# Eukaryotic phosphate homeostasis: the inositol pyrophosphate perspective

Cristina Azevedo and Adolfo Saiardi*

Medical Research Council Laboratory for Molecular Cell Biology, University College London, London, WC1E 6BT, UK.

*Correspondence addressed to:
Adolfo Saiardi
MRC-LMCB; University College London, Gower Street, London WC1E 6BT, UK. Email: dmcbado@ucl.ac.uk.Tel: +44-20-7679-7254; Fax: +44-20-7679-7805

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**Abstract**

Phosphate, as a cellular energy currency, essentially drives most biochemical reactions defining living organisms, thus its homeostasis must be tightly regulated. Investigation into the role of inositol pyrophosphates has provided a novel perspective on the regulation of phosphate homeostasis. Recent data suggest a metabolic and signalling interplay between inositol pyrophosphates, ATP and inorganic polyphosphate (polyP), that influences and is influenced by cellular phosphate homeostasis. Different studies have demonstrated that the SPX protein domain is a key component of proteins involved in phosphate metabolism. How inositol pyrophosphates control some aspects of phosphate homeostasis has become clearer with the recently acquired crystal structures of SPX domains. Here we review recent studies on eukaryote phosphate homeostasis and provide insights into future research.

**Importance of studying phosphate homeostasis**

The importance of phosphate ($PO_4^{3-}$, $P_i$) is perhaps underappreciated in biology: it is not an overstatement to note that the large majority of biochemical reactions involve phosphate [1]. Only water participates in more reactions than adenosine triphosphate (ATP), and the release of energy by the hydrolysis of ATP virtually drives life as it currently is known. However, what drives life is not the hydrolysis of the chemical bonds of adenine or the ribose but the hydrolysis of the phosphoanhydride bonds between two phosphate groups. For this reason, the three phosphates of an ATP molecule are the most important components. Phosphate's tetrahedral molecular geometry gives the oxygen atoms the three-dimensional structure to participate in hydrogen bonds, allowing phosphate to be a key component of macromolecules such as nucleic acids and phospholipids. Furthermore, being negatively charged at physiological pH provides phosphate with signalling properties, including the attachment of a phosphate group to a specific amino acid, thereby altering the overall charge of the protein and affecting its functions [2]. In fulfilling these varied roles, phosphate moieties circulate between organic molecules, generally being passed along via phosphotransfer reactions involving nucleotide triphosphates. Eventually the cascade of events generates free phosphate that mitochondrial ATP synthases subsequently utilise to regenerate ATP, completing the cycle. Consequently, phosphate homeostasis is closely interwoven with energy metabolism. Given the fundamental functions played by phosphate in molecular structure, signalling and as an energy currency, its cellular homeostasis must be tightly regulated.

Investigation into eukaryotic phosphate homeostasis came alight in the ‘90s with several seminal papers where the phosphate signal transduction (PHO) pathway of the budding yeast, *Saccharomyces cerevisiae*, was defined (Box 1) [3, 4]. In fact Trends in Biochemical Sciences contributed to cementing the yeast PHO pathway with a most influential 1996 review [5]. However, with the availability of complete genome sequences for an ever-growing number of eukaryotic species, it became evident that the PHO pathway as discovered in *S. cerevisiae* is not evolutionarily conserved: many of its components, including Pho81 and Pho4, have no clear homology in other genomes. These key components are even absent in the related ascomycete, the fission yeast *Schizosaccharomyces pombe*, where the phosphate-dependent transcriptional response is present but differs extensively from the budding yeast model [6]. Thus it becomes important to define other regulatory mechanisms able to supervise cellular phosphate homeostasis.

**The association between SPX domains and phosphate metabolism**

In today’s post-genomic era help often comes from the systematic comparative analysis of sequenced genomes. The identification of evolutionarily conserved homology sequences defining specific ‘domains’ and their architecture in proteins through bioinformatics has become the *de facto* standard in protein analysis, providing hints on protein functions. One of these conserved regions, the SPX domain (Pfam: PF03105; see Glossary), named after the yeast proteins Syg1 and Pho81 and the mammalian Xpr1, has emerged in the past decade as a key region/signature of proteins involved in regulating aspects of phosphate metabolism [7].

