]. The phosphoinositides are always facing the cytoplasmic side of the membranes [28]. The only characterized mechanism to flip them across the membrane is that needed for the use of GPI anchors for some proteins. This occurs in the ER which involves a PI without additional phosphorylation[9], although PI3P has been found in the lumen of *Plasmodium falciparum*'s ER and been associated with secretion[29]. This perhaps reflects an adaptation to be exposed to the cytoplasm of the host upon vesicle fusion with the plasma membrane of the parasite. There is also an observation of PI3P present in the outside membranes of some mammalian and plant cells but this remains largely unexplored[30].

1.2. Synthesis, distribution, regulation and function of eukaryotic phosphoinositides

Despite the relative low abundance of phosphoinositides, they perform a wide variety of essential functions. Phosphoinositides function as signaling intermediates[31] and in overall membrane biology[32], including cell division[33], cortical actin organization[34], and membrane curvature control, either directly or through their protein interactors[35]. Additional roles for phosphoinositides have been reported in the nucleus in chromatin maintenance[36]. This functionality is given by all 8 possible combinations of phosphorylation at the 3rd, 4th or 5th position of PI, creating the phosphoinositide code. Figure 2 shows a schematic representation of phosphoinositide location and abundance in a mammalian cell. Given the tight regulation of the phosphoinositides both in localization and phosphorylation state I will discuss the synthesis, distribution and functions of them simultaneously.

Regulation of phosphoinositide abundance, and therefore their functions, is very complex. At the basic level it represents a complex balance between synthesis and degradation, where it's important to consider that for the most part this means another type of phosphoinositide is formed either by addition or removal of a phosphate. The synthesis might be under more stringent control than the catalysis since the phosphoinositide phosphatases tend to be more promiscuous than phosphoinositide kinases regarding their substrates[37].

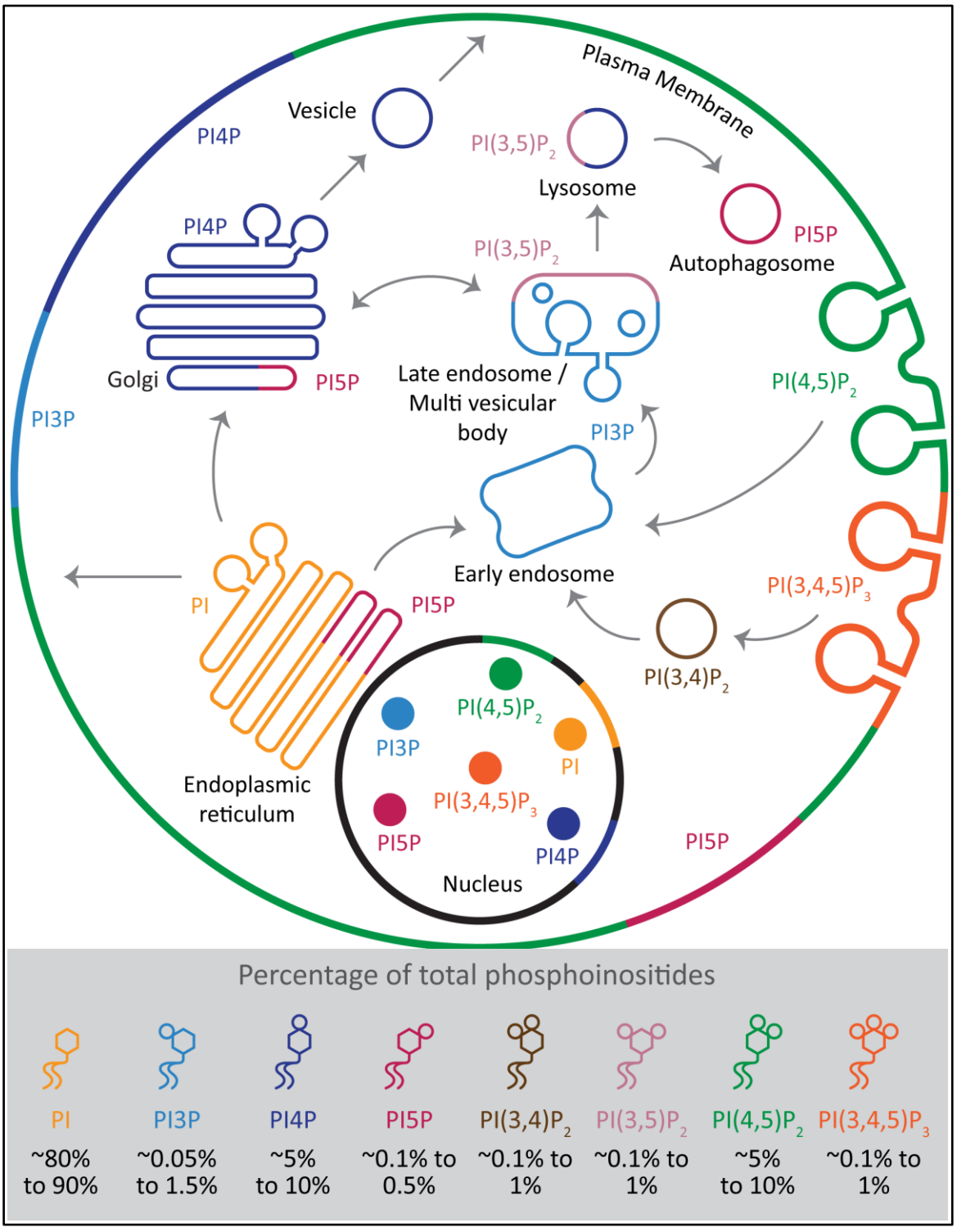


Figure 2. Schematic representation of phosphoinositide location and abundance in a mammalian cell

Figure adapted from the works of Shah and coworkers[38] and Viaud and coworkers[6].

The higher substrate promiscuity of the phosphatases does not mean in any way that they are not regulated, in fact tyrosine phosphorylation is a well characterized form of regulation for the phosphatases and it's been proposed that serine/threonine phosphorylation is relevant as well[39]. The exception for this phosphoinositide phosphorylation/dephosphorylation balance is degradation via separation of the head-group from the fatty acids by phosphoinositide specific phospholipase C (PLC), creating diacylglycerol (DAG) and the inositol phosphate moiety corresponding to the phosphoinositide of origin[40] compounds that are in turn major signaling molecules[11].

Regulation extends beyond synthesis and degradation with sequestration of phosphoinositides representing an alternative form of regulation[41] and the segregation of phosphoinositides across organelles[42] or sections of the same membrane being another important factor. It has been shown for example that different pools of the same phosphoinositide being present in the same membrane but linked to different kinase isoforms are important in aspects of immune cell calcium regulation and signaling[43] as well as vesicle and overall organelle trafficking[44]. Perhaps the best studied system is the sequestration of PIP2 by proteins to release these stored pools upon Ca^{+2} binding[45]. But as in most phospholipid studies the validity of results indicating any sort of "rafts" or segmentation between membranes has been questioned functionally, in this case by showing two proteins with very distinct localizations on the membrane use the same PIP2 pool[46]. This picture is much more complex since it is now clear that some of the enzymes involved in phosphoinositide synthesis have preferences for certain fatty

acid moieties creating another way to regulate the system[47 48]. This type of difference is functionally relevant as shown with the differences between regular and stress induced phosphoinositide pools in *Arabidopsis*[49], where it's also been shown that differences in the fatty acid tails propagate from the synthesis of PI to different PIPs selectively[50]. Whether this is an explanation that applies for all observed differences between pools of the "same" phosphoinositide in cells is still an open question although it wouldn't be a surprising observation.

The length of the lipid tails varies significantly between yeast, mammals and plants. Yeast phosphoinositides possess fatty acid tails in the range of 26 to 36 C atoms per DAG molecule (which includes two fatty acids) and a maximum of 2 double bonds with several moieties being completely saturated. Mammals have between 34 and 40 C atoms per DAG, up to 6 double bonds, and there is always at least one double bond between the 2 lipid tails[25]. Plants showing a similar composition to that of mammals with a slightly larger range, including molecules with only 32 C atoms but the same range of double bonds[49]. Additionally there is evidence for ether linked inositol phospholipids in *Dictyostelium*, although their function seems to remain the same as in mammals[51]. This suggests that the lipid moieties might not be as relevant for the function of phosphoinositides, supporting the role of protein domains that bind to their head-groups as the major effectors of phosphoinositides. Also, since the lipid composition of membranes is controlled as a way to regulate fluidity[52], the changes in the fatty acid tails of phosphoinositides could just reflect the overall status of the cells

phospholipids. The evidence for some differences between different phosphoinositides and other lipids during stress in *Arabidopsis*[49] suggest this might not always be the case. Overall, the extent over which phosphoinositide fatty acids play a role in their functions beyond affecting their membrane mobility and localization is still an open question.

As mentioned earlier, PI is synthesized at the ER from CDP-diacylglycerol, a common precursor for other lipids, by PIS which is a membrane bound protein found in the tubular ER[23]. PI can then be transferred to other membranes by proteins specialized on its transfer [53] and potentially by the normal vesicular transport between ER and Golgi [54]. The transference of PI is however not fully characterized, and given its essential role both as a structural phospholipid and as the precursor of PIPs, which are quickly replenished upon their depletion on signaling activation, a more complex mechanism has been proposed. This mechanism involves a special ER derived organelle that has contact with numerous other membranes and acts independently of the main ER bound PIS, with the different population of PIS having different activities. The experiments that characterized this organelle did not however provide a clear picture as to how it interacts with the ER showing only that it is separate and not continuous with other membranes during the experiments[55]. However, no further support for this organelle has been reported. The function of PI transfer proteins is also now thought to be more complex than initially conceived. The transfer proteins have recently been proposed to have a role in presenting PI to the PI-kinases[56]. There are no protein motifs with specific binding to PI and its function is therefore assumed to be mostly structural or as a

precursor of PIPs but, while these are definitively important roles for it, this might reflect more a lack of tools for its study than the reality in cells[55].

PI can then be phosphorylated at its 3rd, 4th, or 5th positions generating PIP, but of these, PI4P is the most abundant form[26]. There are 4 different kinds of PI-4 kinases in mammals and 3 in yeast. These show different cellular localization and act on the maintenance of different pools of PI4P, although several occur simultaneously at the plasma membrane or Golgi[57]. PI4-kinases have also been shown to occur at secretory vesicles[58] and are known to shuffle between the Golgi and nucleus being essential for viability of yeast in both locations[59]. Synthesis of PI4P is a complex process that is linked to the overall maintenance of ER-Golgi lipid transfer where the gradient in PI between the membranes is used to transfer other lipids; the conversion of PI to PI4P in the Golgi maintains this gradient[60]. PI4P is also an important component of the plasma membrane with many proteins showing erroneous localization upon selectively depleting this pool of PI4P[61]. The function of PI4P is then shown to be not only as a precursor of PIP2, for which different pools have different relevance, but also directly as a scaffold for the binding of proteins to the appropriate membranes. This interaction has been shown to be essential for Golgi vesicle transport[62]. It is common for proteins binding PIPs to present an interaction as a combination of specific and non-specific electrostatic interactions[63] providing a marker for different membranes. This “membrane code” is not exclusively phospholipid based and in many cases an interaction with both PI4P and one or more proteins is required[64], a situation that is common to all phosphoinositides.

There is also evidence of the importance of PI4P in a counter-transport of phospholipids between ER and plasma membrane, in this case helping to enrich phosphatidylserine in the plasma membrane using the PI4P gradient as the driver[65, 66]. This PI4P is in turn dephosphorylated to PI in the ER in a manner that allows for the regulation system based on PI4P abundance[67]. PI4P dephosphorylation into PI is catalyzed by phosphatases with the SAC1 domain[68].

The next major step in PIPs synthesis is the phosphorylation of PI4P in its 5th position to make PIP2. While PI kinases share some homology with protein kinases, the PI4P-5-kinases show no homology to any other kinases and constitute a separate family of kinases[24]. Their active site does however have structural similarity to protein kinases[69]. There are two types of PIP kinases known to generate PIP2, type I and II. Since type II have a preference for PI5P as their substrate[70], the bulk of PIP2 is synthesized by PI4P-5-kinases type I at the plasma membrane directly from PI4P synthesized there[24]. However, there is evidence supporting an important role of Golgi PI4P as a precursor of PIP2[71]. If the pool of PI4P available as a precursor is disrupted the PIP2 synthesis occurs in other cellular compartments from other PI4P pools and is not localized correctly to the plasma membrane[61]. From this plasma membrane PIP2 is where most other PIPs, which are generally short lived, are synthesized either directly or through PI(3,4,5)P_3 (PIP3) as an intermediate. The head-group of PIP2 can also be cleaved by phospholipase C releasing inositol triphosphate (IP_3) and DAG as a signaling molecules[72, 73].

PIP2 is directly involved in a very diverse array of functions at the membrane that include structural effects on membrane curvature[35], cortical actin organization[34], acting ring binding at the cleavage furrow during cell division[74] and overall actin regulation at the membrane[75], endocytosis[76], vesicle recruitment and docking[77], exocytosis[78], ion channel gating[79], basement membrane polarization maintenance[80], ER-plasma membrane interactions[81], regulation of dopamine transporter activity[82], regulation of other lipids through ceramide kinase localization[83], membrane potential sensing through changes in its localization[84], regulation of the phospholipase D family (PLDs) through both localization and activation of the enzymes[85], localization of signaling proteins and complexes to the plasma membrane[86], regulation of mitogen-activated protein kinase (MAPK) signaling though localization of scaffold proteins[87]. Thus, many of the functions that involve plasma membrane identity are in one way or another dependent on PIP2.

In the functions already mentioned activity is mainly dominated by phosphoinositides acting as a platform for the recruitment of proteins, however, functions of phosphoinositides much more complex. Interconversion between phosphoinositides is perhaps the rule when it comes to their diverse functions. As an example PIP2 is excluded from cilia and hypothesized to be dephosphorylated into PI4P as a way to maintain the transition zone and compartmentalization of cilia[88] and it's been shown that this conversion is essential for ciliogenesis[89].

The major function of phosphoinositide turnover might however be in signaling pathways. As an example, the cold sensitive TRPM8 Ca^{2+} channels are activated by a combination of PIP2 and cold or menthol, and in turn the Ca^{2+} input activates Ca^{2+} sensitive phospholipase C which degrades PIP2 and releases IP_3 and DAG providing both a negative feedback and a signaling event[90]. In metazoans DAG is active in the membrane itself as an activator of ion channels, protein kinase D, members of the protein kinase C family, RasGRP1 (which is involved in the Ras/MEK/ERK signaling pathway), and neurotransmitter secretion[91]. IP_3 is soluble and acts on specific receptors in the ER leading to Ca^{2+} release[92]. In plants DAG is phosphorylated before it has a signaling function and it is also phosphorylated derivatives of IP_3 that are signaling effectors[93]. Currently it is considered that direct signaling by DAG and IP_3 from PIP2 through phospholipase C is a metazoan innovation[7].

PIP2 is therefore considered a signaling molecule in itself. Not only is it the precursor of major signaling pathways, but by changing the availability of PIP2 the recruitment of proteins complexes to the plasma membrane and the activation of ion channels and receptors can be controlled. This emphasizes the still unsolved question as to how are so many functions and the possible crosstalk between them regulated[94]. The function of PIP2 as the major precursor of PIP3 adds an extra level of complexity to the system as does the positive feedback loop that PIP2 can have on the activation of PIP-kinases[95].

Although the other phosphoinositides show very low abundance in the cells the diversity and relevance of functions they perform make them essential cell components. Perhaps the most studied one is PIP3, which is a major signaling hub involved in a variety of functions related to cell movement such as wound healing, directional movement of cells, neuronal patterning and embryogenesis, but also in the immune response[96]. This PIP3 signaling pathway seems to be metazoan innovation[93], in it PIP2 is phosphorylated by a class I PI3-kinase upon activation by G-protein-coupled receptors (GPCRs) and/or receptor tyrosine kinases generating PIP3. As expected from the involvement in GPCR signaling the PI3-kinases are regulated through binding to other proteins of which G-proteins, and GTPases in the Rho and Ras families are common interactors[97]. Given the importance of these signaling pathways and the low basal abundance of PIP3 in cells it's not surprising its half-life is quite short, of less than 5 seconds[98]. PIP3 acts by the very fast recruitment of proteins with binding domains specific to it, of which the serine/threonine protein kinase Akt (known as well as protein kinase B, PKB)[99], and the phosphoinositide-dependent kinase 1 (PDK1)[100] are the most studied. Both kinases are involved in a variety of essential signaling networks related to cellular migration, proliferation and metabolism. The signaling pathways involving PIP3 are grouped as PI3K signaling pathways, named for the class I PI3-kinase that synthesizes it. PIP3 can be degraded through two pathways. It can be dephosphorylated at its 3rd position to generate PIP2 again, a reaction catalyzed by the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)[101], or by dephosphorylation of its 5th position by phosphatases synaptojanin or src homology 2-containing inositol

phosphatase (SHIP) producing PI(3,4)P_2 which is another of the minor phosphoinositides[102].

PI(3,4)P_2 can be synthesized in two ways: by dephosphorylation of PIP3 or by phosphorylation of PI4P in its 3rd position by a class II PI3-kinase. The roles of PI(3,4)P_2 are still mostly unknown with very few protein interactors identified[103]. It has been shown to be involved in lamellipodia[104] and podosome[105] formation where several of the proteins relevant for these processes have domains that bind specifically to this phosphoinositide. In a more detailed experimental setup it has been shown to be used as a negative regulator of PI3k signaling through its interactions with tandem pleckstrin homology-domain-containing protein 1 and 2 (TAPP1 and TAPP2) in the control of insulin signaling with deregulation occurring upon genetic ablation of the interaction with TAPP1 and TAPP2[106]. The best studied role of PI(3,4)P_2 is in clathrin mediated endocytosis, where it's been shown to control the enrichment of late stage endosome proteins, and is absence causes the endosome to become an elongated tubular structure before fission[107]. This is mediated by phosphorylation of PI4P in its 3rd position, and this PI4P is also been shown to be the product of PIP2 dephosphorylation[108] providing a precise mechanism for the control of clathrin mediated endocytosis by phosphoinositide transition and the different proteins they recruit[109]. PI(3,4)P_2 has also been shown to be involved in clathrin-independent endocytosis activated by receptor activation of the PIP3 pathway[110]. It is unclear whether PI(3,4)P_2 really has just these functions or if its low abundance and fast dynamics have precluded the discovery of

more. It nonetheless has been proposed as an important molecule downstream of the PI3K signaling pathway and hypothesized to have more functions, with the current lack of understanding being a product of lack of interest in the research community[103]. Given the wide variety of functions involving PIP3 and the known importance of endocytosis (where $PI(3,4)P_2$ role is well characterized) for receptor signaling and recycling it would not be surprising for $PI(3,4)P_2$ to have a central role in PI3K signaling as proposed.

The other three minor phosphoinositides PI3P, PI5P and $PI(3,5)P_2$ have all low abundance but are still very important in the cell. It's been proposed that as a group they allow for the regulation of some types of vesicular traffic and cytoskeletal processes[6]. PI3P is generated by the phosphorylation of PI in its 3rd position by a PI3-kinase or by the dephosphorylation of $PI(3,4)P_2$ or $PI(3,5)P_2$ in their 4th and 5th position respectively. There are 3 classes of PI3-kinases all of which phosphorylate the 3rd position in phosphoinositides but of which class I and class II show substrate promiscuity in vitro, although respectively preferring PIP2 and PI as their substrates. Class III PI3-kinases do show specificity only for PI as a substrate[111]. The generation of PI3P by synthesis from PI seems to be common to all eukaryotes while its synthesis by dephosphorylation of PIPs might be exclusively metazoan[112]. As with all phosphoinositides PI3P can be degraded through lipases, and by being dephosphorylated or phosphorylated. Lipases represent the main pathway for its degradation in yeast and mammals, a process that occurs in the endosomal pathway[113]. It is precisely the endosomal pathway that presents the best known function of PI3P

where it serves as an anchor for the endosomal sorting complex required for transport (ESCRT) machinery, in particular for ESCRT-0 and ESCRT-II [112], although in vitro the interaction of the ESCRT complex with vesicles proceeds in the absence of phosphoinositides at a lower rate[114]. PI3P is considered to be important for endosome recycling where sorting nexin (SNX) protein 1 has been shown to bind PI3P and $PI(3,5)P_2$. There is evidence for the involvement of PI3P in regulated exocytosis in neurons[115] but the mechanism is still unknown[112]. PI3P has also been shown to be essential for autophagy where its synthesis at the ER helps to segregate the membrane that will become separated to form the autophagosome[116] and there is some more detailed mechanistic understanding of the process in yeast, although it is known that the protein binding domains that interact with PI3P in autophagy also bind to $PI(3,5)P_2$[117]. Finally there is some mechanistic evidence for PI3P involvement in cytokinesis[118] and signaling although this role might be indirect due to the role it has in endosome sorting[112].

$PI(3,5)P_2$ is a poorly studied phosphoinositide given its low abundance and the lack of tools for its precise localization. It can be produced by the phosphorylation of PI3P in its 5^{th} position or of PI5P in its 3^{rd} position with the first pathway dominating its production[6]. Theoretically it could also be synthesized through dephosphorylation of PIP3 in its 5^{th} position but there seems to be no evidence for this pathway. The PAS (PIKfyve/ArPIKfyve/Sac3) protein complex localizes to the endosomes and contains both the kinase PIKfyve that phosphorylates the 5^{th} position of PI3P and the phosphatase Sac3 that dephosphorylate it back to PI3P[119]. $PI(3,5)P_2$ is essential for vesicle recycling

from vacuoles and lysosomes[120] and endosome to trans Golgi network recycling[121]. Given the promiscuity in some of the protein binding domains that recognize both PI3P and PI(3,5)P_2[117], and their closely related synthesis and localization[119], it would not be surprising if they share functions or some of their ascribed functions are actually performed by the other phosphoinositide, with both possibilities likely happening. It is nonetheless clear that both PI3P and PI(3,5)P_2 are important factors for vesicle sorting in cells[6].

Finally PI5P was the last phosphoinositide to be discovered and the one with the lowest abundance in cells although, as is usual for phosphoinositides, variability exists[122]. It is localized to the plasma membrane, ER, Golgi apparatus, endosomes, and nucleus[123]. It can be synthesized by phosphorylating PI, or dephosphorylating PI(3,5)P_2, or PIP2, but its degradation seems to be coupled mostly to its phosphorylation leading to PIP2 synthesis[6]. Most of its functions remain poorly studied but it's been associated with cell motility[124], glucose metabolism upon insulin activation in adipocytes[125], negative feedback loops in T-cell signaling[126], and in autophagosome biogenesis in a way independent of PI3P but which shares some of the effectors[127]. The best characterized functions of PI5P are however in the nucleus where it has been shown to bind ING2, (a candidate tumor suppressor) in-vivo, and regulate its ability to activate p53 and apoptosis[128] as well as have a role in chromatin structure[129].

Functions of phosphoinositides in the nucleus are not exclusive of PI5P and involve at least PI, PI5P, PI4P and PIP2 in the most characterized models. PI3P and PIP3 have also been detected but their functions remain obscure[38]. There are several lines of evidence for Ca^{2+} signaling in the nucleus through phospholipase C hydrolysis of PIP2 into IP_3 and DAG as occurs in the cytoplasm[130]. In this case with the Ca^{2+}being released from the ER and nuclear envelope[131]. This system has been associated with cell cycle progression and differentiation[132]. Also, another function that seems to be shared with the cytoplasm is the binding of PIP2 to actin or other cytoskeletal related proteins inside the nucleus[133]. The most surprising observation is that the nuclear phosphoinositides aren't all in the nuclear envelope membrane, but in the nucleoplasm itself with the key initial observation being that of a significant fraction of PIP2 (35%) remaining in nuclear preparations that had been stripped of their membranes by treatment with detergent[134]. It was shown that PIP2 co-localizes with PIPKIα, a PI4P-5-kinase to nuclear speckles[135] which are dynamic structures involved in RNA processing and maturation[136]. The essential role of PI-4-kinases in the nucleus of yeast[59] as well as their known localization to the nucleus in mammalian cells[137], make the PI-PI4P-PI5P-PIP2 axis clearly relevant in the nucleus where it's been implicated into mRNA processing and export[138]. As mentioned before, PI5P has a more characterized role in P53 dependent apoptosis[128]. PI5P has also been shown to be in the chromatin fraction and its amount increases upon UV damage supporting a role in chromatin structure[129].

An additional level of complexity is given by the fact that a variety of inositol polyphosphates (just the inositol head-group lacking any lipid tails) can be generated from IP_3 in the nucleus and have associations with proteins involved in RNA editing[13], RNA export[139], chromatin remodeling[140], telomere length[141], non-homologous end joining[14], and potentially a novel type of protein modification by adding the pyrophosphorylated bond from IP_7 or IP_8 to a serine that is already phosphorylated[15]. But for all these functions it is unknown if inositol polyphosphates act simply as cofactors or are actually regulated and the changes in their abundance serve as ways to control these processes[38]. In the case of *Trypanosoma brucei* the control of the antigen switch and regulation of telomeric expression sites, which is essential for its escape from immune detection, is directly linked to phosphoinositides cycling including the PI4P-PIP2-PIP3 axis but also $PI(3,4)P_2$, and the soluble head groups IP_3, IP_4, and IP_5, reinforcing the notion of a code that is shared both by the soluble head-groups and the phosphoinositides themselves[142].

1.3. How phosphoinositides' functions are performed and how we study them

The very wide array of functions in which phosphoinositides have been implicated makes a detailed description of the evidence for each of them too lengthy for the scope of this thesis. I will instead provide an overview of the mechanistic patterns on these functions as well as the methods useful for the study of phosphoinositides, emphasizing particular methods or discoveries when appropriate.

1.3.1. Phosphoinositides in membranes

Phosphoinositide functions in maintaining/altering the membrane curvature can be direct through their inverted conical shape for the phosphorylated forms (big head group volume relative to a small lipid tail volume) which leads to positive curvature in membranes, but most of their known role in altering membrane seems to be mediated through their interactions with proteins which can also modify the membrane properties by inserting domains into it upon phosphoinositide binding[35]. Given the very high complexity of living cells and the intrinsic difficulty of controlling membrane composition in-vivo, studies of this kind of activity are done on artificial membranes. A good example of how this type of study is performed is provided by the work of Rusinova and collaborators[143]. In it they worked by creating lipid bilayers over 1.5 mm diameter pores and varying the lipid composition using gramicidin A channels as reporters. The use of gramicidin A channels relies on their apparent independence on specific lipid binding for their function, but their requirement for a bilayer compression for their activity. This occurs because they function as dimers, one monomer on each leaflet of the membrane, thus the probability of dimerization depends on the properties of the membrane they reside on. As a dimer they allow the flux of monovalent cations and water allowing electrophysiological measures to be taken. There are gramicidin A channel variants with different lengths allowing for a more detailed control of the system[144].

In the work of Rusinova and collaborators[143] the composition of the lipid bilayers was maintained at PIP2 levels that are physiologically relevant but changing the type of PIP2 that was used from endogenous PIP2 purified from brain, to exogenous PIP2 with short (carbon chains 8 long all saturated) or long (carbon chains 18 long with one double bond) fatty acid tails. The rational for this was to evaluate if there are differences between endogenous PIP2 and the exogenous ones that are more commonly used in research with artificial membranes. The main result of this analysis was that the properties of the bilayer are not affected at low concentrations of phosphoinositides while direct effects on the membrane are observable at high concentrations (>10 μM). This conclusion is important because it highlights the potential that the enrichment of a phosphoinositide has to alter membrane properties. It implies that when binding to proteins in a membrane, if the proteins have a tendency to aggregate, or if they bind to areas already rich in phosphoinositides and stabilize them, phosphoinositides can not only alter the proteins upon binding to their specific binding sites but the properties of the section of membrane. No clear pattern was observable when it came to the comparison between exogenous and endogenous phosphoinositides.

The use of artificial vesicles and membranes has been instrumental for the study of phosphoinositides. While these systems do not present the complexities of a living membrane or cytoplasm (for example not allowing membranes with asymmetric distribution of lipids to be used) being able to control precisely the lipid composition allows for a wide variety of experiments. As mentioned earlier this approach has been

useful to test the effects of PIP2 on membrane properties[143]. The use of unilamellar vesicles has allowed some very detailed studies such as the role of the ESCRT-III Complex in membrane scission, although in these experiments the phosphoinositide involved, PI3P, was kept constant[114]. The concept of lipid domains or "rafts" is also relevant here since artificial vesicles have also been used to test hypothesis about the distribution of phosphoinositides. Lipid rafts rich in PIP2 have been proposed as a mechanism involved in actin dynamics at the cell cortex[145]. In order to test how such rafts could regulate the neuronal Wiskott-Aldrich Syndrome Protein (N-WASP), which normally has a self-inhibitory mechanism until bound to PIP2, vesicles with different concentrations of PIP2 were used showing that the affinity of N-WASP for PIP2 depends directly on PIP2 concentration. These results provided evidence for a cooperative binding model that involves several molecules of PIP2 thus explaining the need for a locally high concentration of the phosphoinositide for N-WASP activity on actin polymerization, such as in a hypothetical raft. In this study the authors went further showing that increasing the amount of basic residues in the phosphoinositide binding patch allowed the interaction to take place at lower PIP2 concentrations[146].

1.3.2. Phosphoinositides and proteins

Knowledge of phosphoinositides in the realm of membranes is very limited compared to that of their functions interacting with proteins[2]. This is likely the result of difficulties studying the biophysical properties of membranes in-vivo combined with the relatively low abundance of phosphoinositides. The interactions of phosphoinositides with proteins are mediated through their roles as scaffolds for protein binding with varying specificities. There are many protein domains specialized in phosphoinositide binding including PH, ENTH, CALM or ANTH, PTB, PHD, C2, BAR, PX, PDZ, FERM, Tubby, and FYVE domains[2, 147-149] (the names of these all come from acronyms of the original proteins they were found and have little relevance). Most of these domains are also present in plants and yeast suggesting they have an ancestral eukaryotic origin[93]. Of these the PH domains and C2 domains are notable because they are some of the most abundant protein families in the human proteome[150] while the PHD domain seems to be particularly common in proteins involved in chromatin maintenance and histone interactors[38]. The C2 domain has been studied phylogenetically showing a very interesting pattern in which the last universal eukaryotic ancestor is hypothesized to have at least 6 families of C2 domains. This study also showed that the calcium dependent membrane binding is exclusive to the protein kinase C-C2 domain family and no other C2 domains[151]. Given the well characterized importance of phosphoinositide signaling for calcium regulation[152], this calcium conditional C2 domain represents a very useful evolutionary adaptation to provide feedback in signaling networks.

The charge of phosphoinositides is an important factor for their interaction with protein domains specialized in binding to them. Nuclear magnetic resonance studies have determined that PIP2 charge is -4 at pH 7.0[153], but based on vesicle mobility in a potassium buffer at pH 7.0 its charge seems to be -3 since it can bind both a K^+ and H^+ ion[154]. It has been proposed that the H^+ ion can be removed by protein interactions thus giving PIP2 a possible charge of -3, -4 or -5 depending on its interactions. For many of the protein domains that interact with phosphoinositides the interaction is given in a non-specific way by this charge and other membrane properties that phosphoinositides impart. Clusters of basic and aromatic residues are common among domains that bind to phosphoinositides[95]. In an experimental set up using phosphatases targeted to the plasma membrane it was shown that this polybasic cluster is important for protein localization to the plasma membrane of a large proportion of the proteins that localize to this compartment. An unexpected result of the study was however the necessity for PIP2 and PIP3 removal suggesting that PIP3 shares the plasma membrane identity role with PIP2[155].

The binding of proteins to phosphoinositides can be very promiscuous or specific depending on the specific domain[156]. It is not uncommon to find that the domain binds the phosphoinositide with similar affinity as the isolated inositol phosphate head-group; the PH domain from phosphor lipase C-δ (PLC-δ) for example binds both PIP2 and IP_3 with high affinity[157]. This property has been exploited for crystallographic studies which

are commonly performed with the proteins bound to only the head-group of the phosphoinositide[158, 159]. Evidently this methodology is very useful when studying inositides phosphates (the naturally occurring head-groups that lack lipid tails).

Specific interactions can also be more complex with the binding sites for a phosphoinositide forming between two interacting proteins, as is the case between phospholipase γ1 and the ion channel TRPC3, which form a PH domains that binds PIP2, localizing the channel to the plasma membrane. This result is interesting not only because of the potential for more complex interactions it illustrates but because it was found through an in-silico approach to identify phosphoinositide binding partners[160]. A well characterized non-specific protein-phosphoinositide interaction is the myristoylated alanine-rich C kinase substrate (MARCKS), which has a patch of basic residues in an unstructured domain that allow it to bind PIPs in a non-specific manner. This binding occurs through electrostatic interactions aided by the insertion of the myristoyl moiety into the membrane. This interaction is regulated by the phosphorylation of serine residues in the basic path causing cytosolic localization of the protein[161]. It's important to highlight that this is an interaction of the MARCKS phosphoinositide binding domain with 3 molecules of PIP2 allowing it to both sequester PIP2 more effectively and bind preferentially to areas of the membrane rich in PIP2[45].

While most of our knowledge about the interaction of protein domains and phosphoinositides seems to indicate it is their head groups that drive the interaction,

the lipid tails are an important part for a few known cases. The most obvious one is that of phosphoinositide transport proteins, which need to house the hydrophobic lipid tails in order to transport them successfully[162]. This does not imply that the lipid tails play an important part for their functions, since it is simply a requirement for their transport by burying the hydrophobic lipid tails in a pocket in the protein and leaving the hydrophilic head-group exposed to the cytoplasm. However this mechanism has been hypothesized as a way in which phosphoinositides could perform functions in a way independent of the membranes they normally are part of. The argument is that since most protein interactions seem to work directly with the head-group, a protein that carries phosphoinositides with the head-groups exposed could lead to successful phosphoinositide-protein interactions. This could be a particularly useful mechanism to explain the functions of phosphoinositides in the nucleus[138]. Recently this was shown to be the case for the human steroidogenic factor-1 ligand (SF1) binding domain (a nuclear receptor), which can bind to both PIP2 and PIP3, hiding the lipid tail in the hormone binding pocket (common to nuclear receptors) and exposing the head groups to the cytoplasm. The surface that is created by this binding, and which includes the phosphoinositide head-group, matches the binding site of a protein interactor of SF1 providing a mechanistic model for the effect of some mutations to SF1 which were previously not understood at the structural level[10]. Whether variation of lipid tails in the phosphoinositides allow for regulation of this type of process is still an open question, but it is now clear that phosphoinositides can have functions that aren't linked to membranes though this "protein presenting" mechanism.

The study of phosphoinositide binding to proteins has used the expected biochemical assays including crystallography[163], nuclear magnetic resonance[164], radioactively labeled phosphoinositides in in-vitro binding assays[70], and calorimetry[156]. Assays specific to phospholipids such as protein lipid overlays[165], and protein binding to vesicles with known lipid compositions have also been used[156]. Protein lipid overlays are particularly relevant because they are analogous to dot blots. In them purified lipids are blotted and incubated with proteins which are then detected with a secondary antibody. This provides a straight forward way to detect the binding preferences of proteins[165], and has been transformed into a system commercialized for the quantification of some phosphoinositides and as the basis for ELISA assays for phosphoinositide quantification.

As expected, there is a wide range of affinities for protein binding to phosphoinositides and whether in vitro measures reflect the reality in cells is still an open question[95]. The K_D of PH domain from pleckstrin (which gives the name to the specialized phosphoinositide binding PH domain as an acronym to Pleckstrin Homology) has been measured at 30 μM through binding to vesicles[166], while that of the PH domain of PLC-δ has been measured at 2 μM through several independent techniques[95, 157, 167]. The affinity and specificity of some phosphoinositide binding domains has made them a very useful tool for the study of phosphoinositides in-vivo. By combining a phosphoinositide binding domain and a fluorescent protein reporter a genetic reporter for phosphoinositide location is created. This has been an instrumental tool for the in-vivo

study of phosphoinositides allowing studies of their dynamics as well as quantification of their abundance in some cases[168]. Antibody staining against phosphoinositides has also been used but in the majority of cases antibodies are used only for immunohistochemistry and have therefore limited utility given the very dynamic nature of phosphoinositides[169].

Phosphoinositide binding domains have also been used in more complex schemes involving fluorescent resonant energy transfer (FRET) as a way to increase sensitivity[170] and a similar approach has been done using a split luciferase[171]. Overexpression of a phosphoinositide binding domain has been used as an approach to sequester the phosphoinositide in order to tests its importance for a specific function, such as overexpressing the pH domain of PLC-δ to inhibit receptor mediated endocytosis[172]. In the specific case of PI3P the use of a FYVE domain that dimerizes with the addition of the rapamycin derivative, AP20187, has the advantage of allowing overexpression of the protein without side effects. This is because it remains cytoplasmic as an inactive monomer, avoiding any negative effects, until AP20187 is added causing the FYVE domain dimerization and sequestration of all PI3P available[173].

Despite the widespread use of phosphoinositide binding domains for their study there are significant caveats to their use. The most commonly mentioned problem is the possibility that their study interferes with the normal processes in which phosphoinositides are involved. This occurs because by binding to proteins the

phosphoinositides are in effect sequestered, precluding the activity of any enzymes that would act upon them or their binding of effectors[6]. This is well illustrated with the measured half-life of PIP3 which was estimated to be less than 10 seconds when measured without using phosphoinositide binding domains (in this case a PH domain) but when measured using a PH domain it was close to 1 minute[98]. The promiscuity of the binding domains can be an important problem too, especially since our knowledge of their binding affinities came from in vitro studies[31]. The pH domain of PLC-δ is widely used for the detection of PIP2 because of its K_D of just 1.66 μM, but its K_D when binding IP_3 is just 0.21 μM [157], making interpretation of results obtained with it very complex since an increase in soluble IP_3 would give the same result as a decrease in PIP2[174].

However, the biggest problem for the use of phosphoinositide binding domains to study phosphoinositides might come from the assumption that these domains only interact with phosphoinositides and/or that different domains interact in the same way[174]. As an example, the PH domains of both Bruton's tyrosine kinase and Cytohesin-1 bind to PIP3 and therefore bind to the plasma membrane upon activation of Jurkat cells. However overexpression of cytohesin-1 PH domain inhibits the cell adhesion of stimulated Jurkart cells while that of Bruton's tyrosine kinase does not[175]. Since their binding and localization is the same this observation is most likely the result of differences in their interactions with other molecules. Different phosphoinositide binding domains have also been shown to have different localization or even miss entirely some pools of phosphoinositides[31]. This problem is not exclusive of phosphoinositide binding domains,

the PTEN phosphatase has phosphatase activity on both phosphoinositides and polypeptides through the same active site[176], so interpretation of results from modifications to PTEN activity, localization, or abundance is very complex.

This "problem" when interpreting experimental results illustrates an important aspect of phosphoinositide behavior. Given that most binding domains do not show high affinity for their preferred phosphoinositides and are promiscuous in their binding partners[174], the functions of phosphoinositides have been proposed as the result of coincidence detection in which it is the simultaneous binding of proteins to phosphoinositides and other proteins that allows for their functions to occur[31]. In this way the "membrane code" is not given exclusively by the phosphoinositides but by a combination of phosphoinositides and proteins that do not necessarily interact directly with them. Of the proteins that share this scaffolding/code function the Rab-GTPases are particularly relevant given their importance for membrane and vesicle trafficking across the cell[177]. The coincidence detection is not limited to phosphoinositides and/or proteins, membrane properties are also employed. The best example might be that of the sorting nexin-1 (SNX1) protein which has both a PX domain that binds to PI3P and PI(3,5)P_2, and a Bin-Amphiphysin-Rvs (BAR) domain that allows it to form dimers that sense membrane curvature, as determined by its binding to vesicles of the same components but different sizes. In this way both the phosphoinositide binding and the membrane curvature allow targeting of SNX1 to the early endosome[178].

The study of phosphoinositide location by using labeled phosphoinositide binding domains or antibodies has been instrumental to our knowledge of their functions despite the caveats of each method. More classical cell biology approaches have also been used successfully. While the fine scale details are lost in these approaches the fractionation of membranes and quantification of the phosphoinositides afterwards gave the first suggestion for non-membrane-bound nuclear phosphoinositides as mentioned earlier[134]. Radioactive labeling, using ^{3}H labeled inositol, has also provided a way of quantifying phosphoinositides through thin layer chromatography or high performance liquid chromatography coupled to mass spectrometry. This approach however is complicated because the three mono-phosphorylated and the three di-phosphorylated isomers are not distinguishable by these methods[169]. A chemical modification that allows for the distinction of isomers in mass spectrometry has been developed[179] but it remains available only to specialized laboratories since it requires a different configuration of the machines involved in the process from that used for protein identification.

1.3.3. The egg and chicken problem

A problem with the interpretation of any result linked to the presence of phosphoinositide rich domains is the experimental determination of the cause for such a domain. These could potentially come from membrane properties that enhance phosphoinositide localization to the areas with the appropriate curvature, but could also

be driven through protein interactions with phosphoinositides and posterior protein aggregation driving the phosphoinositide enrichment[31]. Both situations can occur simultaneously and several proteins can be involved, thus determining a specific cause for an area rich in a phosphoinositide can be very complicated, especially since the processes do not occur in isolation from other processes involving phosphoinositides.

1.3.4. Manipulation of phosphoinositide abundance

Genetic modifications to the proteins that interact with phosphoinositides have been one of the most commonly used tools for their study. Genetic modifications can be very simple and provide clear results such as the demonstration of the osmo-protective role of *myo*-inositol-1-phosphate (MI1P) for growth in elevated salinity environments. This work was done by expressing the gene for the synthesis of MI1P (PcINO) obtained from *Porteresia coarctata* (a wild plant related to rice) in *E. coli*, *Schizosaccharomyces pombe*, and plants from the genus *Oryza* (rice) and *Brassica* (mustard and cabbage). The experiment showed that the expression of the MI1P-synthase allowed them to growth under normally inhibitory salt concentrations. Since *E. coli* does not have the ability to synthesize *myo*-inositol this allowed to confirm it was MI1P that provided the salt tolerance and not the synthase itself since the growth of the bacteria showed a dose response to the *myo*-inositol added[22].

Genetic labeling with fluorescent proteins of the enzymes involved in phosphoinositide synthesis or degradation is a common practice. In the best cases co-localization of the labeled phosphoinositide binding domain and the enzyme responsible for its synthesis can be evaluated simultaneously by using a labeled enzyme and a labeled phosphoinositide binding domain. PIP2 and a PI4P-5-kinase were shown to co-localize to nuclear speckles[135] suggesting that PI4P should also be localized there. This approach has the same caveats as using the phosphoinositide binding domains since the enzyme might interact with a lot more proteins thus their localization does not necessarily imply the presence of phosphoinositides. As mentioned earlier the tumor suppressor PTEN is a good example of an enzyme which active site is known to dephosphorylate both peptides and phosphoinositides[176].

Basic approaches including knock-outs have been particularly fruitful in yeast. By selectively deleting the parts of the sequence that control the nuclear or cytoplasmic localization of Pik1, a PI4-kinase, it was possible to show that it is necessary for viability in both locations, and this was confirmed by the rescue of viability only when constitutively cytoplasmic and constitutively nuclear forms were expressed simultaneously[59]. Mouse knock-downs have also been carried out and there are a significant number of human diseases linked to loss of function of enzymes involved in phosphoinositide metabolism. The interpretation of these is however hampered by the difficulty in ascribing specific functions given the possibility of compensation for the

production of a phosphoinositide from an alternative pathway or the lethality of the mutations[5, 169].

Overexpression of enzymes involved in phosphoinositide metabolism is an approach that has also provided useful information. While the approach suffers from the same problem most genetic screens do when dealing with phosphoinositides, the possibility of alternative compensating pathways, very important insights have been gained in this way. The characterization of a second PIS in *Arabidopsis* was done in this way for example. Through fluorescent labelling it was shown that both PIS enzymes (PIS1 and PIS2) localized to the ER and in a lower amount to the Golgi apparatus. In vitro activity showed a difference in the preference for different fatty acids in the CDP-DAG from both synthetic and plant derived origins with PIS2 preferring unsaturated fatty acids while PIS1 used mostly saturated or mono-unsaturated ones. Overexpression of each of the enzymes showed very different patterns in other lipids. While PIS2 generated significantly higher levels of PI4P and PIP2 the levels of these remained normal with overexpression of PIS1 which instead led to higher levels of phosphatidylethanolamine and DAG[50]. This study is exceptional because it suggests a mechanism of regulation of phosphoinositide metabolism by the fatty acid tails of the phospholipid, and because the tests were performed in-vivo.

Changes in the localization of enzymes involved in phosphoinositide metabolism can be achieved with the use of rapamycin and dimerization domains that depend on it for

their interaction. As an example, localization of the PI4-phosphatase Sac1 to the Golgi was shown to inhibit exit of cargo to the plasma membrane and endosomes and change the localization of some proteins and dynamics of PIP2 at the plasma membrane[180]. A caveat of the use of rapamycin is the fact that its target is mTOR (mammalian target of rapamycin) is already involved in phosphoinositide metabolism through the PI3K pathway, thus it has the potential to produce significant undesirable side effects[169]. Nonetheless this has been a very useful tool providing insights into many of the phosphoinositides roles in the cell.

Electrogenetic and optogenetic manipulations present a new set of tools that allow the same type of approach as rapamycin induced localization but with potentially more control on the system. A voltage sensitive phosphoinositide phosphatase from *Ciona intestinalis* opened the door for the manipulation of some phosphoinositides upon membrane potential changes. Its specificity is controlled by the magnitude of the change in membrane potential allowing for a more precise tuning of its phosphatase activity from the 5^{th} position of the inositol ring upon activation to the 3^{rd} position with a higher voltage (0 mV and 60 mV respectively)[181]. The mechanism for this activation and change on preferred substrate has been recently described by showing there are two sequential active states on which the protein can be depending on voltage[182]. The approach however has the caveat that it can only be used at the plasma membrane, and it is not really specific to a phosphoinositide but a position in the phosphoinositide ring. This has been addressed in one case by coupling it to a native phosphatase to acquire a

more realistic control of its activity using the PTEN phosphatase[183]. Optogenetic manipulations rely on the use of protein domains that dimerize upon illumination from specific wavelengths, allowing the localization of a phosphoinositide kinase or phosphatase domain to the cellular localization of interest in a relatively fast (seconds) and reversible (few minutes) manner[184, 185]. This approach has also been used to activate signaling pathways involving phosphoinositide and GPCRs[186]. The main problem with them, which is also common to the use of rapamycin, is the potential activity the soluble phosphatases or kinases might have before being localized, creating an abnormal basal cell state. Additionally the use of some wavelengths to control the system restricts those available for visualization with fluorescence microscopy but that is only a minor caveat[169].

Pharmacological manipulation of the enzymes involved in phosphoinositide metabolism is also possible in several cases, with the majority of attention focusing on PI3-kinases since PTEN is a PI3-phosphatase that is implicated in a variety of cancers[187]. The main problem with inhibitors of PI3-kinases is the lack of specificity and the presence of resistant isoforms[188]. The problem with lack of specificity is shared by PI4-kinase and PI5-kinase inhibitors, and the drugs specificity is also only present in very narrow concentration ranges thus their value is mostly for in vitro studies[169]. Nonetheless pharmacological inhibitors of phosphoinositide metabolizing enzymes present a very active area of research given their potential for treatment of several cancers and immune diseases[187, 189].

Small molecules that directly bind to phosphoinositides preventing their interactions with proteins while minimizing undesired effects on other proteins or phosphoinositides are starting to be researched. A membrane soluble molecule that binds to PIP2 was developed and shown to be well tolerated by fibroblasts (3T3 cell line) in culture modifying both cytoskeletal dynamics and receptor endocytosis. This molecule shows preference for PIP2 over IP_3 (the head-group of PIP2 without lipid tails) which is the opposite of the behavior in PIP2 binding proteins[190]. While this is an isolated approach it might represent a new avenue for the creation of tools to control phosphoinositide abundance.

On the reverse approach, membrane permeable phosphoinositide analogs in which the phosphate and hydroxyl groups are respectively masked with acetoxymethyl esters and butyrates have been developed. Once in the cytosol unspecific carboxyhydrolases turn the molecules into "normal" phosphoinositides[191]. This approach has been extended to unnatural phosphoinositides which are also phosphorylated at the 6^{th} position, although the results obtained with this approach couldn't be interpreted mechanistically[192]. Since the carboxylases are relatively slow to release the phosphoinositide analog, a caged version was developed in which the molecule can diffuse and accumulate in the cytoplasm in the caged state until photoactivated and released to go into membranes[193]. This approach allows for more precise control at both the temporal and spatial scales. Nonetheless there is no control over which membrane the

phosphoinositide analogs integrate potentially complicating the interpretation of results obtained using them[169]. The fatty acid tails used in these phosphoinositide analogs are also typically short[192] and while the regulation of phosphoinositide activity by the lipid tails remains largely unexplored this could add another level of complexity to the interpretation of experiments using the analogs.

Finally pathogens have provided alternate evidence for some of the functions of phosphoinositides. The intracellular pathogen *Shigella* uses several protein effectors in order to remodel the actin cytoskeleton and successfully carry out infection. Of this IpgD is a phosphoinositide phosphatase that produces PI5P showing a preference for PIP2 as its substrate but also from other phosphoinositides[194]. *Salmonella*, another intracellular pathogen has a homolog of IpgD called SopB which prefers $PI(3,4)P_2$ and PIP3 as its substrates[195]. In the case of SopB the function of the protein seems to be a general one depending on changes of the charge of the membrane of the vacuole containing the bacterium since it not only alters phosphoinositides but also reduces phosphatidylserine. The change in the membrane prevents the vacuole from fusing with lysosomes[196]. It is however complex to adjudicate this results exclusively to charge since there is no methodology available to in-vivo separate the specific binding of proteins to phosphoinositides to that based only on charge interactions.

IpgD however seems to be more specific in its pathogenic role and was shown to regulate the trafficking of ICAM-1 (intercellular adhesion molecule-1) in a manner

dependent of its phosphatase activity and PI5P production. This in turn leads to less neutrophil adhesion reducing the immune response to the pathogen[197]. The role of PI5P in endosomal trafficking has been described for other receptors, such as the epidermal growth factor receptor (EGFR), and shown to be dependent on the adaptor protein TOM1(target of myb-1 originally described from chicken) by using IpgD as a tool for the enrichment of PI5P[198]. PI3P has been shown to be important for *Plasmodium* pathogenesis[29], both for its own trafficking of ingested hemoglobin and in the host cell where one of its effectors is a promiscuous PI3-kinase but which functions are less well characterized[199]. There is evidence suggesting PI3P is present on the outer leaflet of the plasma membrane of some plant and animal cells where it is used by fungal and oomycetes effectors for internalization[30]. The authors of this study used phosphoinositide binding domains that are specific for PI3P labeled with fluorescent proteins for their assays and as a negative control showed that this construct didn't bind to erythrocytes which act as a control for unspecific binding. A PI4P binding domain also showed no accumulation on the evaluated cells outer membrane leaflet suggesting that the results are valid. How PI3P would be localized to the plasma membrane in the first place remains unexplored and highlights the fact that our knowledge of phosphoinositide biology is still limited.

Given the vast array of functions phosphoinositides are part of, and the complexity in studying them, it has been proposed that a network approach that includes both proteomic and interatomic analyses might help elucidate their functions better. This

would help build a clearer picture about the different functions of isoforms of the same enzymes. Such an approach would also allow using the phenotypes observed in the diseases linked to mutations in the enzymes involved in phosphoinositide metabolism to better inform knowledge of their functions and potentially identify medical targets[5]. While this definitively seems to be a very promising avenue for research it has remained largely unexplored.

1.4. Reason and goal for this thesis

The study of phosphoinositides represents an area of science where the intrinsic complexity of the system requires the slow tuning of our mechanistic models through many independent lines of evidence. It is clear that the phosphoinositides are involved in a wide variety of functions that they perform though their interactions with proteins in a finely tuned manner, and through many detailed experiments we have several mechanistic models for some of the processes that involve phosphoinositides. Direct evidence is however very hard to obtain for most functions since the controls available are not ideal and, given the multiplicity of functions phosphoinositides have, it is not possible to demonstrate sufficiency of a set of components for a process. This is because of the possibility of interference with other pathways in the cell that involve some of the same phosphoinositides or proteins. An additional problem is the possibility of compensation for any experimental alteration by alternative phosphoinositide metabolic or trafficking pathways.

While in vitro experiments present a good alternative to demonstrate the sufficiency of the components of a model, in vitro systems lack the complexity of the highly diverse living membranes. Artificial membranes do not fully reproduce the dynamic processes and complexity observed in living cells[200]. With this in mind I set to develop a new platform for the in-vivo study of phosphoinositides without the possibility of cross talk or interference with other processes that involve them.

In order to do so I have developed a system that takes advantage of the lack of any phosphoinositides in the bacterium *Escherichia coli*[201] to prevent any interference from other processes while allowing researchers to perform experiments in-vivo. By engineering this bacterium to synthesize the phosphoinositides of interest it is possible to build new systems based on mechanistic models of phosphoinositide functions and demonstrate the sufficiency of a set of components for a process in-vivo. While this platform does not address the necessity of the set of components used for the actual process being evaluated it provides with a fully independent line of evidence to complement other types of experiments and advance our knowledge of phosphoinositide biology.

1.5. Design decisions for the new platform

To systematically test all of the possible combinations for designing such a platform would require an enormous amount of work. Thus I made several design decisions at the start of the project, most of which worked satisfactorily.

The first one was to use the gram negative bacterium *Escherichia coli* as the cell upon which to build the system. The reason for this choice was the assumption that by being one of the most well characterized organisms the tools available to build the system, and thus its utility would be maximized. *E. coli* is known not to have any endogenous phosphoinositides since classical experiments intended to find them[202] and this remains the case in recent experiments[201], thus it is perhaps the best candidate organism to not have any phosphoinositides. While this sounds like an extreme precaution it is not without reason; until very recently it was thought that phosphoinositides only occurred on few bacteria while now they are known to be present in a significant number of bacterial clades[3]. Another advantage of using *E. coli* is the availability of tools for protein expression, and in particular for multiple protein co-expression[203].

The second design decision was to focus on PIP2 as the phosphoinositide of interest with PI, PI4P as its precursors. While the system can be expanded in the future to other phosphoinositides limiting it to just these 3 allowed for a simpler biochemical pathway

to be needed. Additionally given these are the most abundant phosphoinositides in mammalian cells[25] the potential applications for the system are maximized.

The other design decisions refer to the expression of the enzymes necessary and the control of phosphoinositide abundance. Since the goal of the system is to build a platform that can be used to recreate diverse models for cellular processes I decided to minimize the use of any protein expression tools such that most would be available to potential users of the system. To do so I chose to express the enzymes required for the phosphoinositide synthesis in a constitutive manner, using inositol as a way to control the system since it can be added to growth media. This choice means all inducible protein expression systems will be available to any potential user of the system. All enzymes were labeled with the same peptide tag to ease their identification with a single antibody, thus also leaving all other commonly used tags free for use by users of the system. Additional by putting all enzymes on the same plasmid only one antibiotic marker and only one of the 4 families of compatible plasmids[203] is used leaving 3 plasmid origin families and all but one antibiotic resistant markers available for users of the system.

2. Materials and Methods

2.1. Description of the system

Because of the nature of the system I have combined its description with the methods section in order to better illustrate why certain optimization steps were required. The designed metabolic pathway is illustrated in Figure 3A. The first step, synthesizing PI, is catalyzed by phosphatidyl inositol synthase (PIS) from the lipid cytidine diphosphate-diacylglycerol (CDP-DAG) and *myo*-inositol. Cytidine diphosphate-diacylglycerol is present naturally as the precursor of *E. coli*'s phospholipids[204] and *myo*-inositol can be added to growth media of *E. coli* expressing *Trypanosome brucei* PIS to synthesize PI in-vivo[205]. This enzyme was kindly provided by Dr. Terry K. Smith of The University of St Andrews, UK. Using this enzyme gave me the advantage of starting from a system that was already characterized for the production of PI, and in which inositol was known to be a viable way to regulate the system in vivo. While it is not clear how inositol will reach the cytoplasm of the bacterium, I did not try to investigate the mechanisms since the previous work had shown its addition to the media leads to the production of PI. Thus for the purposes of my system the relevant factor is that inositol is incorporated, although the mechanisms is unknown.

The next two steps involve the phosphorylation of PI at the 4^{th} and 5^{th} positions, by phosphatidylinositol 4- kinase and phosphatidylinositol 4-phosphate 5-kinase

respectively[206]. To my knowledge these enzymes have not been shown to be active in-vivo when expressed in *E. coli*, so I used enzymes that had been previously expressed in this bacterium and showed activity when purified. I used *Bos taurus* (cow) phosphatidylinositol 4-kinase β [207] (PI4Kβ, PI4K henceforth) kindly provided by Dr. Tamas Balla of the Program for Developmental Neuroscience at NIH, and human phosphatidylinositol 4-phosphate 5-kinase type-1 α isoform 2 (PI4P5Kα henceforth PI4P5K)[208] kindly provided by Dr. Richard A. Anderson of the University of Wisconsin - Madison. When choosing these enzymes I assumed that given they are active after production and purification from *E. coli* they should also show activity in the bacterium.