The yeast *S. cerevisiae* possesses ten proteins containing an SPX domain, all with N-terminal localisation (Figure 1). In these proteins the SPX domain is associated with other functional regions, defining six diverse groups. The SPX domain is present in four components of the vacuolar transport complex (VTC) that is responsible for synthesis of inorganic polyphosphate (polyP; see Glossary) in yeast [8-10], polyP is a linear polymer of phosphate groups [11, 12] whose main function is to buffer free phosphate (see below). Other proteins containing SPX domains are the low-affinity phosphate transporters Pho87 and Pho90, in which SPX deletion revealed an inhibitory function: the truncated transporters allowed increased phosphate uptake [13]. Detailed analysis on the role of the SPX domain in other yeast proteins is missing, but virtually all other SPX domain-containing proteins have been associated with phosphate metabolism. This is the case for the CDK inhibitor Pho81, for the vacuole phosphate exporter Pho91, and for the glycerophosphocholine phosphodiesterase

Gde1, whose enzymatic activity generates glycerol-phosphate to be utilised as a phosphate source [14]. The SPX domain is also present in Syg1, which additionally contains an EXS domain (see Glossary) (Figure 1). The EXS domain, named after the yeast protein Erd1, human Xpr1 and yeast Syg1 (Pfam: PF03124), contains several predicted transmembrane helices [15] that likely form a channel for transferring phosphate. The association between the N-terminus SPX domain and the C-terminus EXS is a common architecture also found in plant phosphate exporters and in the human phosphate exporter, Xpr1 (see below and Glossary) [16]. Therefore, the uncharacterised yeast Syg1 protein is likely to be also a phosphate exporter.

In recent years, great interest has been drawn to the expanded family of the plant SPX domain-containing proteins (reviewed in [17, 18]). Phosphate is one of the main nutrients in plants, and agricultural ecosystems heavily rely on the application of phosphate fertilizers. The benefits (high yield) and drawbacks (limited reserves of phosphate rock, pollution) that arise from the usage of phosphate fertilizers have divided opinion over time [19]. This has led to concerted global efforts to understand the molecular mechanisms of phosphate homeostasis in plants. The aim is to create a more sustainable agriculture by improving phosphate acquisition in crops. The *Arabidopsis thaliana* (thale cress) genome encodes 20 proteins containing SPX domains, while rice (*Oryza sativa*) has 15 such proteins [17]. Here we briefly review the *A. thaliana* literature. Most of these studies rely on genetic analysis of mutants, and while very important and informative, they often lack the complementary biochemical analysis to elucidate the mechanism of action of the SPX domain. Plant SPX-containing proteins can be grouped into four subfamilies (Figure 1).

The simplest of these comprises proteins containing exclusively the SPX domain. It has four members in *A. thaliana* (SPX1-4). All but *SPX4* expression are induced under phosphate starvation in roots and/or shoots, and are localized to the nucleus. The individual knockouts, grown under either high or low phosphate, do not show an apparent phenotype, but *SPX1* overexpression increased expression of some of the phosphate starvation-inducible genes independent of the phosphate status [20, 21].

The second subfamily is SPX-RING (Really Interesting New Gene). One member of this subfamily is the nitrogen limitation adaptation protein, NLA. It is an E3 ubiquitin ligase that acts on, amongst others, PHT1 (Phosphate transporter 1, homologous to the yeast high affinity phosphate transporter Pho84). PHT1 is localized at the plasma membrane of root cells, where it imports phosphate from the soil. Its activity is tightly regulated, and when phosphate is in abundance, PHT1 is targeted for degradation via ubiquitination by NLA [22].

The SPX domain of NLA is likely to directly interact with PHT1 since it is sufficient for plasma membrane targeting and interaction with PHT1 [22].

The subfamily SPX-MFS (Major Facilitator Superfamily) includes transporters that facilitate the translocation of small solutes, including phosphate [23-25]. In *A. thaliana* there are three members (SPX-MSF1-3). The only characterized member is SPX-MFS1, also known as PHT5, which belongs to the class of vacuolar phosphate transporters [26]. The SPX-MFS1 loss of function mutant has low vacuolar-to-cytoplasm phosphate ratio compared to wild-type plants. Conversely, SPX-MFS1 overexpression leads to misregulation of phosphate starvation-responsive genes and growth retardation as a consequence of phosphate sequestration into vacuoles [26]. The exact function of the SPX domain in this protein is not fully known.