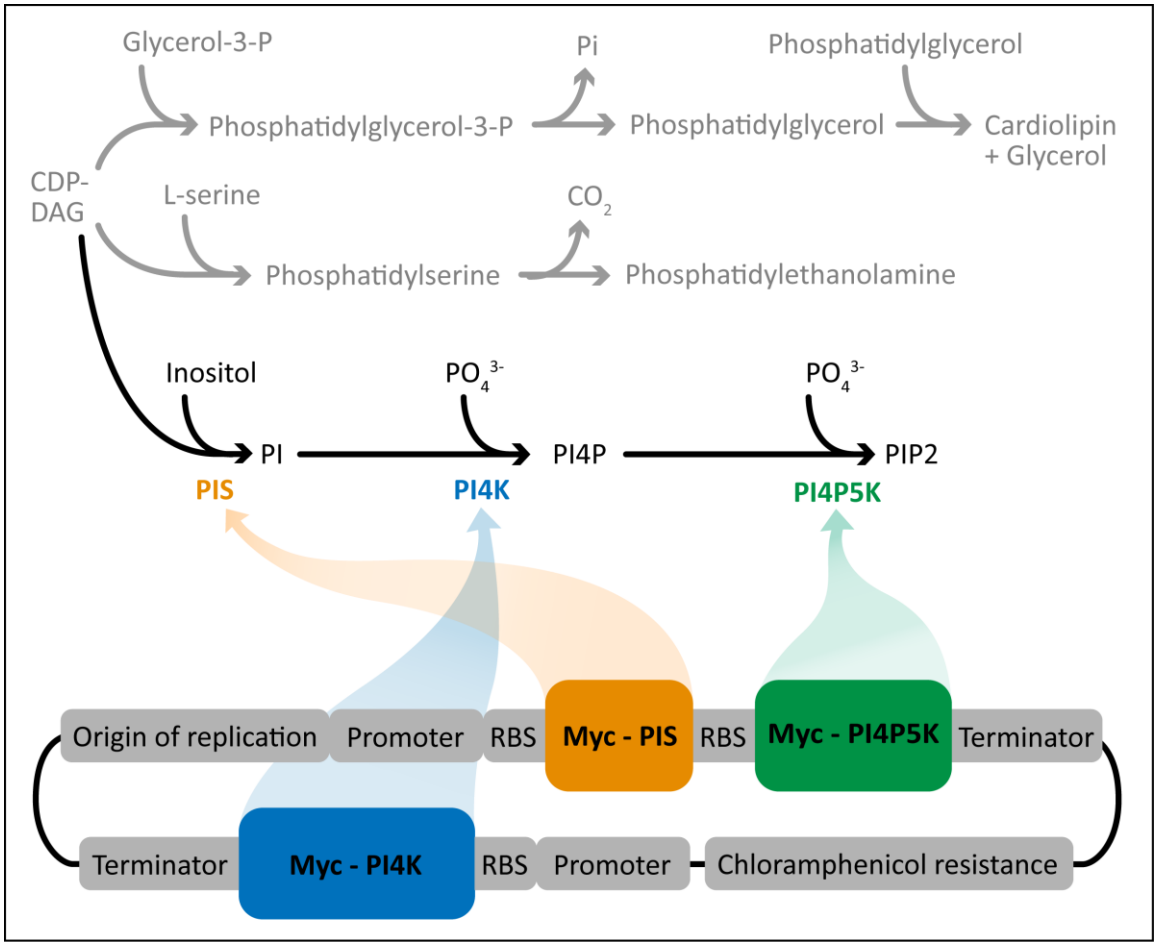


Figure 3. Phospholipid metabolic pathway in *E. coli* and modifications for phosphoinositide production

A) *E. coli* phospholipid metabolic pathway and designed PIP2 synthesis pathway.

B) Construct for the expression of the enzymes required for PIP2 synthesis.

2.2. Laboratory methods

To build the system I used a combination of traditional restriction enzyme cloning, site directed mutagenesis, artificial synthesis of DNA, and assembly PCR. For the site directed mutagenesis I used the QuikChange Lightning site directed mutagenesis kit from Agilent Technologies according to the manufacturer protocol but scaling down the volume to 25, 15, or 10 µl per reaction instead of the recommended 50 µl. All primers used were obtained from Integrated DNA Technologies and designed manually. Restriction enzymes were acquired from New England BioLabs and used according to their protocol. Ligations were performed with the Quick Ligation Kit also from New England BioLabs following their protocol. PCRs were performed using the Platinum PCR SuperMix according to manufacturer protocol or the AccuPrime Pfx SuperMix when blunt ends were required or for the assembly PCR; both of these mixes were from Invitrogen (now Thermo Scientific). Artificial DNA synthesis was obtained as a service from GENEWIZ. All purification steps were performed using the DNA Clean & Concentrator-5 from Zymo Research, and agarose gel purifications were performed using Zymoclean Gel DNA Recovery Kit from the same company. Plasmid purification was performed with the PureLink Quick Plasmid Miniprep Kit from Invitrogen.

No DNA sequence optimization was performed on the enzymes except on PI4K, which had 3 translation pause sites specific to *E. coli*[209] which I removed. On PI4K I also modified the 5' end to eliminate possible loops in the RNA that could affect translation. I

identified the loops using the RNAfold web server from the Institute for Theoretical Chemistry at the University of Vienna (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The changes amount to a total of 10 synonymous point mutations to the enzyme, focused on its N terminus. Since the expression and activity of this enzyme was satisfactory after all these changes I did not attempt to isolate the effects of each individual change or to fully codon optimize the sequence and moved forward with the modified enzyme. A file with the final construct fully annotated is provided as the Supplementary File 1, and the sequence will be uploaded to NCBI upon publication of the paper describing this work. All constructs were fully sequence verified by Sanger sequencing using GENEWIZ services.

Originally the plasmids used for this study were built in lab based on plasmid pET151 from Invitrogen, which has an origin of replication from the ColE1 family. Because during optimization I had to switch to a lower copy plasmid the final constructs were built based on an origin of replication from the P15A family, obtained from the plasmid carried by *E. coli* BL21-CodonPlus(DE3) from Agilent Technologies. This strain was not used in my experiments and only the origin of replication obtained from the plasmid it carries was used. When a second compatible origin of replication was needed for the experiments, I used a plasmid built with an origin of replication from the ClodF13 family obtained from plasmid pCDFDuet-1 MKK4(EE)-MKK7a1(EE) which was constructed by Dr. Kevin Jane's research group and purchased from Addgene (plasmid # 47580). All plasmids used are presented in Table 1.

Cloning procedures were performed in *E. coli* strain DH5α since this is the routine strain for this purpose, using chemically competent cells. For all the experiments the *E. coli* strain BL21 DE3 was used since this is a strain commonly used for the expression of exogenous proteins in this bacterium taking advantage of the inducible T7 polymerase[210] and thus is likely to be most useful to users of the system. Note that the system I developed does not require the T7 polymerase and should work in any *E. coli* strain. In order to make chemically competent cells for my experiments I used the Mix & Go *E. coli* Transformation Kit from Zymo Research according to manufacturer's protocols which follows a standard chemical competence protocol[211]. Strains of *E. coli* were obtained from Invitrogen (Thermo Scientific). All bacteria were grown in LB media at 37C in an orbital shaker at 260 rpms.

In order to make the measures most consistent, BL21 DE3 cells for experiments were transformed with the appropriate plasmids and plated on LB agar with the appropriate antibiotics. After a day a single colony was picked and grown overnight in LB media with antibiotic, and then diluted to an OD600 of 0.05 in fresh media with the appropriate antibiotics and inositol if required for the specific treatment. Liquid cultures were grown in 14 ml tubes filled with 6ml of media unless indicated otherwise. Except for time course experiments, cells were always grown for 3h or less for experiments after the initial dilution to guarantee that they would be in the exponential growth phase, thus diminishing cell to cell variability.

For lipid extractions a volume of culture was pelleted to be equivalent to 10 ml of OD600 equal to 1.0. For protein extractions the same protocol was used but using only 1 ml volumes and only one fourth of the extraction was loaded to the polyacrylamide gels. Protein extraction was performed using the BugBuster 10X Protein Extraction Reagent from Millipore supplemented with rLysozyme Solution and Benzonase Nuclease, Purity > 90%, also from Millipore according to manufacturer protocol. For Western blots 9E10 antibodies were obtained from Acris Antibodies (now part of Origene).

To measure the growth rate of the cells an overnight culture of bacteria was diluted to an OD600 of 0.05 and 300 µl were grown in a 96 well plate incubated at 37C in a plate reader. OD600 was measured every 30 minutes. Each strain was measured in 4 independent wells and a correction was performed for evaporation on the blank well. The slope of the linear approximation to the middle of the exponential phase of growth was measured for each well and this slope is the data gathered to perform the averaging. For morphology observations 1 µl of bacterial culture was spread in a glass slide, covered with a cover slip and bright field images were obtained with a 60x water objective with a 1.5x objective in the light path (90x total).

Table 1: Plasmids used in this work

Plasmid type 1 P15A family origin of replication (10-12 copies per cell), chloramphenicol resistance, constitutive promoter D, N-terminal myc tag		
Name	**Insert**	**Notes**
p15aC	None	Empty plasmid control
p15aC-GFP	GFP	Control
p15aC-1D	PIS	
p15aC-4D1D	PI4K and PIS	Enzymes expressed with separate repeated promoters
p15aC-1D-5	PIS+PI5P5K	Enzymes expressed as an operon in that order
p15aC-4D1D-5	PI4K and PIS+PI5P5K	PI4K expressed separate and PIS+PI5P5K expressed as an operon in that order with repeated promoters
Plasmid type 2 ClodF13 family origin of replication (20-40 copies per cell), ampicillin resistance, dual constitutive promoter D, cytoplasmic mCherry, N-terminal GFP tag		
Name	**Insert**	**Notes**
pClodACherry-PIP2PH-GFP	PLCδ-PH domain	
pClodACherry-PIP2PHmutant-GFP	PLCδ-PH domain with mutant binding site: K30A, K32A, W36A, R40A, E54A, S55A, R56A, K57A	
pClodACherry-PI4PPH-GFP	OSBP-PH domain	Failed construct (see text)

Table 1: Plasmids used in this work (continued)

Plasmid type 3 ClodF13 family origin of replication (20-40 copies per cell), ampicillin resistance, constitutive promoter D, C-terminal NanoLuciferase tag		
Name	**Insert**	**Notes**
pClodANL-Cherry	mCherry	Control
pClodANL-NonBinderFGF2	FGF2 non-binding mutant K127Q,R128Q, K133Q	
pClodANL-wtFGF2	FGF2 wild type	
pClodANL-FGF2-Y82E	FGF2 Y82E phosphomimic	
pClodANL-FGF2-Y82EE	FGF2 Y82EE double phosphomimic	
Plasmid type 4 ColE1 family origin of replication (~40 copies per cell), chloramphenicol resistance, constitutive promoter T7 and promoter D in tandem, N-terminal myc tag, enzymes expressed as operon in order indicated. These were used as optimization intermediates		
Name	**Insert**	**Notes**
pET151Cons1	PIS	
pET151Cons41	PI4K+PIS	
pET151Cons51	PI4P5K+PIS	
pET151Cons54	PI4P5K+PI4K	
pET151Cons541	PI4P5K+PI4K+PIS	Reverse order operon, showed better activity
pET151Cons145	PIS+PI4K+PI4P5K	

Lipid extraction was performed with the Bligh-Dyer protocol[212] adapted specifically for this project (see Supplementary Protocol 1). Lipids were stored at -20C and measured the next day after extraction since degradation was observed over time in the optimization runs. PI4P and PIP2 abundances were measured using a competitive ELISA assay commercially available from Echelon Biosciences. On the day of the ELISA tests an aliquot of 6 µl or less of the extracted lipids was dried by leaving a 1.5 ml microcentrifuge tube open for 10 to 15 minutes and resuspended according to the ELISA protocol.

In order to do the calculations of the percentage of lipids in the cell being phosphoinositides I used a relationship between total biomass of *E. coli* and optical density of the culture determined previously[213]. This equation defines the dry weight of *E. coli* growing in LB media as 0.56 by the OD660 in grams per liter, thus: Dry weight (g/l) = 0.56 x OD660. The percentage of lipids in *E. coli* by dry weight has been estimated at 9.1%[214] so an estimate can be made for the total lipids in the cell given the measured OD. These values were found using the BioNumbers database[215] with their ID numbers being 108127 and 101938 respectively. In order to estimate the quantity of phosphoinositides relative to the overall lipids in the cell, an assumption on the molar weight of the phosphoinositides needs to be made since the precise lipid tails are unknown. I used values of 800 and 850 g/mol for PI4P and PIP2 respectively, which correspond to a phosphoinositide with the shorter lipid tails characteristic of *E. coli*. While this is by no means a precise calculation, it allows for an estimate of the range of

phosphoinositides being produced in the cells. This estimate is good enough to evaluate the potential of the system to build mechanistic models of eukaryotic cell biology.

For the phosphoinositide localization tests I used the PH domain of oxysterol-binding protein (OSBP-PH domain) which binds PI4P [216] and the PH domain of phospholipase Cδ1 (PLCδ-PH domain) which binds PIP2[217]. These constructs were kindly provided by Dr. Tamas Balla of the Program for Developmental Neuroscience at NIH. The expectation for these tests is that the cells should show diffuse fluorescence in the cytoplasm when no phosphoinositides are being produced, and a halo should appear when phosphoinositides are present indicating the localization of some of the labeled protein to the plasma membrane. As a way to have an internal control I also expressed mCherry, a red fluorescent protein, such that in the same cell I could observe the behavior of the tagged phosphoinositide binding PH domain in the green channel and a control in the red channel. To perform these analyses with the appropriate resolution I used a LEICA scanning confocal microscope. I set the aperture of the pinhole to 1 airy unit relative to the fluorescence of GFP at 507 nm (in this case 1 airy = 128 µm), and excited sequentially with a laser at 488nm for GFP and 587 nm for mCherry, acquiring on the range of 500 to 570 nm for GFP and 600 to 670 nm for mCherry. For imaging I used a 100x HC PL APO oil objective of 1.44na. The image size was a square of side 29 µm at a resolution of 512x512 pixels, scanned at 100Hz.

Detection of the enzymes expressed in the system using the myc tag proved problematic initially. Polyclonal antibodies showed poor performance, with many non-specific bands appearing. This is presumably due to imperfect affinity purification combined with the use of proteins expressed in *E. coli* for the immunization of animals. Since the myc tag is a very commonly used tool there are monoclonal antibodies available which should have solved the issue. Of these the clone 9E10 from mouse showed the best results, but surprisingly only when the antibody is directly labeled with horse radish peroxidase (HRP). When the 9E10 monoclonal antibody is used with a secondary antibody a western with very little or no noise is obtained but only PI5K is detected. Figure 4 shows a comparison of the 9E10 antibody with its HRP tagged version when used to detect the construct expressing PIS and PI4P5K in both nitrocellulose and PVDF membranes.

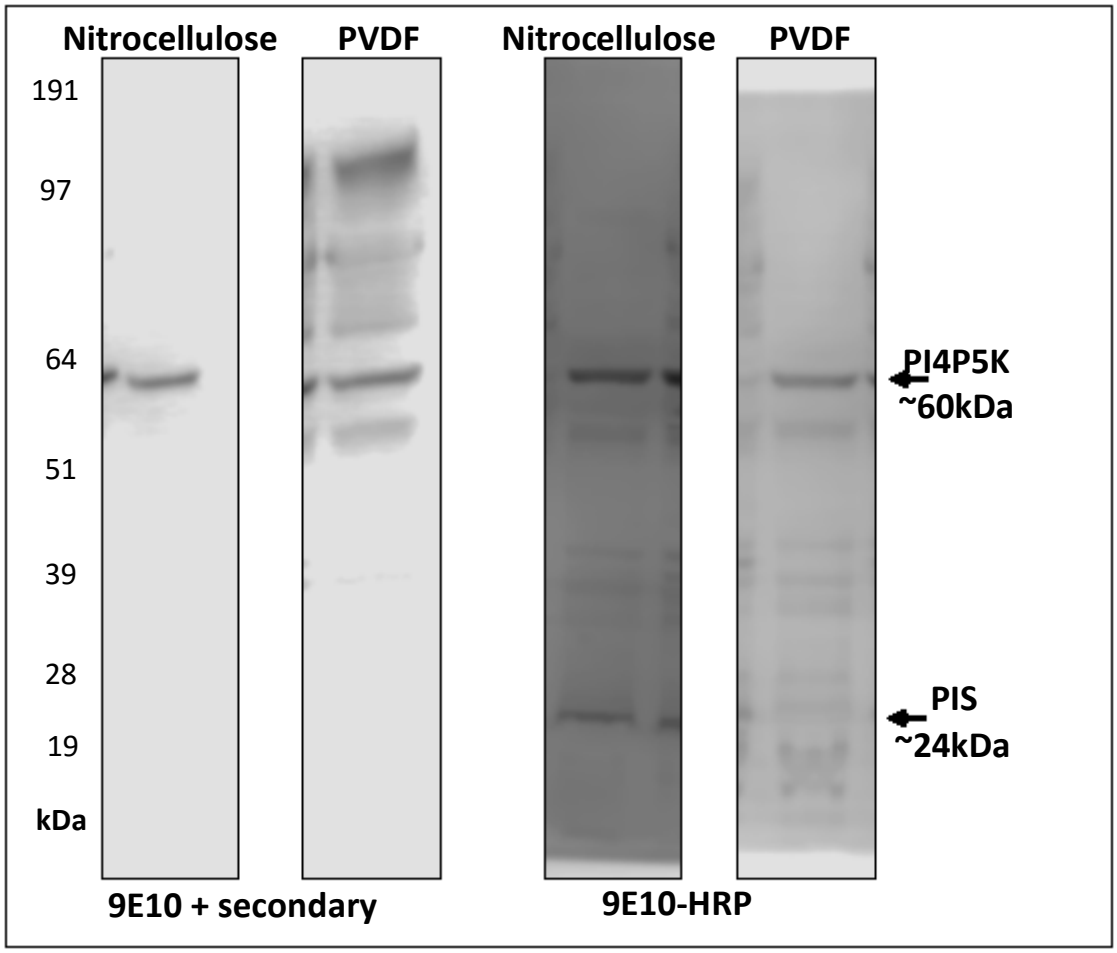


Figure 4. Comparison of the 9E10 monoclonal labeled and unlabeled antibodies

Untagged and tagged versions of the monoclonal antibody 9E10 were used for the detection of PIS and PI4P5K simultaneously in both PVDF and Nitrocellulose membranes. This comparison shows the best detection of the enzymes is obtained when using the HRP labeled antibody in a nitrocellulose membrane.

For NanoLuc Luciferase assays cells were grown in the same way as for the other experiments and at 3h 50 µl of culture were pipetted to a well in a 96-well plate. 30 µl of freshly made PBS pH 7.0 containing 30 µl of substrate (Promega N113A) per 10 ml of buffer were added to each well and luminescence and OD600 were measured. After this initial measure 100 µl of BugBuster 1X prepared in PBS pH 7.0 were added to each well and lysis was allowed to run for 15 minutes. Lysis was confirmed by the decrease in OD600 in the well reading. After lysis 20 µl of PBS pH 7.0 containing 20 µl of substrate were added to each well and luminescence was measured again. All measures were performed immediately after substrate addition to guarantee substrate excess and an appropriate reading.

2.3. Optimization of the system

I initially attempted to build an operon expressing the 3 enzymes required for PIP2 synthesis from the same RNA. However, expression of PI4-kinase was problematic and a system in which PIS and PI4P5-kinase were expressed in that order as an operon, and PI4-kinase was expressed separately was necessary for detection of all 3 enzymes on western blots. This was assembled in the same plasmid, using the same promoter and terminator for both PI4-kinase and the PIS-PI4P5-kinase operon. Each of the enzyme sequences is preceded by a ribosome binding site (RBS) designed with the RBS calculator software to maximize their expression[218, 219], and has an N-terminal myc peptide tag (amino acid sequence EQKLISEEDL) for easy identification in western blots. To prevent

possible recombination between the repeated promoters the origin of replication and the resistance marker were arranged in such a way that if recombination happens the resulting plasmids would lack either a promoter or an origin of replication and be therefore selected against. A schematic of the final expression system is shown in Figure 3B.

In the initial steps of optimization of the system I attempted to use a modified version of the plasmid pET151 (from Invitrogen) changing the resistance marker to chloramphenicol. Plasmid pET151 has a medium-high copy number (~40 copies per cell)[203]. I left the very strong T7 promoter[210] directly followed by the promoter selected for the system (see description of the final system below). This system led to morphological abnormalities in the bacteria (see Figure 5A), presumably due to overly high protein expression levels causing aggregation. An additional abnormal phenotype in which cells became very long also appeared when growing with PIS and PI4P5K together (note the lack of PI4K) in minimal media supplemented with inositol (Figure 5B). Some long cells are observed even in control cells so a small fraction of long cells in any construct is normal, but in this case it is the majority of the cells that are long with most of them being extremely long. This phenotype only appeared as extreme in this media and only for this construct so I suspect it to be an interaction between the level of phosphoinositides present and the amount of the exogenous proteins potentially interfering with correct localization of *E. coli*'s membrane bound proteins under the stress of growing on poor media. In one occasion the high expression of PIS alone did

produce some very long cells in LB media supplemented with inositol, but those were less than 20% of the cells (Figure 5C). Since I did not use these constructs and built a working system using another origin of replication I did not investigate any of these abnormalities further.

I built the final system using a low copy plasmid with an origin of replication from the P15A family, and a chloramphenicol resistance marker. There was no specific reason to choose this over other antibiotics except ampicillin. Since ampicillin is the most commonly used antibiotic I wanted to use one that would allow more of the already available constructs to work in the system without modifications. The origin of replication used has a low copy number of 10-12 copies per cell[203], and I used an insulated constitutive promoter that expresses robustly in *E. coli*, named promoter D in the work of Davis and co-workers[220], henceforth simply referred to as promoter. A schematic of the final expression system is shown in Figure 3B. Since the promoter works in *E. coli* without needing specific exogenous polymerases (such as the commonly used T7 promoter) the system can be used in any strain of this bacterium. In order to make the system most useful and maximize its uses I created versions of the plasmid expressing only PIS, PIS plus PI4K, and PIS plus PI4K and PI4P5K, thus producing PI, PI plus PI4P, and PI plus PI4P and PIP2 respectively. Additionally, I constructed control plasmids expressing only a myc-tagged green fluorescence protein or just the myc tag.

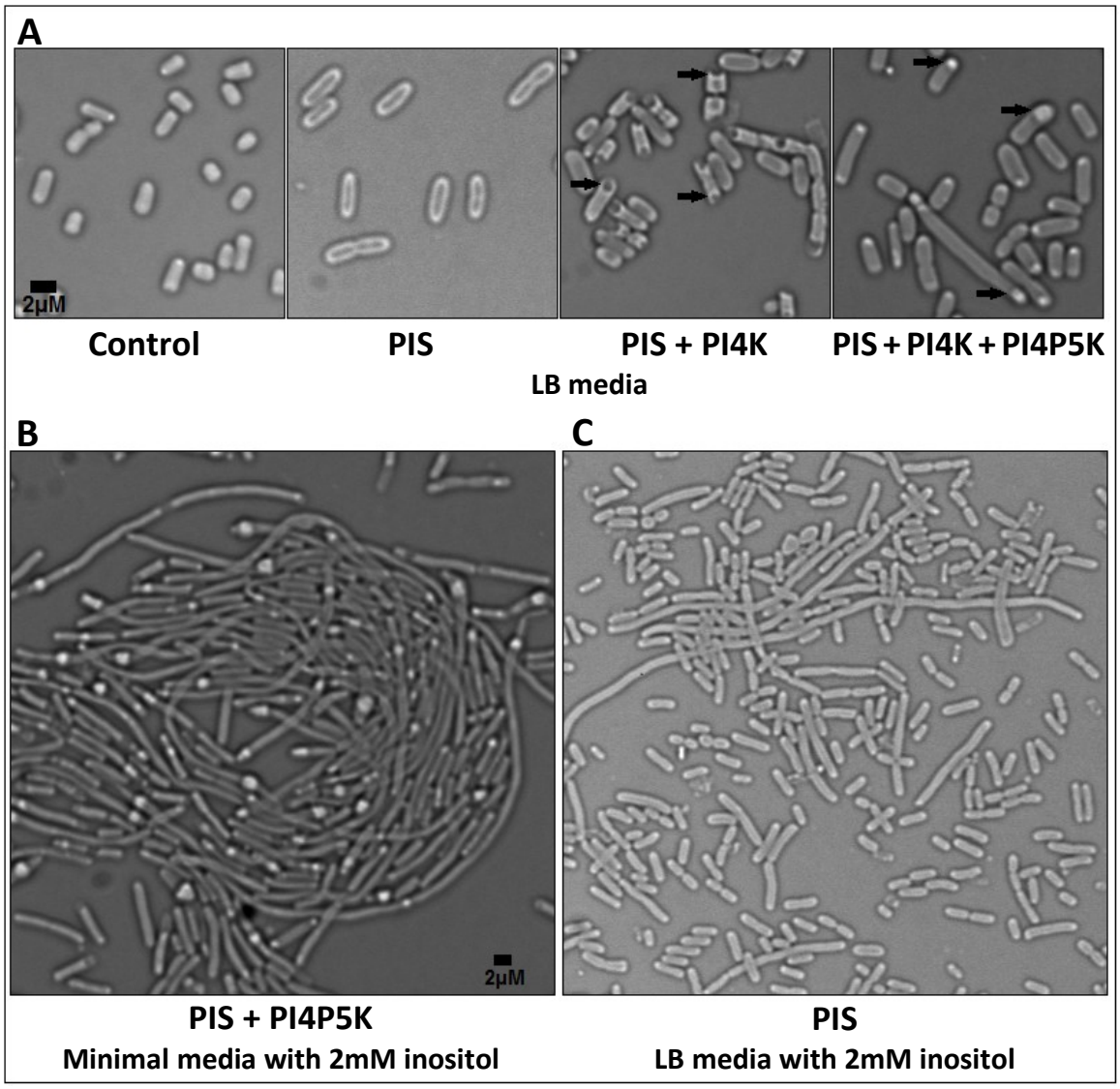


Figure 5. Abnormal morphology of cells with high expression constructs

Overnight cultures were diluted to an OD600 of 0.05 and grown for 3 hours in LB or minimal media supplemented with inositol and the appropriate antibiotics. **A)** Morphology in LB media. While a larger cell size is normal in *E. coli* overexpressing protein, I observed several other abnormalities in LB media even without the addition of inositol. **B)** Extreme abnormal growth observed in *E. coli* expressing PIS and PI4P5K in the grown in minimal media supplemented with inositol. **C)** Low frequency abnormal growth of *E. coli* expressing PIS in LB media supplemented with inositol.

3. Results and discussion

3.1. Characterization of the system

All enzymes were expressed as expected in the final construct (Figure 6, Figure 7 shows the same western on a PVDF membrane) but there are a large number of faint nonspecific bands appearing in the western. While generally these can be thought of as degradation or incomplete translation products some of them increase in size relative to the enzymes expressed and likely reflect a more complex situation like the modification of the enzymes by the bacterium or possibly the induction of expression of endogenous proteins from *E. coli* that share the recognized peptide. Since the pattern of expression is the expected one and the functional tests showed the production of the phosphoinositides of interest I did not try to optimize the system further to reduce these nonspecific/degradation product bands.

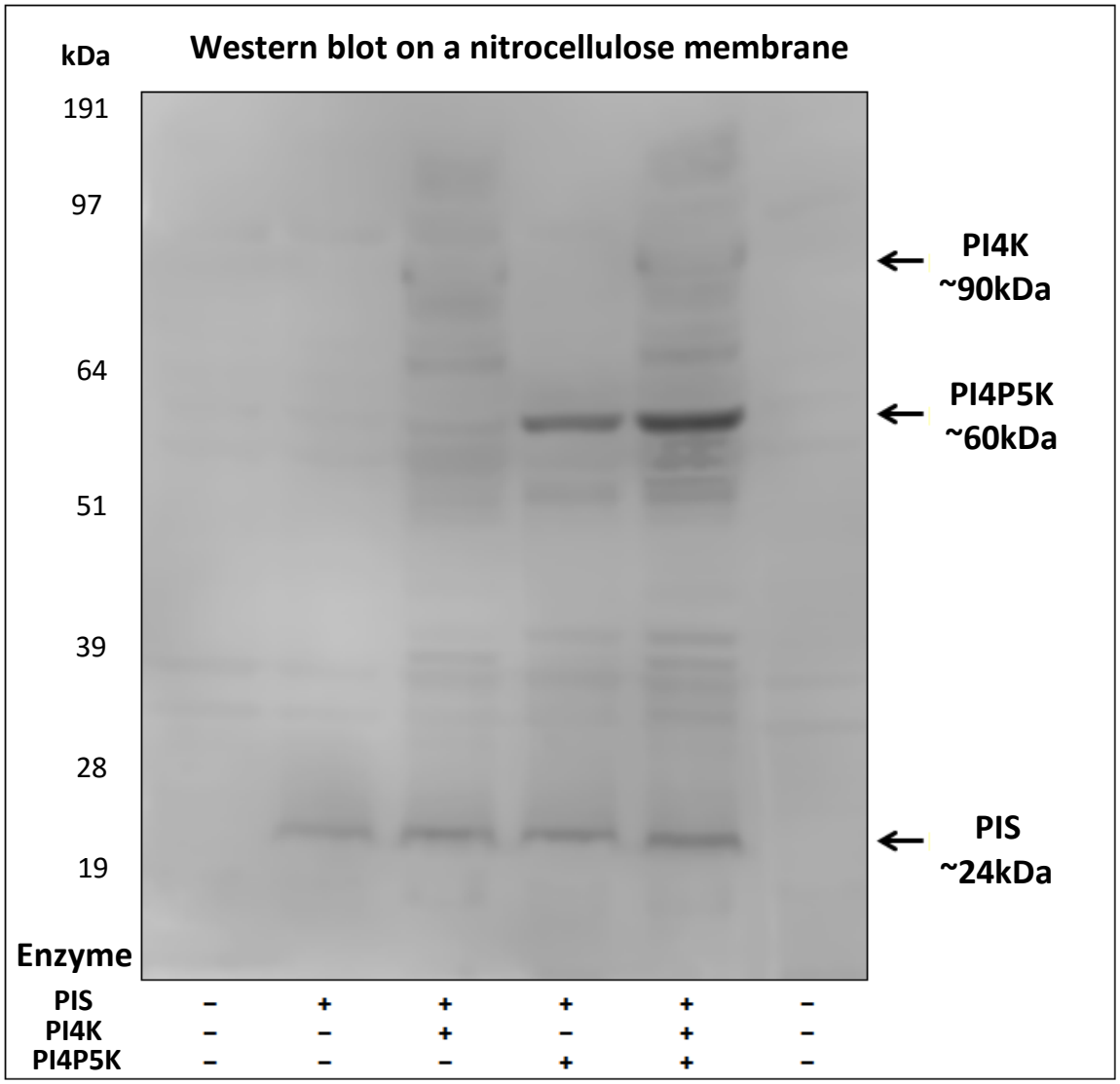


Figure 6. Western blot confirmation of enzyme expression on nitrocellulose

Western blot detection of the enzymes in the final constructs using an HRP labeled 9E10 antibody against myc peptide on a nitrocellulose membrane. Note that constructs expressing PI4K show bands significantly higher than expected and the PI4K band itself is odd showing more signal towards the edges than the center of the lane. The protein sizes indicated include the myc tag and spacers.

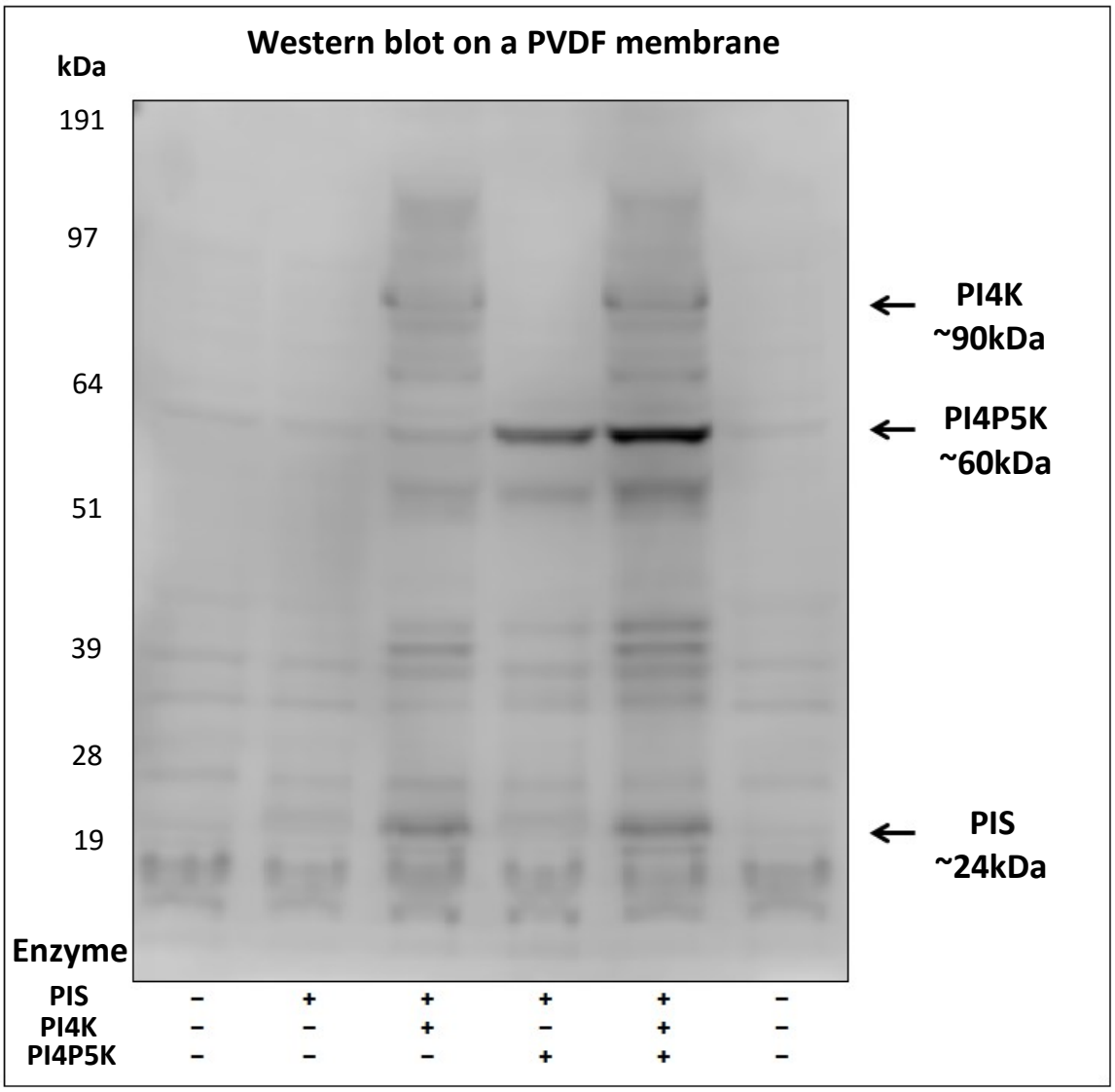


Figure 7. Western blot confirmation of enzyme expression on PVDF

Western blot detection of the enzymes in the final constructs using an HRP labeled 9E10 antibody against myc peptide on a PVDF membrane. Note that constructs expressing PI4K show bands significantly higher than expected and the PI4K band itself is odd showing more signal towards the edges than the center of the lane. Also detection of PIS is not consistent among constructs. The protein sizes indicated include the myc tag and spacers.

The system showed no adverse effects on growth (Figure 8) of the cells expressing the enzymes regardless of inositol addition up to 10mM when growing on LB media. However, 20mM of inositol proved lethal for all constructs except the control. This could be due to a lethal effect of the phosphoinositides themselves or to competition for the CDP-diacylglycerol precursor. Since the effect is observed even when just PI is produced, it should be due to the properties of PI directly or to the competition effect and not to the high charge of PI4P or PIP2. If the effect is due to PI it would likely be due to its bulkier head-group compared to the normal *E. coli* phospholipids. For the purposes of my system this lethality effect imposes a limitation of maximum 10mM inositol in the media. I performed all experiments using at most 5mM inositol in the media with 2mM being the concentration used for most experiments. Larger cell size is normal in *E. coli* overexpressing protein[221], so when judging changes induced by protein expression it is important to consider that a larger size is not an abnormal situation. The morphology of the cells expressing the different constructs was normal when growing in LB media with 2mM inositol (Figure 9). In some of the tests expressing other proteins cell size did increase (see the “no inositol control” in Figure 15 for an example), but the cells never behaved in an aberrant way such as that observed during the optimization runs in the high expression system (see Figure 5).

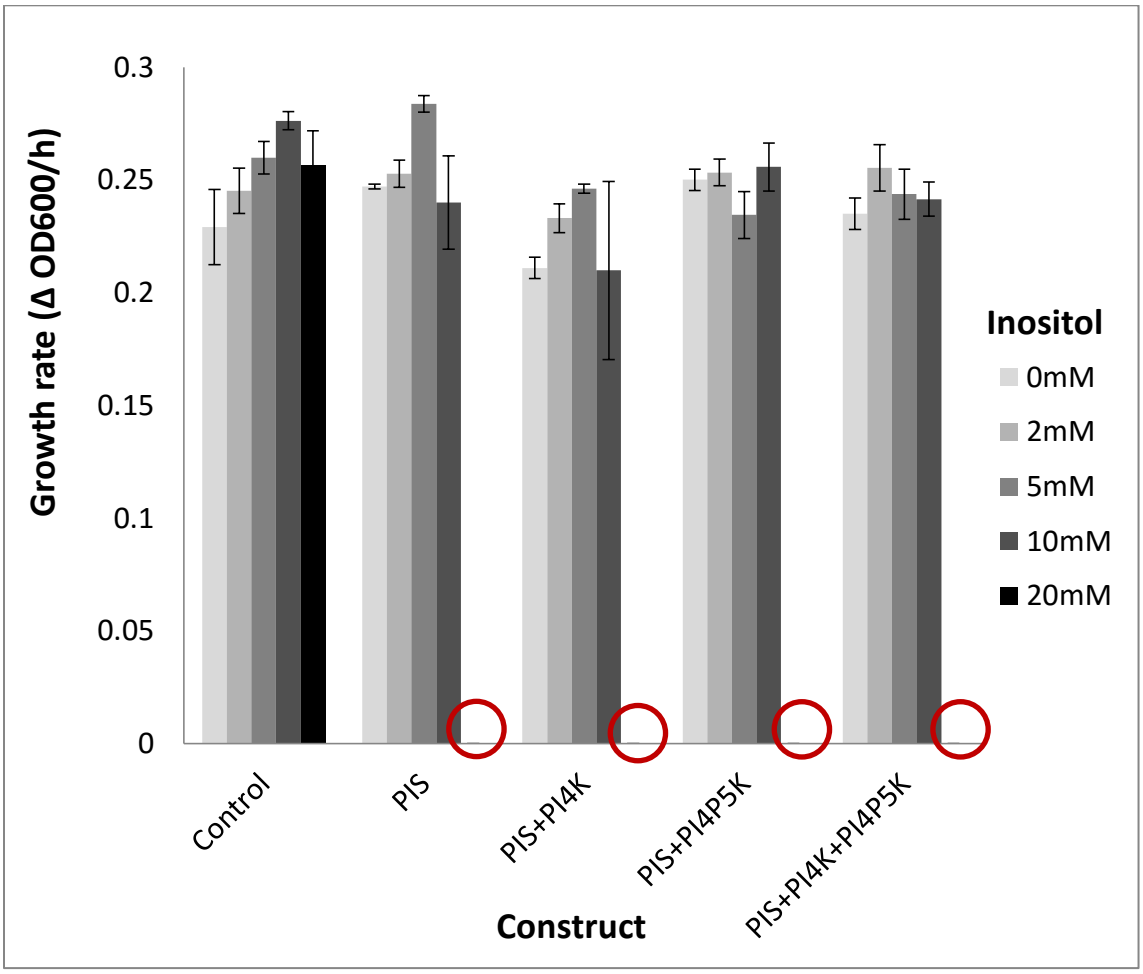


Figure 8. Effects of constructs and inositol on the growth rate of *E. coli*

Cells were grown in 300 µl of media in a plate reader measuring OD600 every 30 minutes. Note the lethality of the treatment of 20mM inositol for all constructs except control (red circles). Data shown is the average of 4 independent measures with error bars showing the standard deviation of the sample.

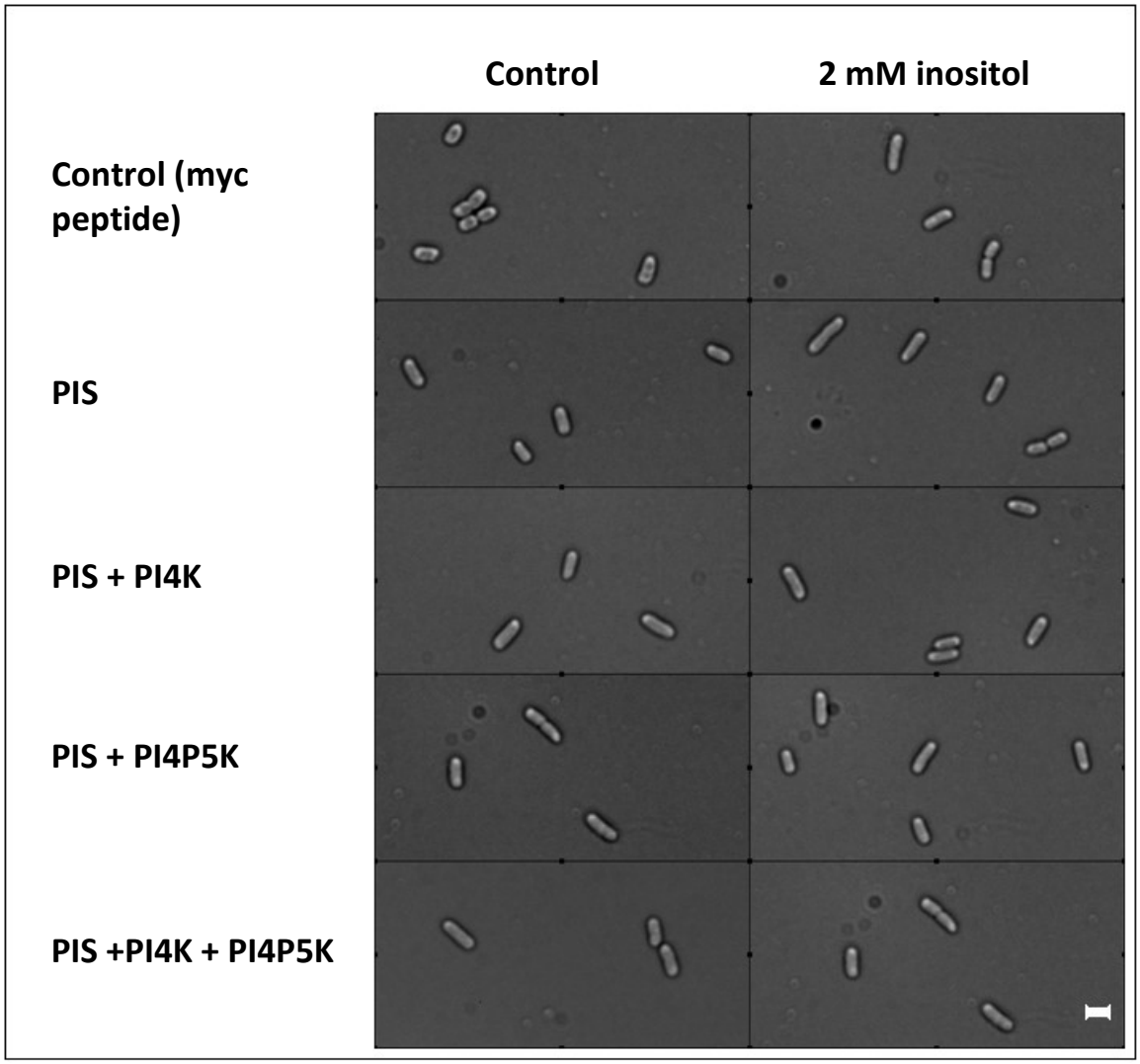


Figure 9. Normal morphology in *E. coli* producing phosphoinositides

Cells were grown for 3h in LB media with chloramphenicol and with or without inositol depending on the treatment. Scale bar is 2µm.

Since PI had been produced and characterized in *E. coli* by other researchers[205] and its role seems to be only structural and as a precursor of phosphorylated phosphoinositides I did not characterize the PI further, and only characterized the production of PI4P and PIP2. The goal of the project is to allow the study of PI4P and PIP2 functions, with a focus on PIP2. Initial tests of the effects of different combinations of the enzymes all showed the expected behavior except for the expression of PIS and PI4P5K together in the absence of PI4K. This construct should produce only PI but it also produced PI4P and PIP2. This shows that PI4P5K is able to phosphorylate PI at the 4th position creating PI4P, its normal substrate, which it then phosphorylates again generating PIP2. The fact that PI4P can be detected implies that the phosphorylation is not carried in tandem but that the enzyme must release PI4P at least a fraction of the time. While it is a surprising finding, it is beyond the scope of this project to investigate it further and I prefer not to speculate about its potential biological meaning. Since the system lacks any of the normal regulators of activity for this enzyme, and there is no spatial segregation by an endo-membranous system, this activity might in fact be specific to my system. Nonetheless this construct was included when characterizing the amount of phosphoinositides produced since it may offer an alternative for users of the system that would like to minimize the metabolic stress of exogenous protein expression to the bacterium. Using this construct it is possible to have a cell with PI, PI4P and PIP2 present while only expressing two enzymes (PIS and PI4P5K).

Figure 10 shows the production of PI4P and PIP2 by the different constructs. Quantities are indicated as percentage of the total lipids by mass since this allows for the most direct comparison to the levels of phosphoinositides Eukaryotic cells. The range observed is on the lower end of the range observed in mammalian cells, in which PI4P or PIP2 each represents about 0.5-1% of the total phospholipids. Considering that in eukaryotic cells phosphoinositides interact with a wide variety of proteins at all times the amount of free phosphoinositides in these cells should be significantly lower, thus the amount produced by the system I developed should be enough to test most cell biology models of interest with levels of available PIP2 equal or higher than those in eukaryotic cells.

Another consideration is the fact that *E. coli* has a double membrane and since phosphoinositides do not spontaneously flip, they most likely would all be on the inner leaflet of the plasma membrane. Although not yet characterized, the unspecific scrambling of phospholipids in the membrane of *E. coli*[222] could cause equal distribution of the produced phosphoinositides on both leaflets of the plasma membrane, but given the high charge of PI4P and PIP2 relative to normal *E. coli* lipids it is unlikely this would be the case since any transporter would not normally act on highly charged phospholipids. If phosphoinositides were to flip to the outer leaflet of the plasma membrane it is also possible, albeit very unlikely for phosphorylated phosphoinositides given their charge, that they could make it to the outer membrane through the normal mechanism for translocation used by the bacterium.

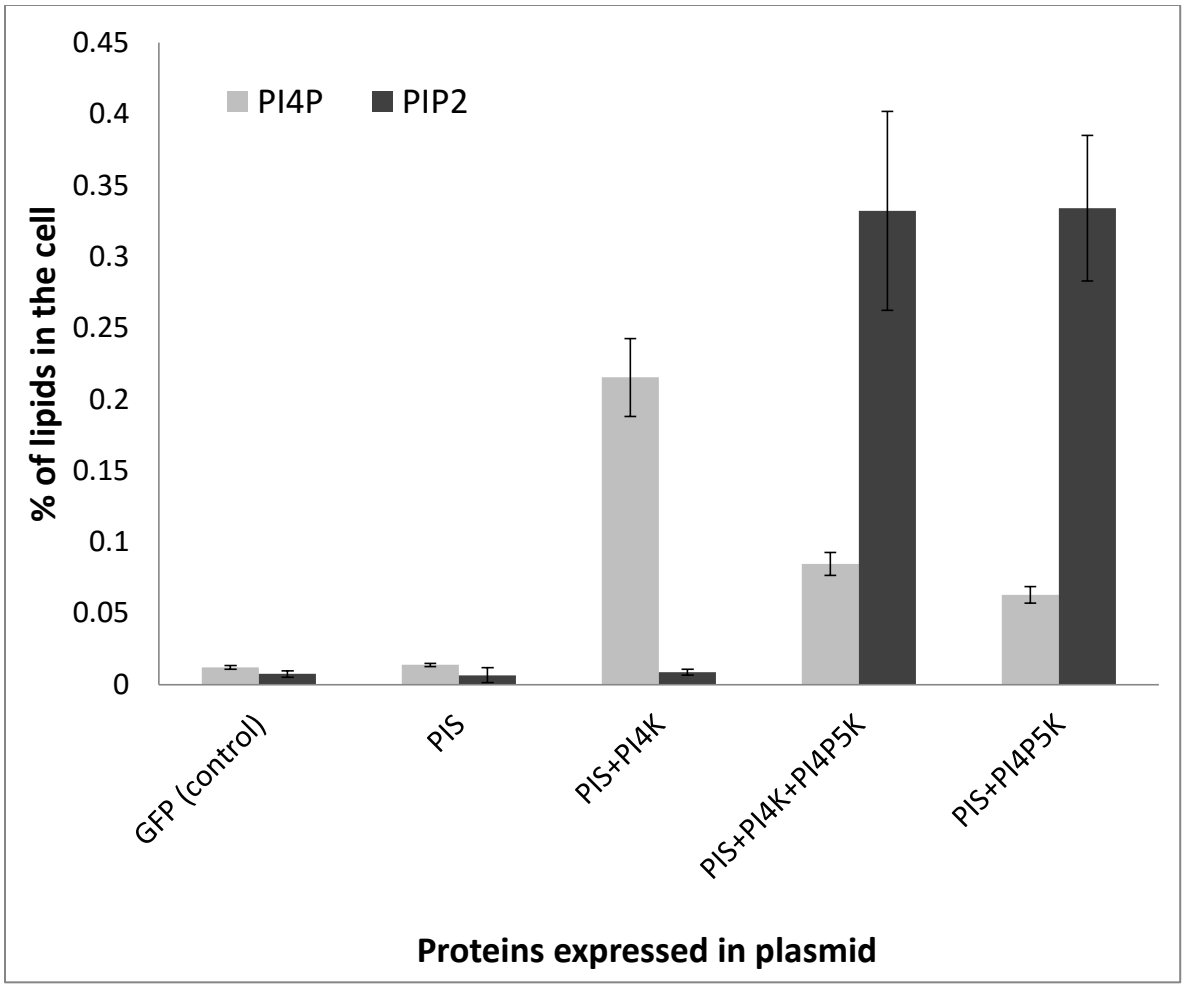


Figure 10. Phosphoinositide production for all constructs

Cells for the analysis were grown for 3h in LB media with 2mM inositol and chloramphenicol, after which lipids were extracted as described in the text. Data shown is the average of 4 independent measures with error bars showing the standard deviation of the sample. Note the unexpected production of PI4P and PIP2 in the last construct where PI4K is absent.

Thus the estimate provided represents a conservative scenario for their abundance with the maximum effective concentration being four times the reported one (assuming an equal split of phospholipids between the inner and outer membranes of the bacterium and all phosphorylated phosphoinositides residing on the inner leaflet of the plasma membrane) and the minimum being the reported one (assuming equal distribution among inner and outer leaflets, of both plasma and outer membranes). It is important to note that PI4P is produced at a lower level than PIP2 even when only PIS and PI4K are expressed. While in the constructs that produce PIP2 a lower amount of PI4P is expected, since it will be phosphorylated to PIP2, one would expect a similar or even higher level of PI4P when no PI4P5K is present since it should accumulate in the cell. The fact that this is not observed suggests that PI4K might autoregulate based on the abundance of PI4P.

The ELISA assay, used to determine PI4P and PIP2 abundances, has a first step consisting on the resuspension of phosphoinositides in aqueous solution and its subsequent binding by a phosphoinositide binding domain specific to the phosphoinositide of interest. Since phospholipids are intrinsically unlikely to be in aqueous solution given their hydrophobic tails this first step is particularly problematic and can cause abnormal behavior in some runs of this assay. The successful runs show consistency across them and within the trends among samples and controls, but the unsuccessful ones show no differences among the samples and either a very low (barely above the detection limit) or very high signal (saturated for the most part) on all samples. Customer support could

not provide any solutions for this problem thus the test was repeated and the results reported are only from successful runs.

As explained above, unsuccessful runs of the ELISA tests are easily detectable by the behavior of controls, thus they can be objectively identified. In particular, the behavior of the control system which is *E. coli* with a control plasmid that doesn't express any of the enzymes, and the control that just expresses PIS allows for the identification of any abnormal runs. An alternative form of evidence confirming this assessment of successful runs was initially done by a protein lipid overlay assay, which is the equivalent of a dot blot in which lipids are dotted on a membrane and then identified with a protein[165]. This overlay assay for the detection of PI4P was also available commercially from Echelon Biosciences but has now been discontinued and the version for the detection of PIP2 was discontinued before I started the project. This test showed that control and PIS expressing constructs produced no PI4P. Figure 11 shows the results of one PI4P strip test performed with some of the intermediate constructs used for the optimization of the system. Unfortunately, these tests were performed before the system was optimized and after the product was discontinued I could not replicate the protein lipid overlay assay successfully even while getting the reagents from the same company. In these failed attempts the calibration curve showed very bad results with little difference across the same range of concentrations used in the commercial kit. The only difference between these optimization assays and the final constructs was the higher expression of enzymes, thus the assessment should be valid for the final optimized system.

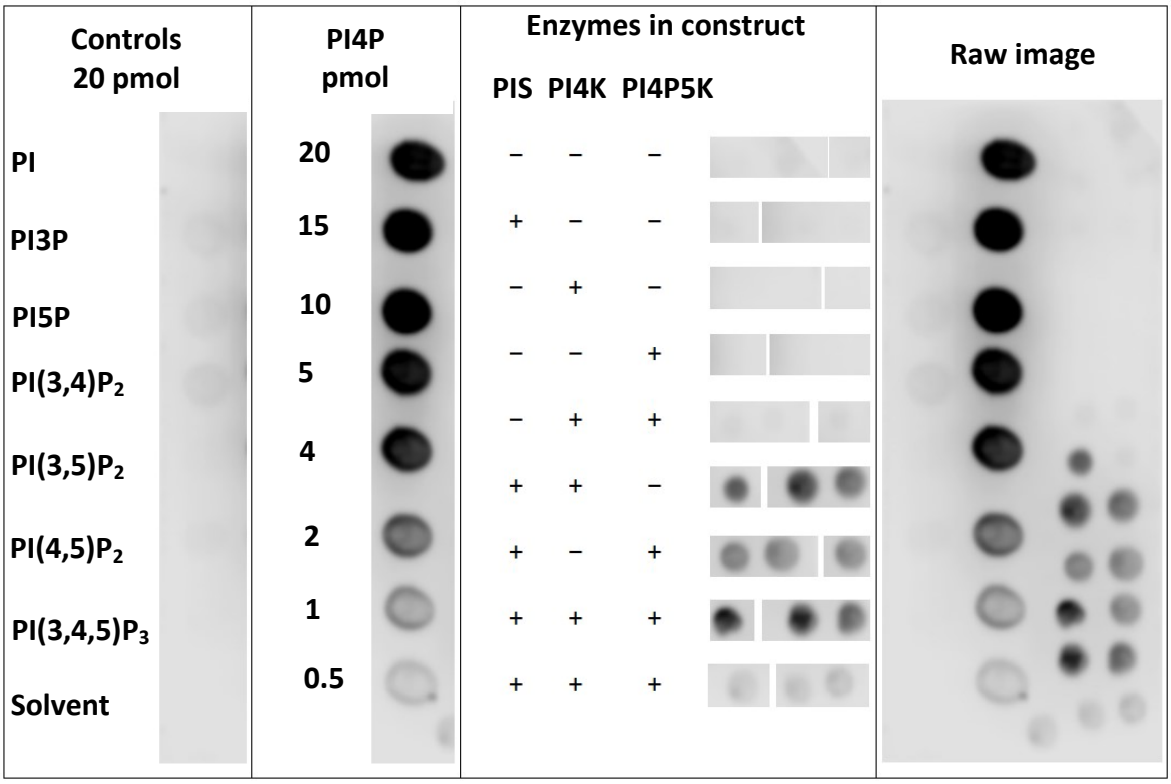


Figure 11. Protein lipid overlay for the detection of PI4P

Detection of PI4P produced by some of the optimization constructs. Since samples were spotted in a complex order the organized data as well as the raw image are provided. Note that at the time I was working with two operon constructs thus there are two tests for a case when all the 3 enzymes are present, but their order of expression in the operon was different.

In order to characterize the system further I performed a time course experiment for the construct expressing all 3 enzymes. The results of this experiment are shown in Figure 12. In this experiment inositol is added at the start of the culture, defining this as time 0. In this way cells should initially accumulate phosphoinositides, but at some point the inositol in the media becomes limiting since it's not being replaces and the phosphoinositides per cell should start to decline by being diluted during cell division. While the result is relatively noisy, it shows the expected pattern in which the amount of phosphoinositide increases over time to a peak around 3 h, after which it declines slowly.

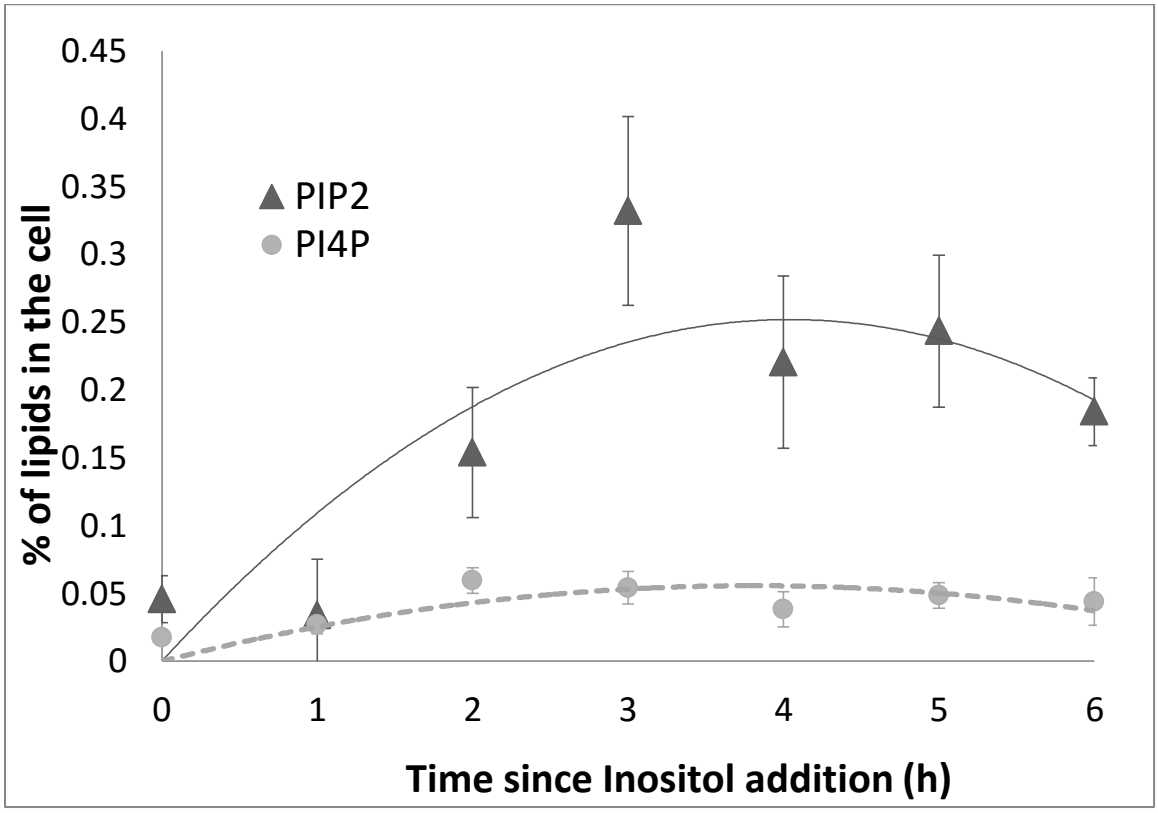


Figure 12. Time course of phosphoinositide production

Cells expressing PIS, PI4K and PI4P5K simultaneously were grown in LB media with 2 mM inositol and chloramphenicol. Aliquots were extracted every hour, and diluted or concentrated to obtain a pellet equivalent to 10 ml of culture at OD600 = 1 for lipid extraction. Trend curves are from a second order polynomial. Data shown is the average of 4 independent measures with error bars showing the standard deviation of the sample.