Members of the subfamily SPX-EXS harbour both SPX and EXS domains (see above). One of the most well-studied is PHO1 (Phosphate 1), a prototypical eukaryotic phosphate exporter found primarily at the Golgi and trans-Golgi network [27]. It is known that the SPX domain is not required for either Golgi/trans-Golgi targeting or for phosphate export itself [28, 29], functions that are performed by the EXS region [30]. However, the SPX domain is required for binding to PHO2, an ubiquitin-conjugating E2 enzyme that helps in the degradation of PHO1 for regulation of phosphate homeostasis [29]. Another SPX-EXS subfamily member is SHB1 (Short Hypocotyl under BLUE1, also known as PHO1;H4) [31]. This protein is not directly associated with phosphate metabolism but instead is a putative transcription co-activator in light signalling, with key regulatory functions in seed development. Overexpression of SHB1, or just its SPX domain, extends its signalling activity towards a broader spectrum of light wavelengths, suggesting that these signalling activities might be directly modulated by SPX, perhaps by interaction with other domains [31]. Interestingly, a link between light sensing and phosphate uptake has been established in algae, where the light-dark cycle directly affects phosphate uptake [32], raising the possibility that these two environmental responses might share components.

While yeast and plants possess several different SPX domain-containing protein architectures, metazoan genomes only encode proteins with the SPX-EXS association (Figure 1). In the fly *Drosophila melanogaster* there are four similar proteins of unknown function (gene names CG10481, CG10483, CG7536, CG2901). The nematode *Caenorhabditis elegans* possesses one poorly characterized gene with SPX-EXS architecture (gene name CELE_Y39A1A.22). The single *Homo sapiens* protein with the SPX-EXS architecture is Xpr1 (Figure 1), originally identified as the Xenotropic and Polytropic Retrovirus Receptor 1,

which is localised on the surface of many cell types [30]. While an initial overexpression study suggested that the SPX domain is not required for Xpr1's phosphate export function [16], a recent human genetic study revealed that several mutations localised within the SPX domain of Xpr1 are responsible for primary familial brain calcification (PFBC), a disease characterized by calcium phosphate deposits in the basal ganglia [33, 34]. Biochemical analysis of SPX-mutated Xpr1 proteins revealed that these mutations do reduce phosphate export, demonstrating a regulatory role for the SPX domain in controlling phosphate efflux activity.

**SPX structure reveals a thought-provoking inositol polyphosphate connection**

All this literature unquestionably links the SPX domain to cellular phosphate metabolism. However, only the recent acquisition of the crystal structure of several SPX domains [35] allows us to appreciate how this region reads the phosphate cellular status. The sequences defining the SPX domain are of variable length, ranging from 135 to >400 amino acids [7], resulting in a tripartite organization with three distinct homology regions of 30-40 amino acids (Figure 2). The resolved SPX structures revealed that the first homology region is normally organised into two small helices (α1 and α2) while the two other homologous regions form long helices (α3 and α4) that constitute the core of the structure. Two linker regions of variable length and with low sequence homology connect these three segments. The structural study included extra C-terminus sequence that resolves into two helices (α5 and α6) contributing to the overall organisational strength of the SPX domain (Figure 2) [35]. The original crystal revealed the presence of a sulphate group, which often replaces phosphate during the crystallization process, that was coordinated by highly conserved amino acids: a tyrosine and a lysine in helix α2, and a lysine in helix α4, that together define the Phosphate Binding Cluster (PBC). In addition, helix α4 was shown to possess three evolutionarily conserved lysines that define a Lysine Surface Cluster (KSC) (Figure 2). Together these two clusters, PBC and KSC, ultimately form a large positively charged surface. While there has been a great deal of speculation on the role of the SPX domain as a direct phosphate sensor, experimental data demonstrating that phosphate specifically and selectively binds to SPX domains were inconclusive [35]. The two PBC and KSC clusters define a large area that might be better suited for a ligand larger than phosphate itself. In fact, it was discovered that SPX domain ligands are the inositol polyphosphates (IPs; Box 2). The crystal structures obtained when co-crystalising the SPX domain with $IP_6$ (inositol

hexakisphosphate or phytic acid; Box 2) revealed that $IP_6$ is coordinated through hydrogen bond interactions with the large basic surface of both the PBC and KSC clusters, which together define the IPs binding site [35].

Diverse binding experiments have demonstrated that several yeast and plant SPX domains bind with submicromolar affinity to $IP_6$ and to inositol pyrophosphates (PP-IPs; see Glossary) [35]. Mutagenesis of PBC and KSC key residues reduced the binding affinity. In some circumstances the SPX domains show a high degree of binding specificity, as in the case of the *O. sativa* SPX4/PHR2 complex, where the inositol pyrophosphate isomer 5PP-$IP_5$ (hereafter called $IP_7$, see below) (Figure 3A,B), has a binding affinity one order of magnitude higher than that of $IP_6$. In other cases, as for the SPX domain of yeast Vtc2, the binding of $IP_6$ and $IP_7$ occurs with similar affinity. However, $IP_7$ was far better than $IP_6$ in stimulating polyP synthesis using purified vacuoles. Mutagenesis of conserved amino acids within the SPX of Vtc3 and Vtc4 impairs polyP synthesis stimulation by $IP_7$ [35]. This gives us a coherent explanation of the genetic evidence where the deletion of PP-IPs biosynthetic pathways (Figure 3A) leads to yeast with no or little polyP [36]. The ability of PP-IPs to control *S. cerevisiae* polyP synthesis was originally reported by two studies. One used $^{31}$P-NMR to determine yeast polyP levels [37] and linked the synthesis of polyP to a specific PP-IPs, PP-$IP_4$, which is generated by the pyrophosphorylation of inositol pentakisphosphate ($IP_5$) (Figure 3A). The second study biochemically extracted and resolved polyP, by gel electrophoresis, from a complete array of inositol phosphate kinase single and double mutants. This study established that the presence of polyP in yeast depends on the presence of any kind of PP-IPs [36], while the presence of $IP_6$ or $IP_5$ does not correlate with polyP synthesis.

Undoubtedly, the regulation of SPX domain by PP-IPs offers a new perspective on cell signalling. However, mechanistically, it is not yet known how PP-IPs binding to SPX domain is transduced to the protein. It is possible that intramolecular conformational changes activate the Vtc4 catalytic domain. However, in other contexts PP-IPs binding could inhibit protein function. For example the PP-IPs activation of Vtc4 and the consequent accumulation of polyP into the vacuole should be complemented by PP-IPs-mediated inhibition of the vacuole phosphate exporter Pho91. Moreover, the binding of PP-IPs to SPX domains might not directly function as an activator/inhibitor but might work as a molecular glue regulating protein-protein interactions. This mode of action has been observed for several proteins: IPs bind in a pocket between histone deacetylases (HDAC) and co-repressor proteins [38] regulating their interaction; similarly IPs bind to TIR1 and COI1-JAZ, the receptor for the

plant hormone auxin [39] and jasmonate, respectively [40]. Different SPX domains may sense different IPs/PP-IPs; however, *in vitro* IPs/PP-IPs binding experiments are difficult due to the exceptionally high charge density of these molecules. Thus studying *in vivo* IP/PP-IP selectivity for SPX domains, while technically challenging, will be essential to properly elucidate SPX domain-regulated events, and to fully appreciate the physiological importance of this domain and the exact roles played by specific IPs/PP-IPs.