As a next step to characterize the system I performed an analysis of the effect of the concentration of inositol in the media for the production of phosphoinositides. The results of this test can be seen in Figure 13. This analysis showed that production of phosphoinositides grew with increasing inositol in the media but it also saturated fast, reaching a plateau at around 2mM inositol. This saturation indicates the system is limited by the speed of PI production since PI4P can be produced at higher levels when PI4P5K is not present and PIP2 is dependent upon this PI4P as a precursor. Evidently these results, as well as the levels of phosphoinositide production and time course of production, will vary when the system is used in different bacterial strains and/or under different growing conditions. Their value lies in showing that the system works as expected, and how inositol can be used to control it.

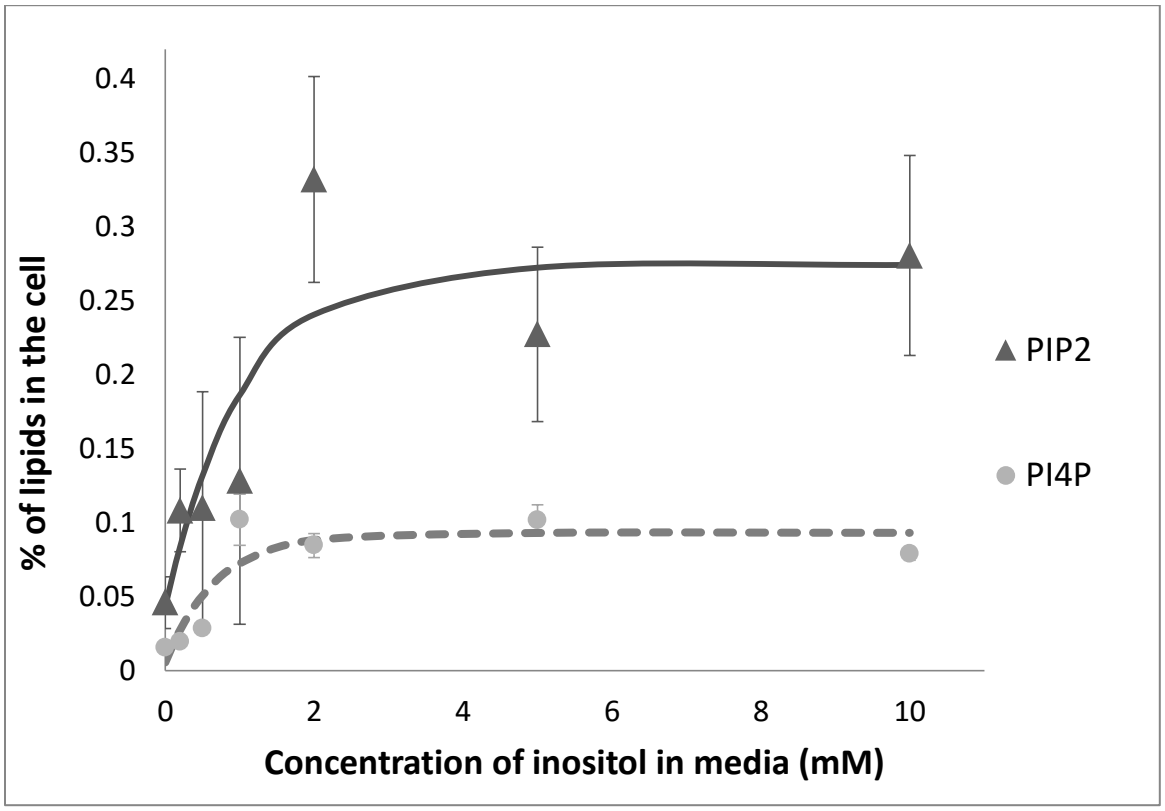


Figure 13. Phosphoinositide production under different inositol concentrations

Cells expressing PIS, PI4K and PI4P5K simultaneously were grown in LB media with chloramphenicol and different concentrations of inositol. At 3 hours OD was measured and cells were diluted or concentrated to obtain a pellet equivalent to 10 ml of culture at OD600 = 1 for lipid extraction. Trend curves are from an exponential fit of the form $y=a+be^{(-c\ x)}$. Data shown is the average of 4 independent measures with error bars showing the standard deviation of the sample. When error bars are not visible it is not because of an absence of variation but because it is too small to be seen at the scale of the graphic.

As a final step in the characterization of the system, and to provide alternative evidence of the production of phosphoinositides, I used a GFP tagged phosphoinositide binding PH protein domain. As mentioned in the introduction these are used routinely to visualize the localization of phosphoinositides in mammalian cells[168]. The advantage is that in my system there is no possible interference by other proteins that also bind specifically to the same phosphoinositide. A result obtained with this system will then allow for a robust in-vivo confirmation of the production of PIP2 in my system.

For PI4P I used the OSBP-PH domain, which binds to PI4P, but this test failed, with no membrane localization appearing even in the presence of the construct that has PIS and PI4K growing in 2mM inositol for 3 h (Figure 14). This could be due to an insufficient amount of PI4P but the ELISA tests showed it is produced satisfactorily under these conditions so it is most likely due to a problem in the folding of the PH binding domain. This domain has been used successfully for this type of experiment in mammalian cells[216], and the fact fluorescence is detected implies the protein is being produced, so the failure is most likely a problem with folding of the PH domain in the *E. coli* cytoplasm.

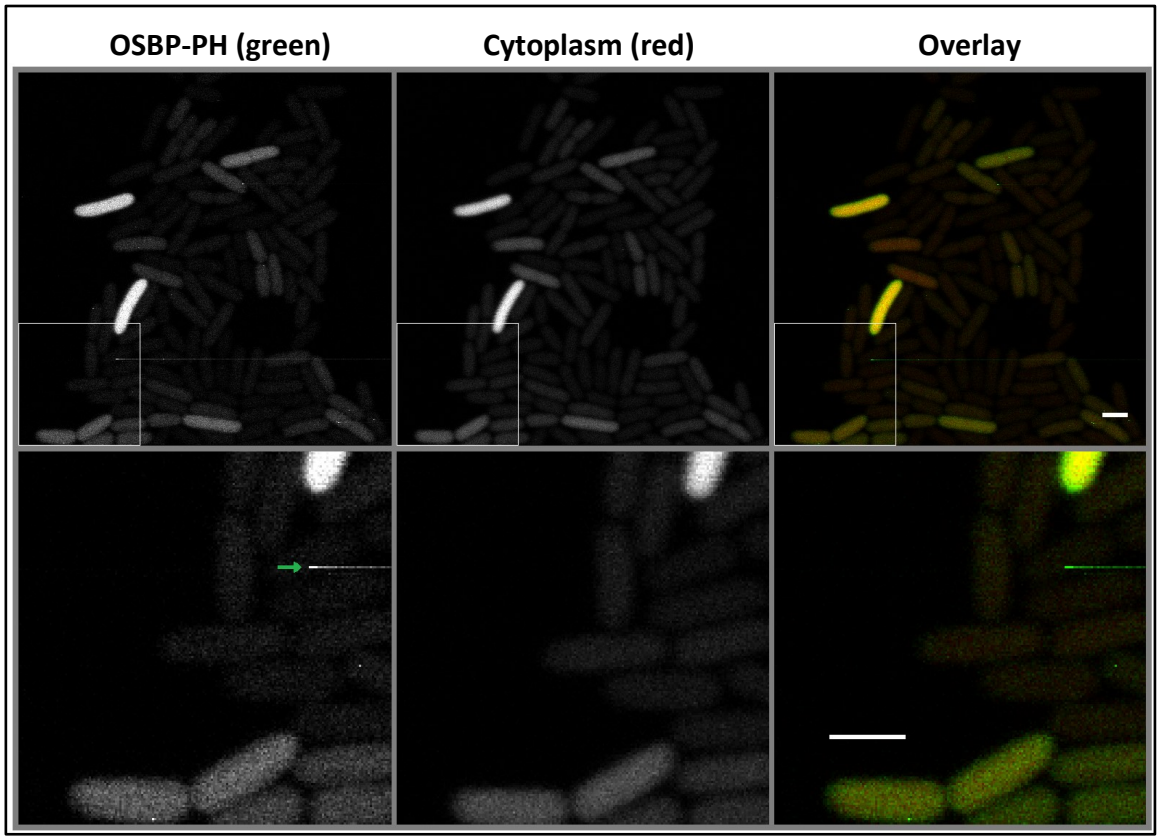


Figure 14. Localization of the GFP tagged PI4P binding OSBP-PH domain

Scale bar is 2 µm. The second row shows a zoom in on the bottom left of the top image as indicated by the square. Note the lack of membrane localization in the green channel. The line observed in the green channel (indicated with an arrow in the lower left panel) is an error of the scanning system during the image acquisition.

For the PIP2 binding test I used the PLCδ-PH domain, which binds PIP2. It is expected that membrane localization of the probe will be observed only in the cells expressing all 3 enzymes required for PIP2 production and growing in LB media with inositol. Several controls can be used for this system in order to make sure that if membrane localization of the probe is observed it is due to the expected interaction. The first control uses the same cells as the main experiment, expressing all 3 enzymes, but growing without inositol. In this control no phosphoinositides should be produced and no halo should form, confirming that the appearance of the halo is not due to some unexpected effect of the enzymes being present. A second control uses a control system that has no enzymes (in this case an empty plasmid) but grown with inositol in the media, showing that inositol on its own also does not cause membrane localization of the probe. A final control in which I mutated the binding site of the PLCδ-PH domain to eliminate its possible interaction with PIP2 also showed no halo even when the enzymes necessary and inositol were present, thus showing that the interaction depends specifically on its binding to PIP2.

This mutation approach had not been attempted before so I used the structural knowledge of the molecule published in the literature[159] and performed 8 amino acid changes to the residues that were implicated in PIP2 binding: K30A, K32A, W36A, R40A, E54A, S55A, R56A, K57A (numbering follows that of Ferguson and coworkers[159]). A representative image of each of these 3 controls and the experimental treatment is presented in Figure 15. In some cells the levels of protein expression are high and it

starts aggregating on the cell poles, in what might be the beginning of, or a fully formed, inclusion body. In this case the red fluorescence is also excluded from this area. It is important to highlight that this does not indicate any interaction with phosphoinositides but simply a known effect of protein overexpression in *E. coli* and analysis should exclude cell poles to avoid confounding the results. In these cases the cells still show diffuse fluorescence in the cytoplasm so there is enough protein to evaluate its localization satisfactorily regardless of a fraction of it being part of these aggregations.

As can be seen in Figure 15 a line scan perpendicular to the major axis, thereby avoiding the poles of the cell, shows a clear difference between the test and the controls. The cytoplasmic signal shows a symmetric mono-modal distribution while that of the membrane bound GFP tagged PLCδ-PH domain shows 2 peaks with a central dip in the signal.

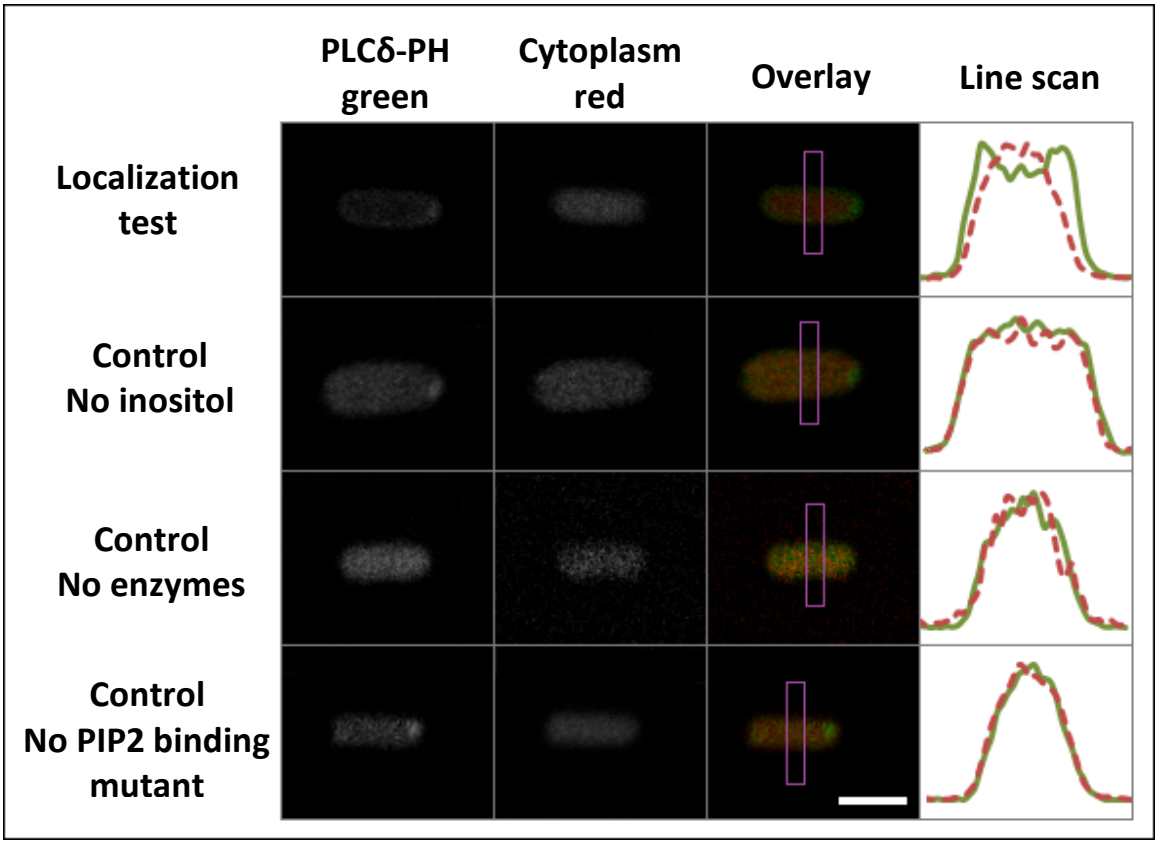


Figure 15. Localization of the GFP tagged PIP2 binding PLCδ-PH domain

Scale bar is 2 µm. Images were modified to leave only one cell in the field. Line scans were 10 pixels wide and are indicated by the purple rectangle on the overlay image. Data from line scans was standardized to show the same amplitude on all channels. Note the aggregation of protein towards the pole in some cells, in a way that excludes the cytoplasmic red marker.

In order to gain better confidence about the data, and avoid a possible selection bias, I created a bimodality index. To calculate this index I split the curves obtained with the line scan into three regions, taking the central 0.5 microns as the center region and the remainder to each site as the left or right side accordingly. Then the maximum of the left and right sides is averaged and divided by the minimum in the center region. In this way a curve that follows a normal or symmetric mono-modal distribution will give a value very close to 1, since the maximum outside the center region is almost the same as the minimum inside. In the case of a bimodal curve this value should be higher than 1 because the average of maximums in the outside regions should be higher than the minimum in the center region where the curve dips.

Since misalignment of the center region would bias the analysis towards values higher than one, I divide the value obtained for the curve corresponding to the green channel by that of the red channel (control channel). In this way any artifact due to misalignment of the center region boundaries will be corrected for, bringing the index value back to 1 for mono-modal curves. Figure 16 illustrates the index calculations. To do the calculations I acquired 4 different fields for each treatment and scored 10 cells per field picking them on the mCherry channel in order to avoid any bias when selecting them. The only consideration when picking the cells was that they were separate enough from other cells so a good curve could be obtained.

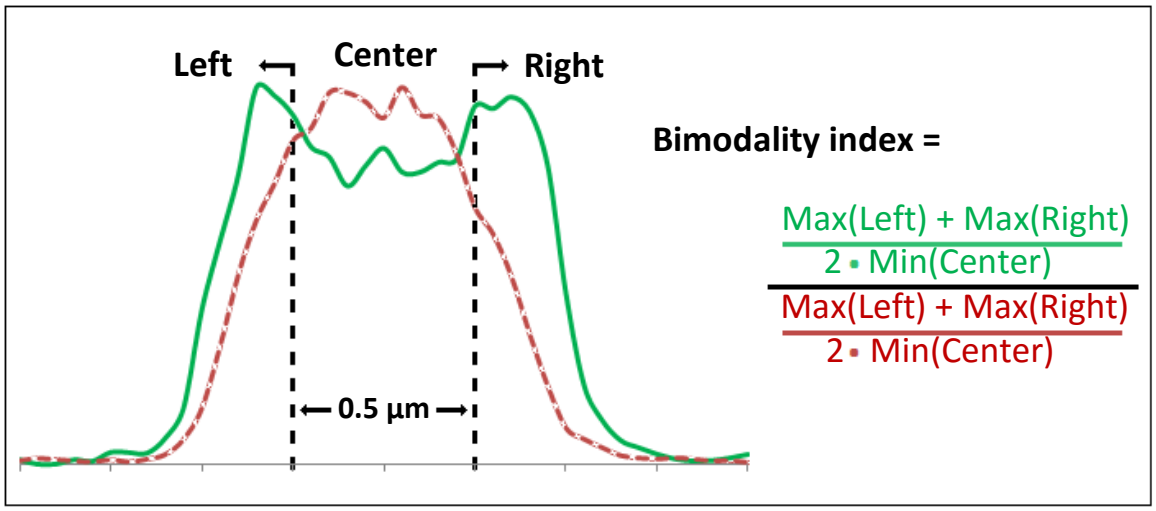


Figure 16. Calculation of the bimodality index

The line scan for each channel is standardized to its maximum so both channels have the same range. The maximum of the values in the outside (left and right) regions is averaged and divided by the minimum in the center region. This value should be very close to 1 for mono-modal distributions and higher than one for bimodal distributions. In order to correct for the effect of misalignment of the center region boundaries, which would lead to values higher than one, the green channel value is divided by the red (dashed) channel value. In this way any misalignment is corrected for, bringing the value back to one for mono-modal distributions. Theoretically this correction would not be necessary if the alignment was perfect but, as can be seen on the right side of the red (dashed) channel in the figure, small variations in the data make the alignment imperfect and the correction is appropriate.

The results obtained with the bimodality index are presented in Figure 17. The data confirms the localization of the PIP2 binding PH domain to the membrane only when PIP2 is present and in a manner directly dependent of PIP2 binding by the PH domain. While this could be observed from the images, being able to do a quantitative test allows for more certainty in the assessment, and confirms in a completely independent way the success of the system I developed in producing phosphoinositides.

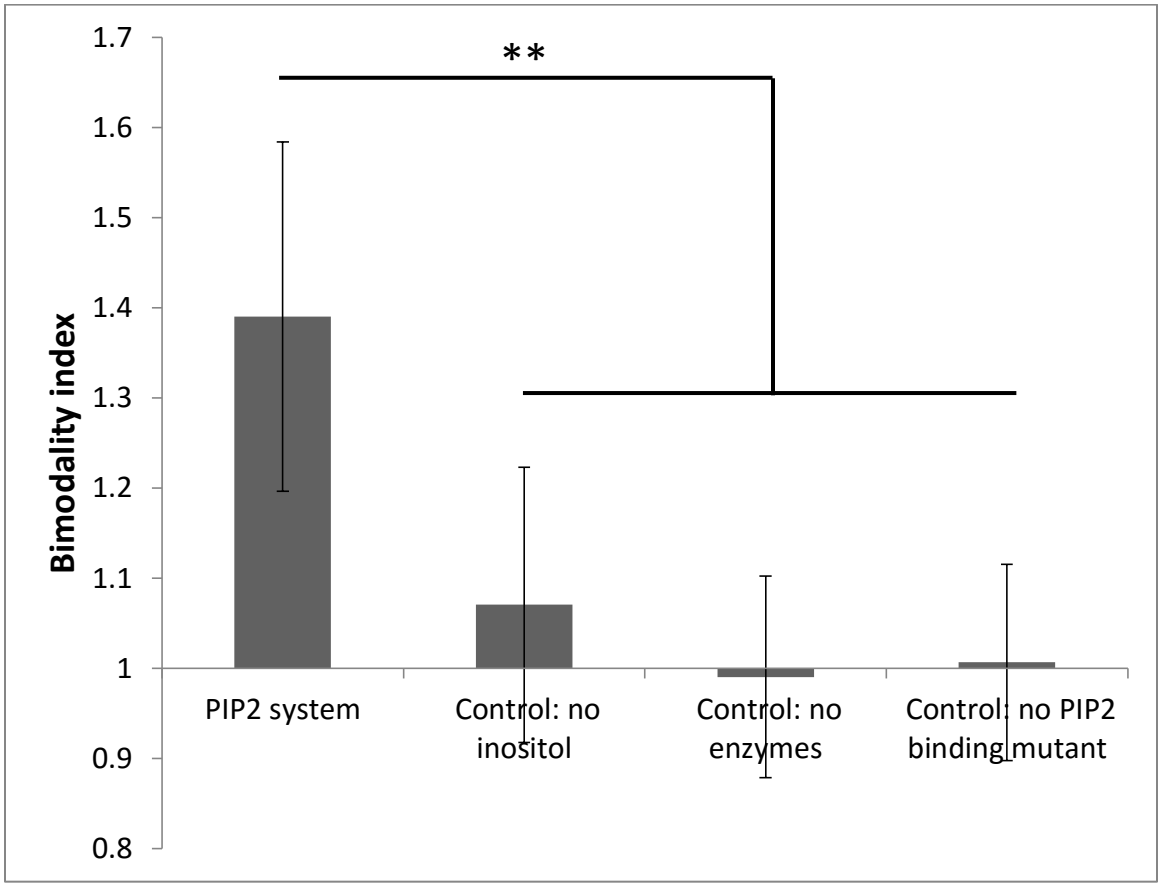


Figure 17. Bimodality index measures for the PIP2 binding PH domain

Each treatment had a sample size of 40 cells that came from 4 different fields, each with 10 cells measured. Graph shows the mean bimodality index and the standard deviation of the sample. All controls show an index value close to 1 as expected for mono-modal curves. There is a statistically significant difference between the localization test and all controls as assessed by a one-way ANOVA analysis and a post hoc Tukey HSD test, both with $p<0.01$ as indicated by **.

3.2. An application for the system

In order to illustrate how my system can be useful I decided to build a test for the current model of non-conventional protein export for human basic fibroblast growth factor (FGF2). FGF2 is a classic example of protein export in a manner independent of the Golgi and endoplasmic reticulum. While several transporters have been proposed to be involved in other models of non-conventional protein export, there is no evidence of their involvement in FGF2 export[223]. The current model proposes that FGF2 is translated and released into the cytoplasm, after which it becomes phosphorylated at tyrosine 82 by Tec-kinase. It is not clear if this phosphorylation occurs in the cytoplasm or at the plasma membrane but it is more likely it occurs at the plasma membrane given that is the normal Tec-kinase localization[224]. This phosphorylation enhances FGF2 oligomerization after binding to PIP2 for which it has a K_D of approximately 1 μM[225]. PIP2 binding drives membrane localization and together with the phosphorylation at tyrosine 82 induces oligomerization of FGF2[226]. While the lack of this phosphorylation diminishes export of FGF2 it does not entirely abolish it[227], suggesting that the system is perhaps more robust than expected. A schematic of the current model is presented on Figure 18.

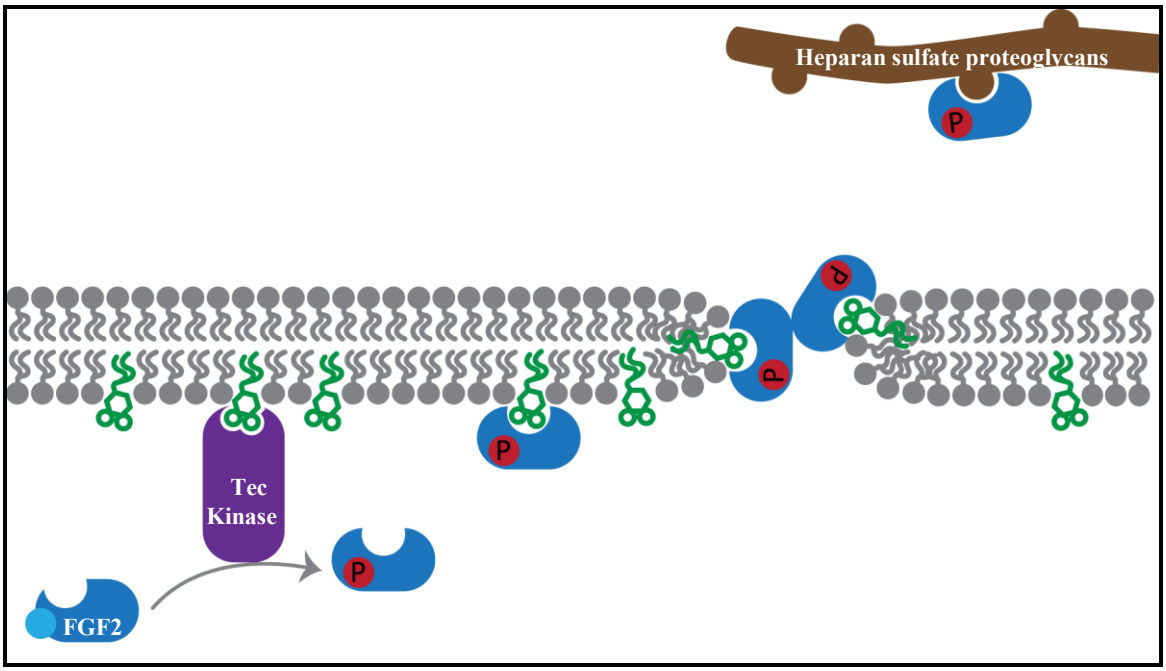


Figure 18. Schematic representation of the current FGF2 export model

FGF2 is synthesized and released to the cytoplasm, it is then phosphorylated in tyrosine 82 by Tec-kinase, this phosphorylation enhances binding to PIP2 and oligomerization forming a temporary channel that allows it to reach the outside of the cell. Once in the outside of the cell it releases PIP2 and binds to heparan sulfate proteoglycans. The binding site for PIP2 is shared with that of heparan sulfate proteoglycans, but the affinity is higher for the later creating a ratchet for accumulation of FGF2 in the outside of the cell.

After oligomerization of FGF2 is it believed that a membrane pore is produced which allows FGF2 to cross through the plasma membrane reaching the extracellular space. This pore allows the diffusion of small molecules, and its hypothesized to be lined with PIP2, although this has only been shown to be the case in vitro[227]. It has been shown that FGF2 exports in a fully folded conformation and in fact that the folded state is required for its export[228]. Once at the extracellular side FGF2 binds to heparan sulfate proteoglycans which act as an anchor. The binding site is partly shared between PIP2 and heparan sulfate proteoglycans[226] but the affinity for heparan sulfate proteoglycans is significantly higher than for PIP2 with a K_D of approximately 10 nM[229]. This anchoring mechanism generates the directionality of the FGF2 movement without the need for energy consumption in the process[230]. Two surface cysteines have recently been shown to be important for the oligomerization of FGF2 at the pore in a manner that seems to be independent of any accessory proteins, but it is hypothesized that they function directly at the pore since the reducing environment at the cytoplasm would not allow disulfide bridge formation[231].

This model represents a problem on which to try my system because it is otherwise impossible to confirm the sufficiency of the proposed components in-vivo. At the same time there is extensive evidence supporting each step of the process in the model, thus an in-vivo, independent test for the sufficiency of the proposed components would provide very strong support for the model. Additionally, there are several experimental advantages to testing this model. FGF2 export isn't impaired when it is fused to a

protein tag, such as GFP, and a phosphomimic using glutamate substitution for tyrosine 82 (Y82E) completely restored FGF2 export when Tec-kinase activity was experimentally ablated[224]. The binding site for PIP2 can also be eliminated by using 3 point mutations K127Q,R128Q, K133Q[226]. These findings allow me to create a very clear test for the sufficiency of the components since a negative control can be stablished by abolishing PIP2 binding, and the wild type and phosphomimic FGF2 molecules can also be evaluated. Since in some instances it has been shown that a double phosphomimic (replacing the aminoacid that is phosphorylated by two glutamates instead of one) resembles better the behavior of a phosphorylated protein[232, 233] I also included a double phosphomimic, Y82EE, construct for my tests.

Since *E. coli* is a gram negative, if FGF2 expressed in this bacterium were to export successfully it would be trapped between the plasma membrane and the outer membrane in the periplasmic space. However, given that my system does not have heparan sulfate proteoglycans to drive the export of FGF2, under the current model FGF2 would only form pores and remain attached to the plasma membrane. It is unclear if in this state FGF2 will face the outside of the plasma membrane, the inside, or oscillate between the two states. The fact that the binding site is shared between PIP2 and heparan sulfate proteoglycans indicates that FGF2 will have to release PIP2 and be exposed to the extracellular space at least some of the time.

Because of this ambiguity I chose a system that would inform on FGF2 forming a pore regardless of FGF2 separation from the membrane or the direction it faces if attached to it. For this I used the NanoLuc Luciferase assay. This consists of a luciferase enzyme optimized to reduce its size (about 19 kDa) to which I will refer as NanoLuciferase, and an optimized substrate. Upon encountering the substrate NanoLuciferase catalyzes its degradation releasing photons. One important advantage over other luminescent systems is that this reaction does not require ATP as a cofactor, with all energy required stored in the chemical structure of the substrate. This substrate is not membrane permeable so if added to the media it will only be available on the periplasm of an intact *E. coli*, with only a basal amount reaching the cytoplasm. NanoLuciferase can be expressed as a C terminal fusion protein with FGF2, and this fusion should not interfere with export since FGF2 has been shown to export successfully with a GFP fused to its C-terminus, and GFP is a larger protein (27 kDa).

Using this reporter assay it's possible to evaluate the effect of PIP2 on the formation of the membrane pore by FGF2 whether it remains attached to the plasma membrane or released to the periplasm. If FGF2 forms a pore in my system a luminescent signal can be produced in 3 different ways. If FGF2-NanoLuc is released to the periplasm, a signal should be observed since the NanoLuciferase will have access to the substrate added to the media; this would also be the same if FGF2-NanoLuc remains attached to the plasma membrane but facing the periplasm. Finally, in the case that only the pore is formed, but FGF2-NanoLuc is neither released to the periplasm not facing it, some level of substrate

should be able to diffuse through the pore and be accessible to FGF2-NanoLuc in the cytoplasm or inner side of the plasma membrane, producing a signal. An additional advantage of this reporter is that it allows for standardization of the results to the overall level of expression of the fusion protein. A percentage of luminescence can be obtained by measuring the luminescence before lysis (signal of the treatment), and after lysis of the cells (maximum signal possible, a measure directly proportional to the level of protein expression), correcting for differences in the expression levels of the different constructs being compared.

For these tests I used a plasmid with a ClodF13 origin of replication and an ampicillin resistance marker. In this way the plasmid is fully compatible with my system for PIP2 production. I expressed the FGF2-NanoLuc reporter constitutively to avoid problems with variable induction of the protein between constructs. For this I used the same constitutive promoter I used for expression of the enzymes required for PIP2 production. This FGF2-NanoLuc construct constitutes my wild type reporter (FGF2-NanoLuc-wt), and on it I performed site directed mutagenesis to obtain three more constructs: FGF2-NanoLuc unable to bind to PIP2 by including the mutations K127Q, R128Q, K133Q (FGF2-NanoLuc-NoBinding), FGF2-NanoLuc phosphomimic (FGF2-NanoLuc-Y82E), and FGF2-NanoLuc double phosphomimic (FGF2-NanoLuc-Y82EE).

Each of the four constructs is expected to behave differently reflecting its proposed fit with the model of FGF2 export. FGF2-NanoLuc-NoBinding should show the lowest signal,

since it is unable to bind to PIP2 and therefore should not be able to form pores; this will be the background signal for the system. FGF2-NanoLuc-wt should show the same low level of signal or perhaps a little higher since it should bind to PIP2 but it lacks the phosphorylation that has been shown to be important for enhancing its oligomerization and pore formation. Finally, FGF2-NanoLuc-Y82E and FGF2-NanoLuc-Y82EE should both present the highest level of signal since they have a phosphomimic mutation, allowing them to oligomerize successfully, forming the pore. It is possible that the double phosphomimic behaves differently, since in theory it is a better substitute for the natural phosphorylation, but it might also not work as well since the backbone of the protein is altered by extending its length.

Using my system to test these four FGF2-NanoLuc constructs allows testing the effect of PIP2 on the pore forming behavior of FGF2 by using two types of cells for each construct: control and test cells. Control cells do not have the enzymes required for phosphoinositide synthesis, allowing to test the effect of inositol on its own. This is important to make sure that inositol has no effect on the system on its own, confounding the results. The test cells on the other hand express the enzymes required for PIP2 synthesis, and differences observed when inositol is added are due to the production of PIP2. The test can be easily performed and measured on a plate reader where the initial OD600 of the culture as well as its luminescence is measured, and then each well is measured again after lysis of the cells. Results of the experiment are presented on Figure 19.

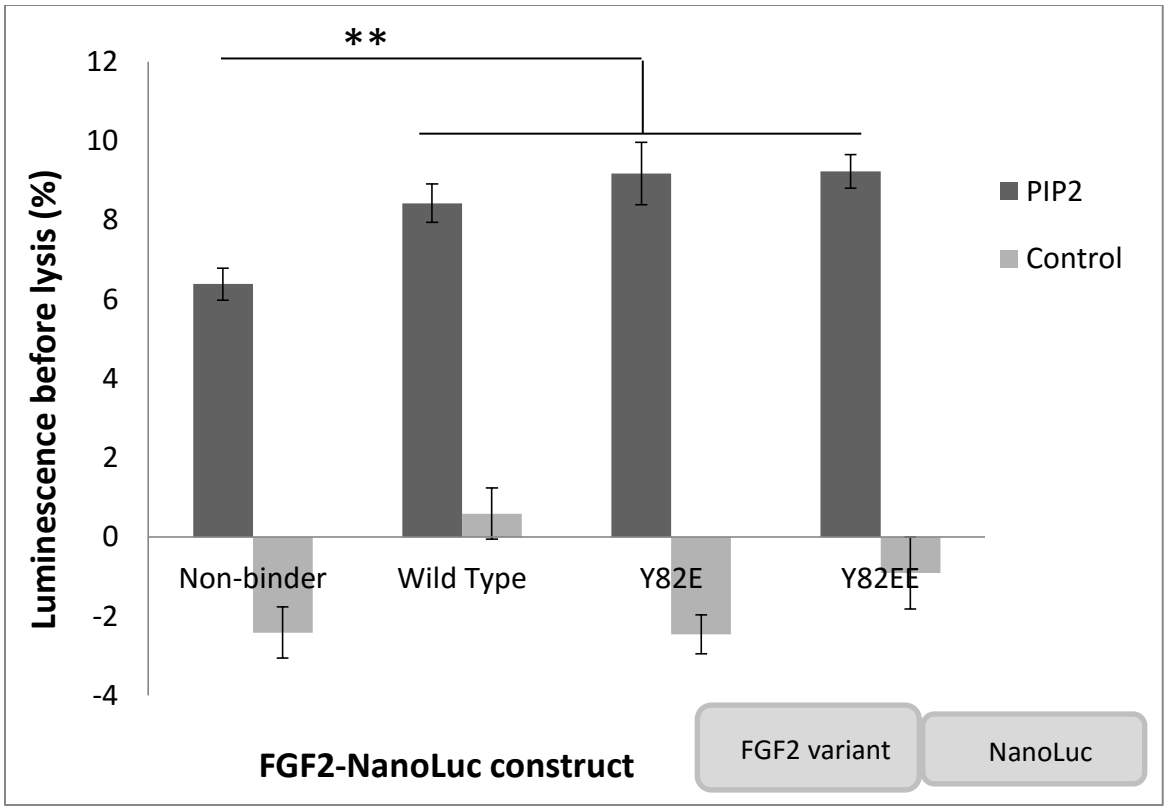


Figure 19. FGF2 export tests with 2 mM inositol

Cells were grown in LB media supplemented with the appropriate amount of inositol for 3h before an initial luminescence measure in a plate reader. After this measure the cells were lysed and the total luminescence was measured. The control treatment lacks the enzymes required for PIP2 production. Data shown is the average of 4 replicates and the distribution is the standard deviation of the samples. A two way ANOVA test found both the constructs, the treatment (PIP2 vs control), and the interaction to have a significant effect on the signal with $p<0.01$. A post hoc Tukey HSD test determined that FGF2-NanoLuc-NoBinding is significantly lower than the other constructs in the PIP2 treatment with $p<0.01$ as indicated by **.

As can be seen in Figure 19, all constructs showed little difference between the treatment of inositol and no inositol in the control system, which lacks the enzymes to synthesize PIP2. This is a basic control and this is the expected result confirming that inositol on its own has no effect on FGF2 export. While the effect sizes observed in the PIP2 treatment are not very large, varying between 6 and 10 % of the total luminescence, they are significant as determined by the two way ANOVA test. This is important because the value observed is too high to be due to the small fraction of cells that might die randomly during the experiments; thus the differences observed are in fact due to the different factors being evaluated (effect of PIP2 and different variants of FGF2). Unexpectedly PIP2 had an effect on all constructs, with an increase in the percentage of signal before lysis observed even for FGF2-NanoLuc-NoBinding when inositol is added to the media. This effect is dependent on PIP2 directly, as it was not observed in the control cells (which do not express the enzymes required for PIP2 synthesis). This suggests that even with the mutations to its binding site FGF2-NanoLuc-NoBinding is able to form a pore in a manner dependent on PIP2. Since its binding site is impaired or possibly entirely non-functional this might be an effect of PIP2 on membrane properties instead of on its oligomerization. This is consistent with the importance of PIP2 at the membrane for the pore formation, where its shape would allow for the curvature deformation required for a hypothesized toroidal pore[227]. All other constructs (wild type and both phosphomimics) showed a statistically significant higher signal which confirms the observation that the binding for PIP2 enhances pore formation. On the other hand the fact that the phosphomimics do not perform

significantly better than the wild type construct suggest that this phosphorylation is not as important as postulated although it shows slightly reduced signal compared to them.

In order to gain more insight into the process I repeated the test adding 5mM instead of 2mM of inositol into the media. While the concentration tests effects on PIP2 production in my system showed the production saturated around 2mM of inositol, if this saturation is the product of feedback on the enzymes the sequestration of PIP2 by FGF2 would induce the production of more PIP2. The increased inositol could also vary the dynamics of production even if the overall maximum level remains the same, reaching the maximum concentration of PIP2 faster. This would allow for more of the FGF2 to form pores during the growth time of the cells, which I kept constant at 3 hours to guarantee the measures are performed during the exponential growth phase of the cells. In this way if the inositol concentration required for pore formation is reached earlier, there is more time for pore accumulation. Additionally I added a new control, in which I replaced the FGF2 in the reporter for mCherry. Since mCherry does not interact with PIP2 in any way this mCherry-NanoLuc construct can determine whether the signal observed in the other treatments is in fact due to the interaction between FGF2 and PIP2 or somehow a passive result perhaps altering the permeability of the membrane to the NanoLuciferase substrate. Results of this experiment are presented in Figure 20.

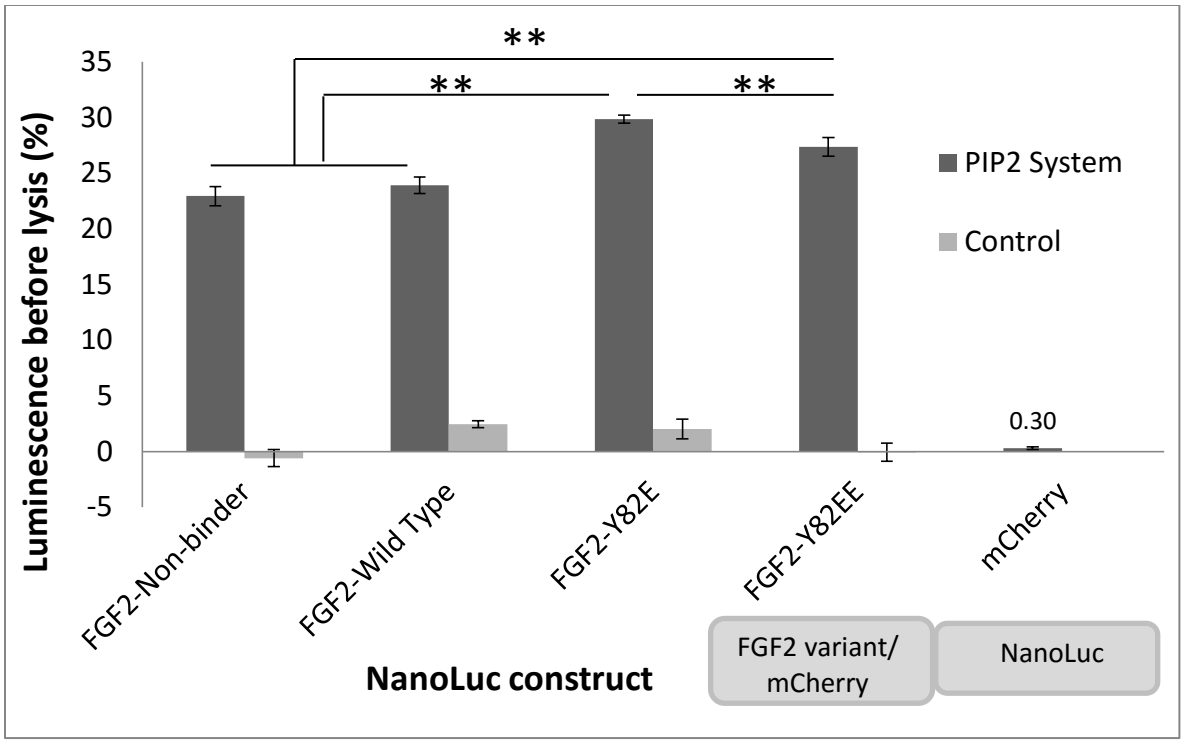


Figure 20. FGF2 export tests with 5 mM inositol

Cells were grown in LB media supplemented with the appropriate amount of inositol for 3h before an initial luminescence measure in a plate reader. After this measure the cells were lysed and the total luminescence was measured. The control treatment lacks the enzymes required for PIP2 production. Data shown is the average of 4 replicates and the distribution is the standard deviation of the samples. A two way ANOVA test found both the constructs, the treatment (PIP2 vs control), and the interaction to have a significant effect on the signal with $p<0.01$. A post hoc Tukey HSD test determined that FGF2-NanoLuc-NoBinding is not significantly different from FGF2-NanoLuc-wt and that these are significantly lower than both phosphomimics which are also significantly different between themselves. All these differences were significant with $p<0.01$ as indicated by **. The very small value obtained for the mCherry construct is indicated on the graph since the scale does not allow visualizing it.

In the new 5mM inositol experiment, the control system, which does not produce PIP2, showed the same lack of effect as in the previous 2mM treatment for all constructs. This confirmed the previous observation of inositol not having an effect on its own. The mCherry control (which was performed only for the system producing PIP2) showed no difference between the inositol and no inositol treatment, confirming that neither inositol not PIP2 alter the background level of signal of NanoLuciferase in the *E. coli* cells used for my studies. Just as in the 2mM inositol treatment, FGF2-NanoLuc-NoBinding showed a significant increase in signal upon inositol addition and in this case the effect size is much higher, increasing from about 6% in the 2mM inositol treatment to about 22% in the 5mM inositol treatment. The implication is the same as in the previous experiment, showing that PIP2 has an effect on FGF2 pore formation (and possibly export) that does not depend fully on the described binding site. In this case the signal in FGF2-NanoLuc-wt wasn't significantly different from FGF2-NanoLuc-NoBinding and these were both significantly lower that the signal of the phosphomimics. The double phosphomimic (FGF2-NanoLuc-Y82EE) showed lower signal than the single phosphomimic (FGF2-NanoLuc-Y82E) but the overall patterns are the same. The double phosphomimic is therefore not an improvement over the single phosphomimic and does not add any new information to the tests.

The lack of a difference between the wild type construct and the non-binding mutant might reflect a saturation effect. Based on the combined data of the 2mM and 5mM inositol tests I propose that a shift in the dominant limiting factor for export is occurring.

At both concentrations the phosphomimic, which can both bind to PIP2 and oligomerize successfully, exports better than the other constructs. At the same time the non-binding mutant, which has impaired PIP2 binding and lacks the phosphorylation therefore showing impaired oligomerization, presents the lowest level of export at both concentrations. The wild type on the other hand should bind to PIP2 successfully but lacking the phosphorylation should have impaired oligomerization, and at lower concentrations (2mM inositol) is able to export at a rate similar to that of the phosphomimic. This implies that in this situation PIP2 might be limiting the system, thus differences in oligomerization are not that important. Once PIP2 is increased (at 5mM inositol) the limiting factor is not binding to PIP2 but oligomerization of the FGF2 molecule. The impaired binding to PIP2 has little effect given an excess of PIP2 in the membrane and the oligomerization, which is dependent on the phosphorylation, becomes the dominant factor limiting the pore formation and export of the molecule.

An important factor to consider is that this binding site of the PIP2 binding mutant (FGF2-NanoLuc-NoBinding) might not be fully impaired. When the mutations to the binding site were described a small fraction of mutant FGF2, less than 1%, would still associate with plasma membrane like liposomes containing PIP2, although surface localization would only be reduced to 20-30% of that of the control when only 2 mutations were present (K127Q,R128Q but not K133Q)[226]. In that same work it was shown that the K133Q mutation impaired PIP2 binding, but more importantly heparan sulfate proteoglycan binding, and this was the decisive factor to virtually eliminate all

export of FGF2. While all mutations affect PIP2 binding and my system has all 3 mutations, the lack of heparan sulfate proteoglycan means that the mechanism of action has to be different. The results observed in my system suggest that FGF2 pore formation is enhanced by PIP2 regardless of the mutations. These results also support the idea that at 5mM inositol PIP2 is in excess, rendering the mutation less relevant and shifting the limiting factor to the oligomerization of the molecule.

While discussing these tests I have analyzed the difference between the no inositol and inositol treatments, but it is also important to consider the absolute values of background observed for them. These are shown for both experiments in Figure 21. The important result in this case is the fact that the FGF2 constructs are able to produce a signal corresponding to a range between 40% and 60% of the total signal. This is the equivalent of saying that 40-60% of the protein produced encounters the NanoLuciferase substrate. While the controls show that this is not dependent on addition of exogenous inositol, and the PIP2 treatment clearly has an effect that depends on the construct (this has been discussed already referring to figures 12 and 13), this very high background implies that FGF2 on its own, regardless of the presence of inositol and/or PIP2, produces a very significant signal. While this is simply the background level for the experiment, and as such one would just use it as a baseline, there is important information reflected in this observation. For instance, the mCherry control shows a background signal level of less than 1%, matching to the expected cytoplasmic localization of the construct. This is not an artifact of protein production

since the mCherry control produces significantly higher protein levels measured as the total luminescence per OD600 unit ($p<0.01$ on a t test).

The very large background level observed for all FGF2 constructs means that a very large fraction of the FGF2-NanoLuc constructs is able to reach, or be exposed to, the periplasm even under control conditions. The mCherry control on the other hand shows almost no signal. This is important because it shows unambiguously that inositol, PIP2, and the enzymes required to synthesize it are not responsible for this signal thus it is an effect of FGF2. An alternative to FGF2 being fully exported could be that a number of pores are being formed on the membrane by the FGF2 constructs allowing the entrance of a significant amount of NanoLuciferase substrate to the cytoplasm. I argue that the magnitude of the effect indicates that FGF2 molecules are either being released to the periplasm or on the outer leaflet of the plasma membrane but only going through a pore state as a short lived intermediate, since having such a high amount of permanent pores on the membrane would lead to major growth defects which I did not observe.

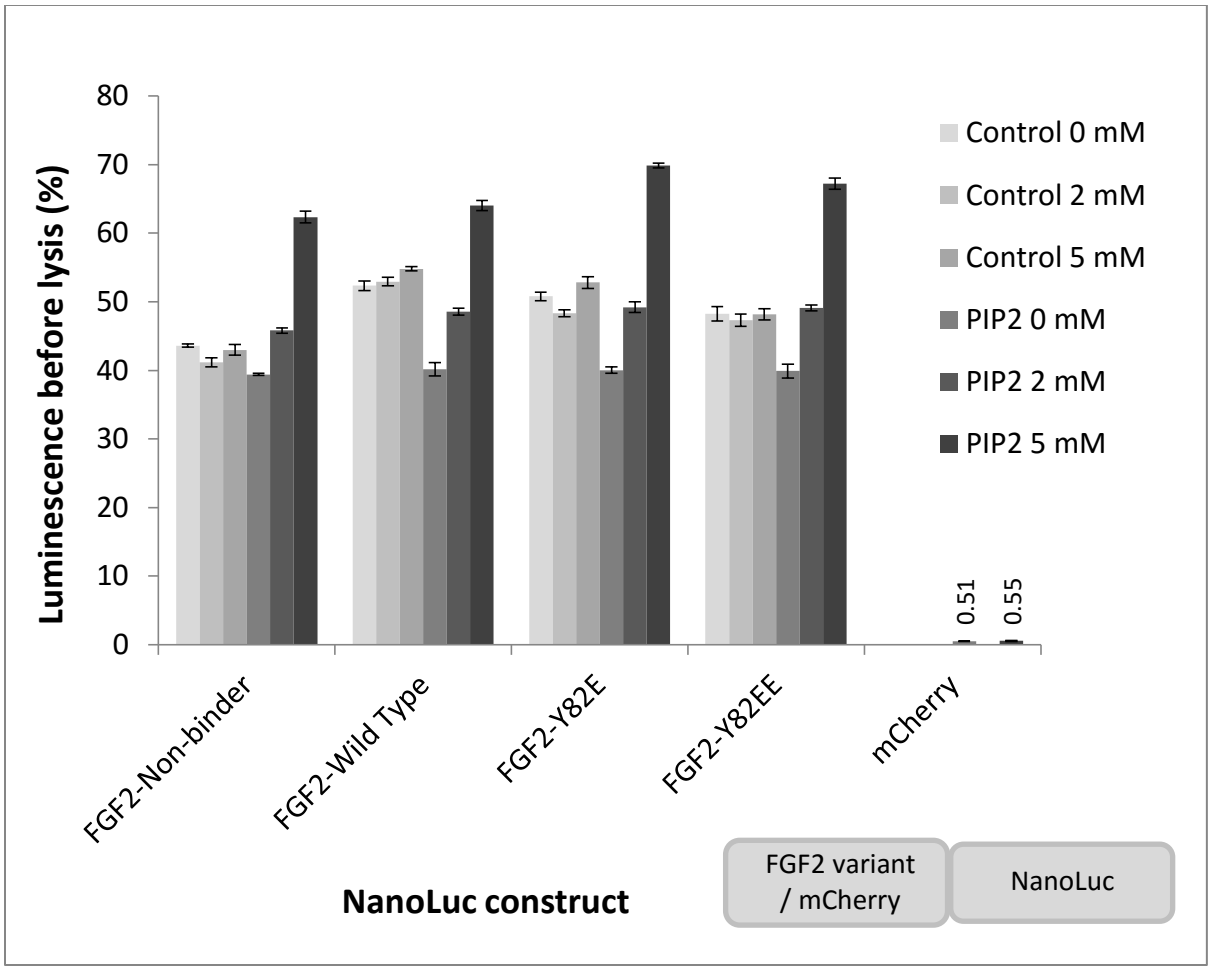


Figure 21. Raw data for the FGF2-NanoLuciferase export tests

Cells were grown in LB media supplemented with the appropriate amount of inositol for 3h before an initial luminescence measure in a plate reader. After this measure the cells were lysed and the total luminescence was measured. The control treatment lacks the enzymes required for PIP2 production. The very small value obtained for the mCherry constructs is indicated on the graph since the scale does not allow visualizing them. Note that the mCherry construct was only evaluated in the presence of the PIP2 system at 0 mM and 5 mM inositol in the media.

FGF2 has been shown to not bind to phosphatidylethanolamine or PI, but to have affinity for any phosphorylated phosphoinositide and for phosphatidylserine although at a lower level than for phosphoinositides[226]. This indicates that it has a strong preference for phospholipids with negatively charged head-groups, thus in the absence of PI4P and PIP2 it should have low affinity for the plasma membrane of *E. coli*, which is composed of phosphatidylethanolamine, phosphatidyl glycerol and cardiolipins (the latter two having neutral head-groups). The fact that it is able to bind and export even in the absence of PIP2 suggest that some hydrophobic interactions with the lipid tails and not just the head-groups might be important for its export. Alternatively, it is possible that phosphatidylserine, which is an intermediate for phosphatidylethanolamine production in *E. coli*, is binding to FGF2 and this interaction allows FGF2 to anchor itself to the membrane and initiate export. However, this is unlikely since phosphatidylserine can only be detected in trace amounts in normally growing *E. coli*[201] and will only accumulate if phosphatidylethanolamine production is impaired, a situation that has significant effects on the bacterium leading to lethality in the extreme cases[204].

FGF2 might also be able to insert at membranes at a low rate even when PIP2 binding and oligomerization are impaired. It is possible that this hypothesized insertion and oligomerization is enough to cross the thinner plasma membrane found in bacteria (which have phospholipids with shorter lipid tails) a fraction of the time but not enough to allow for its export of the mammalian cell. Since my assays are end point assays the observed result could reflect accumulation over the time of the experiment of FGF2 that

exports at a low constant level. At this point this is a purely speculative mechanism and elucidating it would require a variety of independent tests that are beyond the point of this work, which is to demonstrate an application of my system.

Overall my experiments on FGF2 export support two main aspects of the current model; they show that PIP2 enhances export of FGF2 and that the phosphorylation at Y82 also enhances the export of the molecule. My data also shows that export of FGF2 is more robust than expected, and that the effect of PIP2 is not only dependent on the reported binding site, and/or that the binding site is not fully impaired by the mutations that have been described in the literature.

4. Conclusion and outlook

In this work I have built a new platform for the study of phosphoinositides in-vivo using the bacterium *E. coli*, and have shown that the phosphoinositide production can be controlled with the addition of inositol to the media allowing for very detailed experimental manipulations. I have confirmed the production of PIP2 in my system with both a biochemical characterization and an imaging approach, providing fully independent observations as evidence that my system behaves as desired.

I have also applied my system to the study of FGF2 export from cells, confirming the role of PIP2 and a phosphorylation of its tyrosine residue at position 82 in enhancing its export. Additionally I have found some puzzling results suggesting that FGF2 export is more robust than expected in bacteria. This results needs to be confirmed and explored with alternative approaches to provide independent evidence, and this is beyond the scope of my project.

Overall the system I have developed shows the potential I set up to accomplish, and provides a new tool for experimenters interested in phosphoinositide biology. Opportunities are only limited by the boundaries imposed by *E. coli* in terms of protein production. The adoption of the tool by other researchers will be the ultimate indication of its value.

5. Appendix

5.1. Supplementary Protocol: Lipid extraction protocol

This Extraction Protocol is adapted from the Bligh & Dyer protocol. The volumes reported here are for a 10 mL pellet but can be adjusted to other sizes with a linear approximation. The protocol assumed an 80 µl pellet came out of the 10 ml of culture, but this measure does not need to be precise.

-Take 10 ml of culture at OD600 equal to 1 and pellet gently (1-3 min at 8000 rcf)

-Entirely remove the supernatant and store the pellet at -80C until fully frozen. The pellet can be stored like this until the lipid extraction is to be performed

-Add 420 µl of water to the frozen pellet ant thaw while resuspending with pipet mixing. Make sure the pellet is fully resuspended before continuing

-Add 1250 µl of methanol

-Add 625µl of chloroform

-Vortex until fully mixed, no cell clumps should be visible

-Add another 625µl of chloroform

-Vortex until fully mixed, no cell clumps should be visible

-Add 600 µl of ddH20

-Vortex until fully mixed, at this point two phases become obvious and start appearing even without centrifugation

-For total lipid extraction add 25 µl of HCl

-Vortex until fully mixed, the two phases are equally evident here

-Centrifuge at 100-150 rcf for 5 minutes

-Take the bottom phase of this solution, this is the organic phase and contains the lipids. The upper phase contains proteins and other water soluble molecules. Avoid pipetting up any of the top phase by pipetting a bit of air into the pipette and releasing it while moving through to the aqueous top phase. Between the two phases there is normally a layer of white material, mostly protein that is to be avoided

-Dry the bottom phase with argon until there is no solution remaining. These are the dry lipids. This should be done on glass vials

-Resuspend the mix on 100 µl of chloroform:methanol mix 20:9. I the resuspension turns problematic this can be sonicated in ice cold water to help, or a higher volume of chloroform:methanol can be used

Protocol variations:

For a cleaner extraction the obtained bottom phase can be washed with authentic upper phase. The authentic upper phase is obtained from the same protocol but in a tube in which instead of a sample pellet 80 µl of water were used. This cleaning step can be repeated if desired but for most extractions no cleaning is required.