**Phosphate alters inositol pyrophosphate metabolism**

Inositol pyrophosphates, PP-IPs, are an interesting but understudied class of signalling molecules that, as the name suggests, possess one or more pyrophosphate moieties (Figure 3B, Box 2). The presence of highly energetic phosphoanhydride bonds is just one of the distinctive features of these molecules. They have a respectable signalling pedigree [41] as PP-IPs belong to an important family of cellular messengers that includes the calcium release factor $IP_3$ [42]. In yeast, these molecules are synthesized by sequential phosphorylation of $IP_3$ generated by phospholipase C (Figure 3A) [43]. Inositol pyrophosphates are ubiquitously present in eukaryotes, and, remarkably for organic molecules, often contain more phosphate groups than carbons; for example $IP_7$ possesses seven phosphates attached to the six carbon inositol ring (Figure 3B). Besides binding to proteins, PP-IPs can induce the protein post-translational modification protein *pyro*phosphorylation. In this modification, hydrolysis of the pyrophosphate moiety drives the transfer of the β-phosphate to a pre-phosphorylated serine residue, forming a *pyro*phosphoserine [44, 45]. Inositol pyrophosphates appear to regulate a wide range of cell biological processes, and we refer the interested reader to recent reviews [46-48]. Here we highlight PP-IPs importance in the regulation of cellular phosphate homeostasis and basic metabolism.

Inositol pyrophosphates have been defined as ‘metabolic messengers’ [49], sensing the metabolic or energetic status of the cell [48, 50]. The enzymatic synthesis of $IP_7$ is tightly linked to the cellular energetic status since the inositol hexakisphosphate kinases (IP6Ks, see Glossary) that are responsible for the bulk of $IP_7$ synthesis have a $K_m$ for ATP in the milimolar range [51, 52]. Thus, fluctuations in cellular ATP level are transduced into changes in $IP_7$ concentration. The availability of cellular phosphate, which affects ATP synthesis, will therefore also alter the cellular level of $IP_7$. Indeed two independent reports have observed that phosphate starvation in yeast induces a specific decrease in $IP_7$ levels [35, 36]. These fluctuations in $IP_7$ can signal the cellular phosphate status to a downstream signalling cascade

that, at least in part, is likely transduced by SPX domain-containing proteins. At the present time, apart from the yeast VTC complex, there is just one other study indicating that $IP_7$ regulates the function of an SPX containing protein. However, in this study the ability of the $IP_7$ isomer 1PP-$IP_5$, synthesized by a different class of enzymes, the PPIP5Ks, to regulate Pho81 interaction with Pho85-Pho80 was not attributed to the SPX domain [53]. Furthermore this study shows that $IP_7$ increases after phosphate starvation [54], which is in contrast with the current view and analysis [35, 36]. Apart from the yeast experimental model, there are no reported studies in other organisms in which altering the phosphate availability modulates PP-IPs cellular level.

**Inositol pyrophosphates as metabolic regulators**

In humans, phosphate comprises 1% of the total body weight. Phosphate is essential for the mineralization of the bone; indeed 85% of phosphate localises in bone and teeth, complexed with calcium in the form of hydroxyapatite crystals or as amorphous calcium phosphate. Organismal phosphate homeostasis is primarily regulated, by different organs and at multiple levels, by vitamin D, parathyroid hormone, and fibroblast growth factor-23 (FGF-23) [55, 56]. The fact that alterations of human phosphate metabolism are implicated in several pathological states makes this topic very important. In depth discussing of organismal phosphate regulation is beyond the scope of the current essay. We will refer only to a genome-wide study aimed at identifying genetic variations associated with changes in human serum phosphate concentrations [57]. This study identified two independent single nucleotide polymorphisms (SNPs) at locus 6p21.31, localised within the first intron of one of the three enzymes responsible for $IP_7$ synthesis: *IP6K3* [57, 58]. While no functional studies were performed to understand the effect of these SNPs, their localization suggests that they might affect IP6K3 transcription.

The *IP6K2* gene was cloned almost 20 years ago, while searching for a novel mammalian intestinal phosphate transporter and was identified as PiUS (Phosphate inorganic Uptake Stimulator) [59]. When PiUS RNA was injected into *Xenopus* oocytes, it stimulated the cellular uptake of radioactive phosphate. A few years later it was discovered that PiUS was capable of converting $IP_6$ to $IP_7$ and renamed IP6K2 [52, 60]. Importantly, this ability of PP-IPs to control the uptake of phosphate is conserved in yeast. The IP6K null yeast *kcs1Δ* (see Glossary, Figure 3A), with undetectable levels of $IP_7$, exhibits a reduced uptake of phosphate from the culture medium [44]. While these studies clearly link PP-IPs with