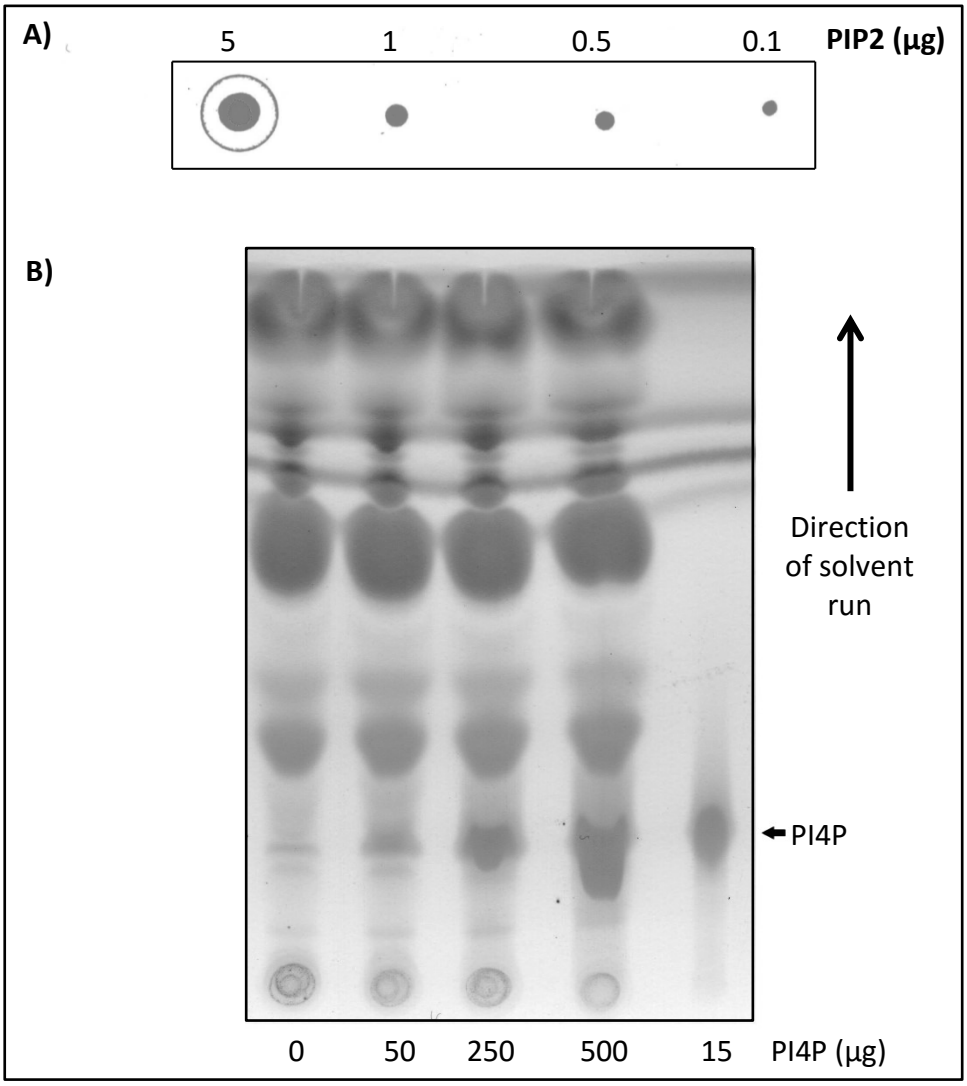
For separation of charged lipids the bottom phase can be obtained before the addition of HCl, saving the upper phase and layer of white material. The bottom phase so obtained is enriched in the uncharged phospholipids. Then the charged lipids can be obtained by adding new authentic bottom phase to the remaining top phase and white material and only then adding the HCl to acidify the mixture. The new bottom phase obtained will be enriched on the charged phospholipids, in this case PI4P and PIP2.

5.2. Supplementary discussion: Thin layer chromatography for phosphoinositide detection

Besides the detection by mass spectrometry mentioned in the main text a common technique for the detection of phosphoinositides consists on the chromatographic separation of the extracted phospholipids. This method is known as thin layer chromatography (TLC). In TLC differences in the affinity of each lipid for the moving phase relative to their affinity for the solid face create a separation of distinct lipid classes allowing for their detection. The detection can be done in a nonspecific manner, for example using iodine vapor, or in some cases with chemical reactions specific to the head-group of the phospholipid of interest, but to the best of my knowledge there is no specific chemical method for the detection of phosphoinositides on TLCs. For this reason it is common to use radioactively labelled inositol; this allows to make a direct measure of the phosphoinositides present in the sample, integrating over the spread of each

class. The method is highly sensitive and there is virtually no interference of other phospholipids since only phosphoinositides will contain the radioactive molecule.

For this project the use of radioactivity was undesirable since it would add a significant cost and regulatory burden, thus I tested the use of non-specific iodine detection. Measuring spots on the solid silica phase I could detect amounts as small as 0.1 μg of PIP2 (Supplementary Figure 1), which was a satisfactory observation. However once in a complex mixture that same level of sensitivity was problematic because the noise left by other classes of phospholipids during the run made it very hard to detect small amounts of control phosphoinositides, with the effective detection limit in the range of 50 μg (Supplementary Figure 2). This occurred because the distribution of any particular phospholipid had long tails, thus the signal of the very small amounts of phosphoinositide was diluted among the tails of the very abundant *E. coli* phospholipids. Given the measured sensitivity in my system, successful detection of the phosphorylated phosphoinositides would require them to be produced in the range of 5% of total phospholipids, which is too high relative to their abundance in eukaryotic cells. In my case the phosphorylated phosphoinositides are produced at very small amounts, a fraction of less than 0.5% of the phospholipids in the bacterium. It is thus clear that TLC would only be useful for my project if combined with radioactive labeling of the inositol added to the media.



Supplementary Figure 1. Sensitivity and effective TLC sensitivity of iodine vapor detection of phospholipids:

A) PIP2 was spotted at different amounts on a Silica Gel Hard Layer plate, and this was developed with iodine crystals in a closed box until signal was detected (approx. 15 min), after which the plate was scanned. Note that no solvent was used for this test.

B) A combination of 375 µg of E coli total phospholipids and varying amounts of PI4P were spotted at the bottom of the plate for each lane, plus a control lane where PI4P was spotted alone. After the run of the solvent to the top of the plate, the plate was allowed to air dry and developed with iodine crystals in a closed box until signal was detected (approx. 15 min), after which the plate was scanned.

An additional problem with the use of TLC is that it cannot differentiate among the different kinds of mono-phosphorylated or di-phosphorylated phosphoinositides since each category will run in the same manner regardless of which of the positions in the inositol ring is phosphorylated. While in my case this shouldn't be a problem, since I only have PI4P as a mono-phosphorylated phosphoinositide and PIP2 as a di-phosphorylated one, this is an important consideration for other projects.

For all TLC test mentioned I used plates with a Silica Gel Hard Layer (HL) 250 µm, using a mixture of chloroform-methanol-acetic acid-water-formic acid (35:15:6:2:0.3) as the solvent. After running to completion plates were allowed to air-dry, once dry the plated were incubated with iodine crystals in a closed box until signal was observed (approximately 15 minutes). The plates were then scanned using a conventional document scanner.

5.3. Supplementary discussion: Alternative reporters for FGF2 export detection

As mentioned in the main text, the use of the NanoLuc reporter allowed a precise measure of FGF2 export that is corrected for differences in protein expression. This is an important factor to consider when doing this type of analysis because the expected response of the system is small, thus small variation in the protein expression among constructs might mask the signal of interest. It is important to recall here that the difference between treatments and control at 2mM inositol in the media is only

between 6 and 10% of the signal. While several alternative approaches could have been used since they have the potential to detect periplasmic localization of FGF2, most of them have caveats that make them less appropriate than the NanoLuc assay employed.

As a basic approach, biochemical enrichment for periplasmic protein could have been employed. This approach has several problems, the main one being the fact that, as enrichment, it is very prone to some level of leakage of cytoplasmic proteins into the enriched periplasmic fraction. While this is a minor problem for proteins that should mostly be in the periplasm, in my system I expect a small signal and a very significant cytoplasmic contribution, thus the small level of contamination with cytoplasmic proteins expected can easily mask the signal. The second problem is the variability of the level of cytoplasmic contamination among samples; again this could easily mask a low level of signal. A final problem consist on the quantitation of the protein in the isolated fractions, since it would require a quantitative western blot, which is not a strength of the western blot technique, or a mass spectrometric approach, which would require the development of several time consuming controls. As a test for this approach I performed periplasmic protein enrichment on bacteria expressing cytoplasmic GFP, in theory the periplasmic fraction should be entirely free of GFP, in practice it is expected that it had a small amount would be present. On a western blot I detected a strong GFP band in the periplasmic fraction, confirming that this approach would not be useful for my FGF2 tests where only small differences were expected.

A second approach could consist on the immune detection of the localization of FGF2. This could be done both with immunohistochemistry and with electron microscopy. I did not attempt either of these approaches for two reasons. First the test would be done in fixed permeabilized cells, thus once the size of the antibodies used is taken into account it would be complex to determine whether the molecules detected are truly in the periplasm or simply associated with the plasma membrane but on the cytoplasm. This problem would favor the use of electron microscopy over that of regular immunohistochemistry since the resolution of the latter would not be satisfactory for this test. The second problem with this approach is purely logistic, because even if the resolution obtained is satisfactory, it would require a significantly larger amount of time to perform the analysis and obtain enough sample size to perform statistical tests. Differences in the efficiency of labeling among samples could also be a big problem, again masking the small levels of signal expected.

Finally, the use of fluorescent proteins would be another valid alternative. Direct measure of the localization of FGF2 could be an alternative, but there are several problems with this approach. The main difficulty would consist in determining the difference between periplasmic localization and localization to the inner side of the plasma membrane. The very small size of the bacterium makes this distinction a very hard problem to solve with conventional fluorescent proteins. This could be circumvented using a pH sensitive fluorescent protein, since the pH of the media can be controlled carefully in a flow chamber. The pH sensitive reporters in the cytoplasm

should vary less upon media pH changes than those in the periplasm, given that the pH in the cytoplasm is buffered by the bacterium. There are several pH sensitive fluorescent proteins that could be used for this purpose. However results obtained with this analysis depend directly on the fluorescent intensity (since it is the factor to vary with pH), and fluorescent intensity depends on the levels of protein expression. Thus here again small variations in protein expression among constructs could mask the small effects expected. There is a special kind of pH sensitive GFP, known as ratiometric GFP, which does not suffer from this caveat. This ratiometric GFP presents a shift of its absorbance spectrum with pH, thus, instead of simply observing a decrease in brightness upon pH change as in regular pH sensitive fluorescent proteins, a pH change creates a decrease in fluorescence intensity when excited at one wavelength (405 nm), but an increase when excited at another (475). This is a very useful property because instead of analyzing the absolute intensity, one can take the ratio of fluorescence at both wavelengths (signal at 405nm/signal at 475 nm). This not only eliminates the problems of differences due to varying amounts of protein between different constructs, but also slightly amplifies the signal making its detection easier.

Compared to the NanoLuc approach the ratiometric pH sensitive GFP has the advantage of allowing single cell analyses, but the disadvantage of being more labor intensive and costly (due to the special microfluidic plates required for the control of the pH in the media) thus reducing the amount of tests possible. The single cell measures do not represent a particularly relevant advantage in my system since there is no reason to

expect significant cell to cell variation, nonetheless, if cell to cell variation is present this would be the ideal test to detect it.

I attempted to use the ratiometric GFP reporter for my system but could not get a positive control to work thus I decided to use the NanoLuc approach which was working satisfactorily. The problem observed was that a cytoplasmic ratiometric GFP would produce the same signal variation (change in the ratio of signal at 405nm/signal at 475 nm) upon pH changes as one that had a twin-arginine translocation pathway signal and should have been localized exclusively to the periplasm (this signal leads to the export of folded proteins to the periplasm). The expectation was that the reporter with the periplasmic localization signal would show more variation than the cytoplasmic reporter. I later detected this problem was due to the failure of the signal to drive periplasmic localization, with the fluorescence being diffuse in the cytoplasm instead of showing periplasmic localization. This reporter remains a viable alternative to detect FGF2 export but it I did not pursue it further since the NanoLuc assay allows me to perform test faster.

6. References

1. Y. Koga, H. Morii, Biosynthesis of Ether-Type Polar Lipids in Archaea and Evolutionary Considerations. *Microbiology and Molecular Biology Reviews* **71**, 97 (2007).
2. T. G. Kutateladze, Translation of the phosphoinositide code by PI effectors. *Nat Chem Biol* **6**, 507 (2010).
3. H. Morii, M. Ogawa, K. Fukuda, H. Taniguchi, Ubiquitous distribution of phosphatidylinositol phosphate synthase and archaetidylinositol phosphate synthase in Bacteria and Archaea, which contain inositol phospholipid. *Biochemical and Biophysical Research Communications* **443**, 86 (2014).
4. Y. Koga, From Promiscuity to the Lipid Divide: On the Evolution of Distinct Membranes in Archaea and Bacteria. *Journal of Molecular Evolution* **78**, 234 (2014).
5. M. Vicinanza, G. D'Angelo, A. Di Campli, M. A. De Matteis, Function and dysfunction of the PI system in membrane trafficking. *The EMBO Journal* **27**, 2457 (2008).
6. J. Viaud *et al.*, Phosphoinositides: Important lipids in the coordination of cell dynamics. *Biochimie* **In press**, (2016).
7. R. H. Michell, Inositol derivatives: evolution and functions. *Nat Rev Mol Cell Biol* **9**, 151 (2008).

8. A. L. Majumder, A. Chatterjee, K. Ghosh Dastidar, M. Majee, Diversification and evolution of L-*myo*-inositol 1-phosphate synthase 1. *FEBS Letters* **553**, 3 (2003).

9. M. Pittet, A. Conzelmann, Biosynthesis and function of GPI proteins in the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1771**, 405 (2007).

10. R. D. Blind *et al.*, The signaling phospholipid PIP3 creates a new interaction surface on the nuclear receptor SF-1. *Proceedings of the National Academy of Sciences* **111**, 15054 (2014).

11. M. J. Berridge, R. F. Irvine, Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* **312**, 315 (1984).

12. N. Borges *et al.*, Biosynthetic Pathways of Inositol and Glycerol Phosphodiesters Used by the Hyperthermophile *Archaeoglobus fulgidus* in Stress Adaptation. *Journal of Bacteriology* **188**, 8128 (2006).

13. M. R. Macbeth *et al.*, Inositol Hexakisphosphate Is Bound in the ADAR2 Core and Required for RNA Editing. *Science* **309**, 1534 (2005).

14. L. A. Hanakahi, M. Bartlet-Jones, C. Chappell, D. Pappin, S. C. West, Binding of Inositol Phosphate to DNA-PK and Stimulation of Double-Strand Break Repair. *Cell* **102**, 721 (2000).

15. A. Saiardi, R. Bhandari, A. C. Resnick, A. M. Snowman, S. H. Snyder, Phosphorylation of Proteins by Inositol Pyrophosphates. *Science* **306**, 2101 (2004).

16. G. Zhang *et al.*, Importance and regulation of inositol biosynthesis during growth and differentiation of *Streptomyces*. *Molecular Microbiology* **83**, 1178 (2012).

17. R. C. Fahey, Novel Thiols of Prokaryotes. *Annual Review of Microbiology* **55**, 333 (2001).

18. J. B. Walker, Enzymatic synthesis of aminocyclitol moieties of aminoglycoside antibiotics from inositol by *Streptomyces* spp.: detection of glutamine-aminocyclitol aminotransferase and diaminocyclitol aminotransferase activities in a spectinomycin producer. *Journal of Bacteriology* **177**, 818 (1995).

19. A. Ongun, W. W. Thomson, J. B. Mudd, Lipid composition of chloroplasts isolated by aqueous and nonaqueous techniques. *Journal of Lipid Research* **9**, 409 (1968).

20. P. M. Vignais, P. V. Vignais, A. L. Lehninger, Restoration of ATP-induced contraction of “aged” mitochondria by phosphatidyl inositol. *Biochemical and Biophysical Research Communications* **11**, 313 (1963).

21. R. H. Michell, Inositol lipids: from an archaeal origin to phosphatidylinositol 3,5-bisphosphate faults in human disease. *FEBS Journal* **280**, 6281 (2013).

22. A. Das-Chatterjee *et al.*, Introgression of a novel salt-tolerant L-*myo*-inositol 1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateoka (PcINO1) confers salt tolerance to evolutionary diverse organisms. *FEBS Letters* **580**, 3980 (2006).

23. B. Antonsson, Phosphatidylinositol synthase from mammalian tissues1. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **1348**, 179 (1997).

24. R. A. Anderson, I. V. Boronenkov, S. D. Doughman, J. Kunz, J. C. Loijens, Phosphatidylinositol Phosphate Kinases, a Multifaceted Family of Signaling Enzymes. *Journal of Biological Chemistry* **274**, 9907 (1999).

25. M. R. Wenk *et al.*, Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. *Nat Biotech* **21**, 813 (2003).

26. A. Shisheva, D. Sbrissa, O. Ikonomov, Plentiful PtdIns5P from scanty PtdIns(3,5)P2 or from ample PtdIns? PIKfyve-dependent models: Evidence and speculation (response to: DOI 10.1002/bies.201300012). *BioEssays* **37**, 267 (2015).

27. C. J. Bonangelino *et al.*, Osmotic stress–induced increase of phosphatidylinositol 3,5-bisphosphate requires Vac14p, an activator of the lipid kinase Fab1p. *The Journal of Cell Biology* **156**, 1015 (2002).

28. R. J. Botelho, Changing phosphoinositides “on the fly”: how trafficking vesicles avoid an identity crisis. *BioEssays* **31**, 1127 (2009).

29. S. Bhattacharjee, Robert V. Stahelin, Kaye D. Speicher, David W. Speicher, K. Haldar, Endoplasmic Reticulum PI(3)P Lipid Binding Targets Malaria Proteins to the Host Cell. *Cell* **148**, 201 (2012).

30. S. D. Kale *et al.*, External Lipid PI3P Mediates Entry of Eukaryotic Pathogen Effectors into Plant and Animal Host Cells. *Cell* **142**, 284 (2010).

31. J. G. Carlton, P. J. Cullen, Coincidence detection in phosphoinositide signaling. *Trends in Cell Biology* **15**, 540 (2005).

32. M. P. Czech, PIP2 and PIP3: Complex Roles at the Cell Surface. *Cell* **100**, 603 (2000).

33. C. Cauvin, A. Echard, Phosphoinositides: Lipids with informative heads and mastermind functions in cell division. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 832 (2015).

34. K. Tsujita, T. Itoh, Phosphoinositides in the regulation of actin cortex and cell migration. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 824 (2015).

35. S. Suetsugu, S. Kurisu, T. Takenawa, Dynamic Shaping of Cellular Membranes by Phospholipids and Membrane-Deforming Proteins. *Physiological Reviews* **94**, 1219 (2014).

36. R. Fiume *et al.*, PIP4K and the role of nuclear phosphoinositides in tumour suppression. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 898 (2015).

37. Y. Liu, V. A. Bankaitis, Phosphoinositide phosphatases in cell biology and disease. *Progress in Lipid Research* **49**, 201 (2010).

38. Z. H. Shah *et al.*, Nuclear phosphoinositides and their impact on nuclear functions. *FEBS Journal* **280**, 6295 (2013).

39. W. s. E. Edimo, V. Janssens, E. Waelkens, C. Erneux, Reversible Ser/Thr SHIP phosphorylation: A new paradigm in phosphoinositide signalling? *BioEssays* **34**, 634 (2012).

40. B. Mueller-Roeber, C. Pical, Inositol Phospholipid Metabolism in Arabidopsis. Characterized and Putative Isoforms of Inositol Phospholipid Kinase and Phosphoinositide-Specific Phospholipase C. *Plant Physiology* **130**, 22 (2002).

41. A. Gambhir *et al.*, Electrostatic Sequestration of PIP_2 on Phospholipid Membranes by Basic/Aromatic Regions of Proteins. *Biophysical Journal* **86**, 2188 (2004).

42. M. A. De Matteis, C. Wilson, G. D'Angelo, Phosphatidylinositol-4-phosphate: The Golgi and beyond. *BioEssays* **35**, 612 (2013).

43. N. Calloway *et al.*, Stimulated association of STIM1 and Orai1 is regulated by the balance of PtdIns(4,5)P2 between distinct membrane pools. *Journal of Cell Science* **124**, 2602 (2011).

44. K. M. Weixel, A. Blumental-Perry, S. C. Watkins, M. Aridor, O. A. Weisz, Distinct Golgi Populations of Phosphatidylinositol 4-Phosphate Regulated by Phosphatidylinositol 4-Kinases. *Journal of Biological Chemistry* **280**, 10501 (2005).

45. S. McLaughlin, D. Murray, Plasma membrane phosphoinositide organization by protein electrostatics. *Nature* **438**, 605 (2005).

46. J. van Rheenen, E. Mulugeta Achame, H. Janssen, J. Calafat, K. Jalink, PIP2 signaling in lipid domains: a critical re-evaluation. *The EMBO Journal* **24**, 1664 (2005).

47. V. Carricaburu, B. Fournier, Phosphoinositide fatty acids regulate phosphatidylinositol 5-kinase, phospholipase C and protein kinase C activities. *European Journal of Biochemistry* **268**, 1238 (2001).

48. Y. V. Shulga, M. K. Topham, R. M. Epand, Study of Arachidonoyl Specificity in Two Enzymes of the PI Cycle. *Journal of Molecular Biology* **409**, 101 (2011).

49. S. König, A. Mosblech, I. Heilmann, Stress-inducible and constitutive phosphoinositide pools have distinctive fatty acid patterns in Arabidopsis thaliana. *The FASEB Journal* **21**, 1958 (2007).

50. C. Löfke, T. Ischebeck, S. König, S. Freitag, I. Heilmann, Alternative metabolic fates of phosphatidylinositol produced by phosphatidylinositol synthase isoforms in Arabidopsis thaliana. *Biochemical Journal* **413**, 115 (2008).

51. J. Clark *et al.*, Dictyostelium uses ether-linked inositol phospholipids for intracellular signalling. *The EMBO Journal* **33**, 2188 (2014).

52. R. Dawaliby *et al.*, Phosphatidylethanolamine is a Key Regulator of Membrane Fluidity in Eukaryotic Cells. *Journal of Biological Chemistry*, (2015).

53. S. Cockcroft, N. Carvou, Biochemical and biological functions of class I phosphatidylinositol transfer proteins. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1771**, 677 (2007).

54. R. V. Stahelin, J. L. Scott, C. T. Frick, Cellular and molecular interactions of phosphoinositides and peripheral proteins. *Chemistry and Physics of Lipids* **182**, 3 (2014).

55. Yeun J. Kim, Maria L. Guzman-Hernandez, T. Balla, A Highly Dynamic ER-Derived Phosphatidylinositol-Synthesizing Organelle Supplies Phosphoinositides to Cellular Membranes. *Developmental Cell* **21**, 813 (2011).

56. S. Cockcroft, K. Garner, Function of the phosphatidylinositol transfer protein gene family: is phosphatidylinositol transfer the mechanism of action? *Critical Reviews in Biochemistry and Molecular Biology* **46**, 89 (2011).

57. A. Balla, T. Balla, Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends in Cell Biology* **16**, 351 (2006).

58. M. R. Wenk, P. De Camilli, Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: Insights from vesicle recycling in nerve terminals. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 8262 (2004).

59. T. Strahl, H. Hama, D. B. DeWald, J. Thorner, Yeast phosphatidylinositol 4-kinase, Pik1, has essential roles at the Golgi and in the nucleus. *The Journal of Cell Biology* **171**, 967 (2005).

60. D. Peretti, N. Dahan, E. Shimoni, K. Hirschberg, S. Lev, Coordinated Lipid Transfer between the Endoplasmic Reticulum and the Golgi Complex Requires the VAP Proteins and Is Essential for Golgi-mediated Transport. *Molecular Biology of the Cell* **19**, 3871 (2008).

61. F. Nakatsu *et al.*, PtdIns4P synthesis by PI4KIIIα at the plasma membrane and its impact on plasma membrane identity. *The Journal of Cell Biology* **199**, 1003 (2012).

62. G. D. Fairn, A. J. Curwin, C. J. Stefan, C. R. McMaster, The oxysterol binding protein Kes1p regulates Golgi apparatus phosphatidylinositol-4-phosphate function. *Proceedings of the National Academy of Sciences* **104**, 15352 (2007).

63. R. V. Stahelin, W. Cho, Differential Roles of Ionic, Aliphatic, and Aromatic Residues in Membrane−Protein Interactions: A Surface Plasmon Resonance Study on Phospholipases A2. *Biochemistry* **40**, 4672 (2001).

64. J. He *et al.*, Molecular Basis of Phosphatidylinositol 4-Phosphate and ARF1 GTPase Recognition by the FAPP1 Pleckstrin Homology (PH) Domain. *Journal of Biological Chemistry* **286**, 18650 (2011).

65. J. Chung *et al.*, PI4P/phosphatidylserine countertransport at ORP5- and ORP8-mediated ER–plasma membrane contacts. *Science* **349**, 428 (2015).

66. J. Moser von Filseck *et al.*, Phosphatidylserine transport by ORP/Osh proteins is driven by phosphatidylinositol 4-phosphate. *Science* **349**, 432 (2015).

67. B. Mesmin *et al.*, A Four-Step Cycle Driven by PI(4)P Hydrolysis Directs Sterol/PI(4)P Exchange by the ER-Golgi Tether OSBP. *Cell* **155**, 830 (2013).

68. T. Ischebeck, S. Seiler, I. Heilmann, At the poles across kingdoms: phosphoinositides and polar tip growth. *Protoplasma* **240**, 13 (2010).

69. V. D. Rao, S. Misra, I. V. Boronenkov, R. A. Anderson, J. H. Hurley, Structure of Type IIβ Phosphatidylinositol Phosphate Kinase. *Cell* **94**, 829 (1998).

70. L. E. Rameh, K. F. Tolias, B. C. Duckworth, L. C. Cantley, A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. *Nature* **390**, 192 (1997).

71. E. J. Dickson, J. B. Jensen, B. Hille, Golgi and plasma membrane pools of PI(4)P contribute to plasma membrane PI(4,5)P2 and maintenance of KCNQ2/3 ion channel current. *Proceedings of the National Academy of Sciences* **111**, E2281 (2014).

72. Y. Nishizuka, Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **258**, 607 (1992).

73. Z. Yuan *et al.*, Na/K-ATPase Tethers Phospholipase C and IP3 Receptor into a Calcium-regulatory Complex. *Molecular Biology of the Cell* **16**, 4034 (2005).

74. S. J. Field *et al.*, PtdIns(4,5)P2 Functions at the Cleavage Furrow during Cytokinesis. *Current Biology* **15**, 1407 (2005).

75. J. Kwik *et al.*, Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. *Proceedings of the National Academy of Sciences* **100**, 13964 (2003).

76. D. E. Klein, A. Lee, D. W. Frank, M. S. Marks, M. A. Lemmon, The Pleckstrin Homology Domains of Dynamin Isoforms Require Oligomerization for High Affinity Phosphoinositide Binding. *Journal of Biological Chemistry* **273**, 27725 (1998).

77. A. Honigmann *et al.*, Phosphatidylinositol 4,5-bisphosphate clusters act as molecular beacons for vesicle recruitment. *Nat Struct Mol Biol* **20**, 679 (2013).

78. T. F. J. Martin, PI(4,5)P2-binding effector proteins for vesicle exocytosis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 785 (2015).

79. L. Chen *et al.*, Migration of PIP2 lipids on voltage-gated potassium channel surface influences channel deactivation. *Scientific Reports* **5**, 15079 (2015).

80. O. Devergne, K. Tsung, G. Barcelo, T. Schüpbach, Polarized deposition of basement membrane proteins depends on Phosphatidylinositol synthase and the levels of Phosphatidylinositol 4,5-bisphosphate. *Proceedings of the National Academy of Sciences* **111**, 7689 (2014).

81. F. Giordano *et al.*, PI(4,5)P_2-Dependent and Ca^{2+}-Regulated ER-PM Interactions Mediated by the Extended Synaptotagmins. *Cell* **153**, 1494 (2013).

82. P. J. Hamilton *et al.*, PIP2 regulates psychostimulant behaviors through its interaction with a membrane protein. *Nat Chem Biol* **10**, 582 (2014).

83. T.-J. Kim, S. Mitsutake, Y. Igarashi, The interaction between the pleckstrin homology domain of ceramide kinase and phosphatidylinositol 4,5-bisphosphate regulates the plasma membrane targeting and ceramide 1-phosphate levels. *Biochemical and Biophysical Research Communications* **342**, 611 (2006).

84. Y. Zhou *et al.*, Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. *Science* **349**, 873 (2015).

85. V. A. Sciorra *et al.*, Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. *The Journal of Cell Biology* **159**, 1039 (2002).

86. M. N. Teruel, T. Meyer, Translocation and Reversible Localization of Signaling Proteins. *Cell* **103**, 181 (2000).

87. L. S. Garrenton, C. J. Stefan, M. A. McMurray, S. D. Emr, J. Thorner, Pheromone-induced anisotropy in yeast plasma membrane phosphatidylinositol-4,5-bisphosphate distribution is required for MAPK signaling. *Proceedings of the National Academy of Sciences* **107**, 11805 (2010).

88. V. L. Jensen *et al.*, Formation of the transition zone by Mks5/Rpgrip1L establishes a ciliary zone of exclusion (CIZE) that compartmentalises ciliary signalling proteins and controls PIP2 ciliary abundance. *The EMBO Journal* **34**, 2537 (2015).

89. Q. Xu *et al.*, Phosphatidylinositol phosphate kinase PIPKIγ and phosphatase INPP5E coordinate initiation of ciliogenesis. *Nat Commun* **7**, (2016).

90. T. Rohacs, C. M. B. Lopes, I. Michailidis, D. E. Logothetis, PI(4,5)P2 regulates the activation and desensitization of TRPM8 channels through the TRP domain. *Nat Neurosci* **8**, 626 (2005).

91. N. Brose, C. Rosenmund, Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *Journal of Cell Science* **115**, 4399 (2002).

92. C. W. Taylor, S. C. Tovey, IP3 Receptors: Toward Understanding Their Activation. *Cold Spring Harbor Perspectives in Biology* **2**, (2010).

93. E. Delage, J. Puyaubert, A. Zachowski, E. Ruelland, Signal transduction pathways involving phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate: Convergences and divergences among eukaryotic kingdoms. *Progress in Lipid Research* **52**, 1 (2013).

94. N. Gamper, M. S. Shapiro, Target-specific PIP2 signalling: how might it work? *The Journal of Physiology* **582**, 967 (2007).

95. Stuart McLaughlin, Jiyao Wang, Alok Gambhir, D. Murray, PIP2 and Proteins: Interactions, Organization, and Information Flow. *Annual Review of Biophysics and Biomolecular Structure* **31**, 151 (2002).

96. A. Tengholm, O. Idevall-Hagren, Spatio-Temporal Dynamics of Phosphatidylinositol-3,4,5-Trisphosphate Signalling. *Vitamins & Hormones* **80**, 287 (2009).

97. R. S. Salamon, J. M. Backer, Phosphatidylinositol-3,4,5-trisphosphate: Tool of choice for class I PI 3-kinases. *BioEssays* **35**, 602 (2013).

98. S.-C. Yip *et al.*, Quantification of PtdIns(3,4,5)P3 dynamics in EGF-stimulated carcinoma cells: a comparison of PH-domain-mediated methods with immunological methods. *Biochemical Journal* **411**, 441 (2008).

99. B. D. Manning, L. C. Cantley, AKT/PKB Signaling: Navigating Downstream. *Cell* **129**, 1261 (2007).

100. A. Mora, D. Komander, D. M. F. van Aalten, D. R. Alessi, PDK1, the master regulator of AGC kinase signal transduction. *Seminars in Cell & Developmental Biology* **15**, 161 (2004).

101. M. S. Song, L. Salmena, P. P. Pandolfi, The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol* **13**, 283 (2012).

102. B. Vanhaesebroeck *et al.*, Synthesis and Function of 3-Phosphorylated Inositol Lipids. *Annual Review of Biochemistry* **70**, 535 (2001).

103. H. Li, A. J. Marshall, Phosphatidylinositol (3,4) bisphosphate-specific phosphatases and effector proteins: A distinct branch of PI3K signaling. *Cellular Signalling* **27**, 1789 (2015).

104. M. Krause *et al.*, Lamellipodin, an Ena/VASP Ligand, Is Implicated in the Regulation of Lamellipodial Dynamics. *Developmental Cell* **7**, 571 (2004).

105. T. Oikawa, T. Itoh, T. Takenawa, Sequential signals toward podosome formation in NIH-src cells. *The Journal of Cell Biology* **182**, 157 (2008).

106. S. Wullschleger, David H. Wasserman, A. Gray, K. Sakamoto, Dario R. Alessi, Role of TAPP1 and TAPP2 adaptor binding to PtdIns(3,4)P2 in regulating insulin sensitivity defined by knock-in analysis. *Biochemical Journal* **434**, 265 (2011).

107. Y. Posor *et al.*, Spatiotemporal control of endocytosis by phosphatidylinositol-3,4-bisphosphate. *Nature* **499**, 233 (2013).

108. F. Nakatsu *et al.*, The inositol 5-phosphatase SHIP2 regulates endocytic clathrin-coated pit dynamics. *The Journal of Cell Biology* **190**, 307 (2010).

109. Y. Posor, M. Eichhorn-Grünig, V. Haucke, Phosphoinositides in endocytosis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 794 (2015).

110. E. Boucrot *et al.*, Endophilin marks and controls a clathrin-independent endocytic pathway. *Nature* **517**, 460 (2015).

111. K. F. Tolias, L. C. Cantley, Pathways for phosphoinositide synthesis. *Chemistry and Physics of Lipids* **98**, 69 (1999).

112. K. O. Schink, C. Raiborg, H. Stenmark, Phosphatidylinositol 3-phosphate, a lipid that regulates membrane dynamics, protein sorting and cell signalling. *BioEssays* **35**, 900 (2013).

113. D. J. Gillooly *et al.*, Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *The EMBO Journal* **19**, 4577 (2000).

114. T. Wollert, C. Wunder, J. Lippincott-Schwartz, J. H. Hurley, Membrane scission by the ESCRT-III complex. *Nature* **458**, 172 (2009).

115. F. A. Meunier *et al.*, Phosphatidylinositol 3-Kinase C2α Is Essential for ATP-dependent Priming of Neurosecretory Granule Exocytosis. *Molecular Biology of the Cell* **16**, 4841 (2005).

116. C. A. Lamb, T. Yoshimori, S. A. Tooze, The autophagosome: origins unknown, biogenesis complex. *Nat Rev Mol Cell Biol* **14**, 759 (2013).

117. K. Obara, T. Sekito, K. Niimi, Y. Ohsumi, The Atg18-Atg2 Complex Is Recruited to Autophagic Membranes via Phosphatidylinositol 3-Phosphate and Exerts an Essential Function. *Journal of Biological Chemistry* **283**, 23972 (2008).

118. A. P. Sagona *et al.*, PtdIns(3)P controls cytokinesis through KIF13A-mediated recruitment of FYVE-CENT to the midbody. *Nat Cell Biol* **12**, 362 (2010).

119. A. J. McCartney, Y. Zhang, L. S. Weisman, Phosphatidylinositol 3,5-bisphosphate: Low abundance, high significance. *BioEssays* **36**, 52 (2014).

120. S. K. Dove *et al.*, Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. *The EMBO Journal* **23**, 1922 (2004).

121. A. C. Rutherford *et al.*, The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport. *Journal of Cell Science* **119**, 3944 (2006).

122. J. Viaud, F. Boal, H. Tronchère, F. Gaits-Iacovoni, B. Payrastre, Phosphatidylinositol 5-phosphate: A nuclear stress lipid and a tuner of membranes and cytoskeleton dynamics. *BioEssays* **36**, 260 (2014).

123. D. Sarkes, Lucia E. Rameh, A novel HPLC-based approach makes possible the spatial characterization of cellular PtdIns5P and other phosphoinositides. *Biochemical Journal* **428**, 375 (2010).

124. J. Viaud *et al.*, Phosphatidylinositol 5-phosphate regulates invasion through binding and activation of Tiam1. *Nat Commun* **5**, (2014).

125. D. Sbrissa, O. C. Ikonomov, J. Strakova, A. Shisheva, Role for a Novel Signaling Intermediate, Phosphatidylinositol 5-Phosphate, in Insulin-Regulated F-Actin Stress Fiber Breakdown and GLUT4 Translocation. *Endocrinology* **145**, 4853 (2004).

126. G. Guittard *et al.*, Cutting Edge: Dok-1 and Dok-2 Adaptor Molecules Are Regulated by Phosphatidylinositol 5-Phosphate Production in T Cells. *The Journal of Immunology* **182**, 3974 (2009).

127. M. Vicinanza *et al.*, PI(5)P Regulates Autophagosome Biogenesis. *Molecular Cell* **57**, 219 (2015).

128. O. Gozani *et al.*, The PHD Finger of the Chromatin-Associated Protein ING2 Functions as a Nuclear Phosphoinositide Receptor. *Cell* **114**, 99 (2003).

129. D. R. Jones *et al.*, Nuclear PtdIns5P as a Transducer of Stress Signaling: An In Vivo Role for PIP4Kbeta. *Molecular Cell* **23**, 685 (2006).

130. L. Cocco *et al.*, Phosphoinositide-specific phospholipase C (PI-PLC) β1 and nuclear lipid-dependent signaling. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1761**, 509 (2006).

131. W. Echevarria, M. F. Leite, M. T. Guerra, W. R. Zipfel, M. H. Nathanson, Regulation of calcium signals in the nucleus by a nucleoplasmic reticulum. *Nat Cell Biol* **5**, 440 (2003).

132. I. Faenza, R. Fiume, M. Piazzi, A. Colantoni, L. Cocco, Nuclear inositide specific phospholipase C signalling – interactions and activity. *FEBS Journal* **280**, 6311 (2013).

133. R. F. Irvine, Nuclear lipid signalling. *Nat Rev Mol Cell Biol* **4**, 349 (2003).

134. R. L. Vann, P. F. B. Wooding, F. R. Irvine, N. Divechia, Metabolism and possible compartmentalization of inositol lipids in isolated rat-liver nuclei. *Biochemical Journal* **327**, 569 (1997).

135. I. V. Boronenkov, J. C. Loijens, M. Umeda, R. A. Anderson, Phosphoinositide Signaling Pathways in Nuclei Are Associated with Nuclear Speckles Containing Pre-mRNA Processing Factors. *Molecular Biology of the Cell* **9**, 3547 (1998).

136. A. I. Lamond, D. L. Spector, Nuclear speckles: a model for nuclear organelles. *Nat Rev Mol Cell Biol* **4**, 605 (2003).

137. P. de Graaf *et al.*, Nuclear localization of phosphatidylinositol 4-kinase β. *Journal of Cell Science* **115**, 1769 (2002).

138. C. A. Barlow, R. S. Laishram, R. A. Anderson, Nuclear phosphoinositides: a signaling enigma wrapped in a compartmental conundrum. *Trends in Cell Biology* **20**, 25 (2010).

139. A. R. Odom, A. Stahlberg, S. R. Wente, J. D. York, A Role for Nuclear Inositol 1,4,5-Trisphosphate Kinase in Transcriptional Control. *Science* **287**, 2026 (2000).

140. X. Shen, H. Xiao, R. Ranallo, W.-H. Wu, C. Wu, Modulation of ATP-Dependent Chromatin-Remodeling Complexes by Inositol Polyphosphates. *Science* **299**, 112 (2003).

141. S. J. York, B. N. Armbruster, P. Greenwell, T. D. Petes, J. D. York, Inositol Diphosphate Signaling Regulates Telomere Length. *Journal of Biological Chemistry* **280**, 4264 (2005).

142. I. Cestari, K. Stuart, Inositol phosphate pathway controls transcription of telomeric expression sites in trypanosomes. *Proceedings of the National Academy of Sciences* **112**, E2803 (2015).

143. R. Rusinova, E. A. Hobart, R. E. Koeppe, O. S. Andersen, Phosphoinositides alter lipid bilayer properties. *The Journal of General Physiology* **141**, 673 (2013).

144. J. A. Lundbæk *et al.*, Capsaicin Regulates Voltage-Dependent Sodium Channels by Altering Lipid Bilayer Elasticity. *Molecular Pharmacology* **68**, 680 (2005).

145. T. Laux *et al.*, Gap43, Marcks, and Cap23 Modulate Pi(4,5)p2 at Plasmalemmal Rafts, and Regulate Cell Cortex Actin Dynamics through a Common Mechanism. *The Journal of Cell Biology* **149**, 1455 (2000).

146. V. Papayannopoulos *et al.*, A Polybasic Motif Allows N-WASP to Act as a Sensor of PIP2 Density. *Molecular Cell* **17**, 181 (2005).

147. T. Balla, Inositol-lipid binding motifs: signal integrators through protein-lipid and protein-protein interactions. *Journal of Cell Science* **118**, 2093 (2005).

148. J. P. DiNitto, T. C. Cronin, D. G. Lambright, Membrane Recognition and Targeting by Lipid-Binding Domains. *Science Signaling* **2003**, re16 (2003).

149. S. Mukhopadhyay, P. K. Jackson, The tubby family proteins. *Genome Biology* **12**, 1 (2011).

150. M. A. Lemmon, Membrane recognition by phospholipid-binding domains. *Nat Rev Mol Cell Biol* **9**, 99 (2008).

151. D. Zhang, L. Aravind, Identification of novel families and classification of the C2 domain superfamily elucidate the origin and evolution of membrane targeting activities in eukaryotes. *Gene* **469**, 18 (2010).

152. J. W. Putney, Formation and actions of calcium-mobilizing messenger, inositol 1,4,5-trisphosphate. *American Journal of Physiology - Gastrointestinal and Liver Physiology* **252**, G149 (1987).

153. P. A. van Paridon, B. de Kruijff, R. Ouwerkerk, K. W. A. Wirtz, Polyphosphoinositides undergo charge neutralization in the physiological pH range: a 31P-NMR study. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism* **877**, 216 (1986).

154. M. Toner, G. Vaio, A. McLaughlin, S. McLaughlin, Adsorption of cations to phosphatidylinositol 4,5-bisphosphate. *Biochemistry* **27**, 7435 (1988).

155. W. D. Heo *et al.*, PI(3,4,5)P3 and PI(4,5)P2 Lipids Target Proteins with Polybasic Clusters to the Plasma Membrane. *Science* **314**, 1458 (2006).

156. J. M. Kavran *et al.*, Specificity and Promiscuity in Phosphoinositide Binding by Pleckstrin Homology Domains. *Journal of Biological Chemistry* **273**, 30497 (1998).

157. M. A. Lemmon, K. M. Ferguson, R. O'Brien, P. B. Sigler, J. Schlessinger, Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 10472 (1995).

158. E. Baraldi *et al.*, Structure of the PH domain from Bruton's tyrosine kinase in complex with inositol 1,3,4,5-tetrakisphosphate. *Structure* **7**, 449 (1999).

159. K. M. Ferguson, M. A. Lemmon, J. Schlessinger, P. B. Sigler, Structure of the high affinity complex of inositol trisphosphate with a phospholipase C pleckstrin homology domain. *Cell* **83**, 1037 (1995).

160. D. B. van Rossum *et al.*, Phospholipase Cγ1 controls surface expression of TRPC3 through an intermolecular PH domain. *Nature* **434**, 99 (2005).

161. S. McLaughlin, A. Aderem, The myristoyl-electrostatic switch: a modulator of reversible protein-membrane interactions. *Trends in Biochemical Sciences* **20**, 272 (1995).

162. B. Sha, S. E. Phillips, V. A. Bankaitis, M. Luo, Crystal structure of the *Saccharomyces cerevisiae* phosphatidylinositol- transfer protein. *Nature* **391**, 506 (1998).

163. P. C. Stolt *et al.*, Origins of Peptide Selectivity and Phosphoinositide Binding Revealed by Structures of Disabled-1 PTB Domain Complexes. *Structure* **11**, 569 (2003).

164. T. G. Kutateladze *et al.*, Phosphatidylinositol 3-Phosphate Recognition by the FYVE Domain. *Molecular Cell* **3**, 805 (1999).

165. S. Dowler, G. Kular, D. R. Alessi, Protein Lipid Overlay Assay. *Science Signaling* **2002**, pl6 (2002).

166. J. E. Harlan, P. J. Hajduk, H. S. Yoon, S. W. Fesik, Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. *Nature* **371**, 168 (1994).

167. K. Hirose, S. Kadowaki, M. Tanabe, H. Takeshima, M. Iino, Spatiotemporal Dynamics of Inositol 1,4,5-Trisphosphate That Underlies Complex Ca2+ Mobilization Patterns. *Science* **284**, 1527 (1999).

168. G. R. V. Hammond, T. Balla, Polyphosphoinositide binding domains: Key to inositol lipid biology. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 746 (2015).

169. O. Idevall-Hagren, P. De Camilli, Detection and manipulation of phosphoinositides. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1851**, 736 (2015).

170. F. Hertel *et al.*, A Genetically Encoded Tool Kit for Manipulating and Monitoring Membrane Phosphatidylinositol 4,5-Bisphosphate in Intact Cells. *PLoS One* **6**, e20855 (2011).

171. L. Yang *et al.*, Bioluminescent Probes to Analyze Ligand-Induced Phosphatidylinositol 3,4,5-Trisphosphate Production with Split Luciferase Complementation. *Analytical Chemistry* **85**, 11352 (2013).

172. M. Jost, F. Simpson, J. M. Kavran, M. A. Lemmon, S. L. Schmid, Phosphatidylinositol-4,5-bisphosphate is required for endocytic coated vesicle formation. *Current Biology* **8**, 1399 (1998).

173. S. Stuffers *et al.*, Time-Resolved Ultrastructural Detection of Phosphatidylinositol 3-Phosphate. *Journal of Histochemistry & Cytochemistry* **58**, 1025 (2010).

174. M. A. Lemmon, K. M. Ferguson, Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochemical Journal* **350**, 1 (2000).

175. W. Nagel *et al.*, Phosphoinositide 3-OH Kinase Activates the β2Integrin Adhesion Pathway and Induces Membrane Recruitment of Cytohesin-1. *Journal of Biological Chemistry* **273**, 14853 (1998).

176. J.-O. Lee *et al.*, Crystal Structure of the PTEN Tumor Suppressor: Implications for Its Phosphoinositide Phosphatase Activity and Membrane Association. *Cell* **99**, 323 (1999).

177. S. Jean, A. A. Kiger, Coordination between RAB GTPase and phosphoinositide regulation and functions. *Nat Rev Mol Cell Biol* **13**, 463 (2012).

178. J. Carlton *et al.*, Sorting Nexin-1 Mediates Tubular Endosome-to-TGN Transport through Coincidence Sensing of High- Curvature Membranes and 3-Phosphoinositides. *Current Biology* **14**, 1791 (2004).

179. A. Kielkowska *et al.*, A new approach to measuring phosphoinositides in cells by mass spectrometry. *Advances in Biological Regulation* **54**, 131 (2014).

180. Z. Szentpetery, P. Várnai, T. Balla, Acute manipulation of Golgi phosphoinositides to assess their importance in cellular trafficking and signaling. *Proceedings of the National Academy of Sciences* **107**, 8225 (2010).

181. T. Kurokawa *et al.*, 3' Phosphatase activity toward phosphatidylinositol 3,4-bisphosphate [PI(3,4)P2] by voltage-sensing phosphatase (VSP). *Proceedings of the National Academy of Sciences* **109**, 10089 (2012).

182. S. S. Grimm, E. Y. Isacoff, Allosteric substrate switching in a voltage-sensing lipid phosphatase. *Nat Chem Biol* **12**, 261 (2016).

183. J. Lacroix *et al.*, Controlling the Activity of a Phosphatase and Tensin Homolog (PTEN) by Membrane Potential. *Journal of Biological Chemistry* **286**, 17945 (2011).

184. J. E. Toettcher, D. Gong, W. A. Lim, O. D. Weiner, Light-based feedback for controlling intracellular signaling dynamics. *Nat Meth* **8**, 837 (2011).

185. O. Idevall-Hagren, E. J. Dickson, B. Hille, D. K. Toomre, P. De Camilli, Optogenetic control of phosphoinositide metabolism. *Proceedings of the National Academy of Sciences* **109**, E2316 (2012).

186. W. K. A. Karunarathne, L. Giri, V. Kalyanaraman, N. Gautam, Optically triggering spatiotemporally confined GPCR activity in a cell and programming neurite initiation and extension. *Proceedings of the National Academy of Sciences* **110**, E1565 (2013).

187. D. A. Fruman, C. Rommel, PI3K and cancer: lessons, challenges and opportunities. *Nat Rev Drug Discov* **13**, 140 (2014).

188. J. G. Foster, M. D. Blunt, E. Carter, S. G. Ward, Inhibition of PI3K Signaling Spurs New Therapeutic Opportunities in Inflammatory/Autoimmune Diseases and Hematological Malignancies. *Pharmacological Reviews* **64**, 1027 (2012).

189. M. D. Blunt, S. G. Ward, Targeting PI3K isoforms and SHIP in the immune system: new therapeutics for inflammation and leukemia. *Current Opinion in Pharmacology* **12**, 444 (2012).

190. L. H. Mak *et al.*, A Small Molecule Mimicking a Phosphatidylinositol (4,5)-Bisphosphate Binding Pleckstrin Homology Domain. *ACS Chemical Biology* **6**, 1382 (2011).

191. C. Dinkel, M. Moody, A. Traynor-Kaplan, C. Schultz, Membrane-Permeant 3-OH-Phosphorylated Phosphoinositide Derivatives. *Angewandte Chemie International Edition* **40**, 3004 (2001).

192. V. Laketa *et al.*, Membrane-Permeant Phosphoinositide Derivatives as Modulators of Growth Factor Signaling and Neurite Outgrowth. *Chemistry & Biology* **16**, 1190 (2009).

193. D. Subramanian *et al.*, Activation of membrane-permeant caged PtdIns(3)P induces endosomal fusion in cells. *Nat Chem Biol* **6**, 324 (2010).

194. J. Lee, H. Park, Y. Park, Molecular Mechanisms of Host Cytoskeletal Rearrangements by Shigella Invasins. *International Journal of Molecular Sciences* **15**, 18253 (2014).

195. S. L. Marcus, M. R. Wenk, O. Steele-Mortimer, B. B. Finlay, A synaptojanin-homologous region of *Salmonella typhimurium* SigD is essential for inositol phosphatase activity and Akt activation. *FEBS Letters* **494**, 201 (2001).

196. M. A. Bakowski *et al.*, The Phosphoinositide Phosphatase SopB Manipulates Membrane Surface Charge and Trafficking of the *Salmonella*-Containing Vacuole. *Cell Host & Microbe* **7**, 453 (2010).

197. F. Boal *et al.*, PI5P Triggers ICAM-1 Degradation in *Shigella* Infected Cells, Thus Dampening Immune Cell Recruitment. *Cell Reports* **14**, 750 (2016).

198. F. Boal *et al.*, TOM1 is a PI5P effector involved in the regulation of endosomal maturation. *Journal of Cell Science* **128**, 815 (2015).

199. A. Vaid, R. Ranjan, W. A. Smythe, H. C. Hoppe, P. Sharma, PfPI3K, a phosphatidylinositol-3 kinase from *Plasmodium falciparum*, is exported to the host erythrocyte and is involved in hemoglobin trafficking. *Blood* **115**, 2500 (2010).

200. M. Rao, S. Mayor, Active organization of membrane constituents in living cells. *Current Opinion in Cell Biology* **29**, 126 (2014).

201. S. Furse, H. Wienk, R. Boelens, A. I. P. M. de Kroon, J. A. Killian, *E. coli* MG1655 modulates its phospholipid composition through the cell cycle. *FEBS Letters* **589**, 2726 (2015).

202. R. H. Michell, Inositol phospholipids and cell surface receptor function. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes* **415**, 81 (1975).

203. N. H. Tolia, L. Joshua-Tor, Strategies for protein coexpression in *Escherichia coli*. *Nat Meth* **3**, 55 (2006).

204. J. E. Cronan, Bacterial Membrane Lipids: Where Do We Stand? *Annual Review of Microbiology* **57**, 203 (2003).

205. Kirstee L. Martin, Terry K. Smith, Phosphatidylinositol synthesis is essential in bloodstream form *Trypanosoma brucei*. *Biochemical Journal* **396**, 287 (2006).

206. M. Bohdanowicz, S. Grinstein, Role of Phospholipids in Endocytosis, Phagocytosis, and Macropinocytosis. *Physiological Reviews* **93**, 69 (2013).

207. X.-H. Zhao, T. Bondeva, T. Balla, Characterization of Recombinant Phosphatidylinositol 4-Kinase β Reveals Auto- and Heterophosphorylation of the Enzyme. *Journal of Biological Chemistry* **275**, 14642 (2000).

208. J. C. Loijens, R. A. Anderson, Type I Phosphatidylinositol-4-phosphate 5-Kinases Are Distinct Members of This Novel Lipid Kinase Family. *Journal of Biological Chemistry* **271**, 32937 (1996).

209. M. H. Larson *et al.*, A pause sequence enriched at translation start sites drives transcription dynamics in vivo. *Science* **344**, 1042 (2014).

210. F. W. Studier, B. A. Moffatt, Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *Journal of Molecular Biology* **189**, 113 (1986).

211. H. Inoue, H. Nojima, H. Okayama, High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**, 23 (1990).

212. E. G. Bligh, W. J. Dyer, A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911 (1959).

213. H. Link, B. Anselment, D. Weuster-Botz, Leakage of adenylates during cold methanol/glycerol quenching of *Escherichia coli*. *Metabolomics* **4**, 240 (2008).

214. F. C. Neidhardt, *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology., (American Society For Microbiology, 1996), vol. 1.

215. R. Milo, P. Jorgensen, U. Moran, G. Weber, M. Springer, BioNumbers—the database of key numbers in molecular and cell biology. *Nucleic Acids Research* **38**, D750 (2010).

216. A. Balla, G. Tuymetova, A. Tsiomenko, P. Várnai, T. Balla, A Plasma Membrane Pool of Phosphatidylinositol 4-Phosphate Is Generated by Phosphatidylinositol 4-Kinase Type-III Alpha: Studies with the PH Domains of the Oxysterol Binding Protein and FAPP1. *Molecular Biology of the Cell* **16**, 1282 (2005).

217. S. A. Watt, G. Kular, I. N. Fleming, C. P. Downes, J. M. Lucocq, Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C δ1. *Biochemical Journal* **363**, 657 (2002).

218. H. M. Salis, E. A. Mirsky, C. A. Voigt, Automated design of synthetic ribosome binding sites to control protein expression. *Nat Biotech* **27**, 946 (2009).

219. A. Espah Borujeni, A. S. Channarasappa, H. M. Salis, Translation rate is controlled by coupled trade-offs between site accessibility, selective RNA unfolding and sliding at upstream standby sites. *Nucleic Acids Research* **42**, 2646 (2014).

220. J. H. Davis, A. J. Rubin, R. T. Sauer, Design, construction and characterization of a set of insulated bacterial promoters. *Nucleic Acids Research* **39**, 1131 (2011).

221. M. Basan *et al.*, Inflating bacterial cells by increased protein synthesis. *Molecular Systems Biology* **11**, n/a (2015).

222. W. E. Watkins, Iii, III, A. K. Menon, Reconstitution of Phospholipid Flippase Activity from *E. coli* Inner Membrane: A Test of the Protein Translocon as a Candidate Flippase. *Biological Chemistry*. (2002), vol. 383, pp. 1435.

223. W. Nickel, The Unconventional Secretory Machinery of Fibroblast Growth Factor 2. *Traffic* **12**, 799 (2011).

224. A. D. Ebert *et al.*, Tec-Kinase-Mediated Phosphorylation of Fibroblast Growth Factor 2 is Essential for Unconventional Secretion. *Traffic* **11**, 813 (2010).

225. K. Temmerman, W. Nickel, A novel flow cytometric assay to quantify interactions between proteins and membrane lipids. *Journal of Lipid Research* **50**, 1245 (2009).

226. K. Temmerman *et al.*, A Direct Role for Phosphatidylinositol-4,5-bisphosphate in Unconventional Secretion of Fibroblast Growth Factor 2. *Traffic* **9**, 1204 (2008).

227. J. P. Steringer *et al.*, Phosphatidylinositol 4,5-Bisphosphate (PI(4,5)P2)-dependent Oligomerization of Fibroblast Growth Factor 2 (FGF2) Triggers the Formation of a Lipidic Membrane Pore Implicated in Unconventional Secretion. *Journal of Biological Chemistry* **287**, 27659 (2012).

228. L. C. Torrado *et al.*, An intrinsic quality-control mechanism ensures unconventional secretion of fibroblast growth factor 2 in a folded conformation. *Journal of Cell Science* **122**, 3322 (2009).

229. S. Faham, R. E. Hileman, J. R. Fromm, R. J. Linhardt, D. C. Rees, Heparin Structure and Interactions with Basic Fibroblast Growth Factor. *Science* **271**, 1116 (1996).

230. C. Zehe, A. Engling, S. Wegehingel, T. Schäfer, W. Nickel, Cell-surface heparan sulfate proteoglycans are essential components of the unconventional export machinery of FGF-2. *Proceedings of the National Academy of Sciences* **103**, 15479 (2006).

231. H.-M. Müller *et al.*, Formation of Disulfide Bridges Drives Oligomerization, Membrane Pore Formation, and Translocation of Fibroblast Growth Factor 2 to Cell Surfaces. *Journal of Biological Chemistry* **290**, 8925 (2015).

232. S. C. Strickfaden *et al.*, A Mechanism for Cell-Cycle Regulation of MAP Kinase Signaling in a Yeast Differentiation Pathway. *Cell* **128**, 519 (2007).

233. Y. Li, F. R. Cross, B. T. Chait, Method for identifying phosphorylated substrates of specific cyclin/cyclin-dependent kinase complexes. *Proceedings of the National Academy of Sciences* **111**, 11323 (2014).