phosphate entry into cells, it remains to be investigated if $IP_7$ regulates phosphate importers/exporters through their SPX domains or by other mechanisms. It is likely that injecting *Xenopus* oocytes with PiUS/IP6K2 mRNA would lead to an increase in $IP_7$ synthesis. The observed increase in phosphate uptake that was originally reported for PiUS/IP6K2 could be explained by an inhibitory effect of $IP_7$ on Xpr1, the only SPX domain-containing protein present in the frog genome (NP_001086930), homologue of the human phosphate exporter. Likewise, the low phosphate concentration in serum associated with specific SNPs could be a result of $IP_7$ action on Xpr1 when IP6K3 transcription is altered. However, until the regulation by $IP_7$ of the Xpr1 protein of either human or frog origin is properly studied, these arguments remain speculative.

The presence of a lone SPX domain-containing protein in metazoan genomes, Xpr1, is puzzling. Metazoan complexity would certainly require a multifaceted cellular phosphate homeostasis. For this reason complex organisms must possess alternative regulatory mechanisms that go beyond the regulation by PP-IPs of the single SPX domain-containing protein. Besides acting as SPX domain regulators, PP-IPs could play a major role in phosphate homeostasis due to their ability to control primary metabolism. To synthesize ATP the mitochondrial ATP synthase requires a proton gradient, ADP, and phosphate. In both *kcs1*Δ yeast and in mouse embryonic fibroblasts derived from ip6k1 knockout mice (*ip6k1*$^{-/-}$), two systems with scarce PP-IPs synthesis, there is a remarkable increase in ATP levels even if the mitochondria are found to be dysfunctional [61]. Mitochondrial metabolic dysfunction has also been reported for the *kcs1*Δ strain of the pathogenic basidiomycete *Cryptococcus neoformans* [62]. Mitochondria, by being able to pump protons, are an important component of the emerging "metabolic" signal mediated by cytosolic pH. A genome-wide screening of *S. cerevisiae* knockout mutants revealed that *kcs1*Δ has one of the lowest cytosolic pH and its metabolic rate is virtually unaffected by lowering medium pH [63]. With this in mind, the altered mitochondria functionality of *ip6k1*$^{-/-}$ mice might explain the inability of these mice to become obese [64]. Further evidence of the evolutionarily conserved ability of PP-IPs to regulate primary metabolism comes from the recent metabolomics analysis of the unicellular green alga *Chlamydomonas reinhardtii*, which demonstrated the importance of PP-IPs in regulating tricarboxylic acid (TCA) cycle and fatty acid synthesis [65] Interestingly, *kcs1Δ* yeast, while possessing an adenylate energy charge close to 1, also have an absolute increase in the three adenine nucleotides (AMP, ADP and ATP) [61]. The nucleotide pools might therefore play an important, but poorly characterised, role in buffering cellular phosphate.

ATP and GTP, which are present at milimolar concentrations, have three phosphate groups each and thus small fluctuations in their cellular levels might substantially alter the cytosolic levels of free phosphate. The signalling as well as metabolic crosstalk between PP-IPs, nucleotide triphosphate and polyP, while still emerging, will be fundamental to appreciate the role of phosphate on cell homeostasis.

**Inorganic polyphosphate as a eukaryote phosphate buffer**

A systematic screening of yeast mutants for polyP levels revealed an interdependence with primary metabolism [66]. This linear phosphate polymer (Figure 3C) contains four to several hundred phosphate residues linked by "high-energy" phosphoanhydride bonds [11, 12]. The abiotic synthesis of polyP by volcanic activity has given rise to an exciting theory: that polyP hydrolysis could have driven early metabolism and preceded ATP as energetic molecule [67]. PolyP is ubiquitously present in all living organisms from bacteria to mammals and has many specific functions. These include: regulation of pathogenicity of human parasites [68]; it functions as a chaperone, helping protein folding [69]; and it drives a new post-translational protein modification, polyphosphorylation, in which polyP is covalently attached to a lysine residue [70]. However, intrinsic to its polymeric nature (Figure 3C), polyP also represents an intracellular phosphate buffer since its synthesis and degradation consumes and releases free phosphate. Furthermore, polyP also functions as a chelator of metal ions, thereby regulating cellular cation homeostasis. Clearly, understanding the regulation of polyP metabolism is instrumental to appreciating phosphate homeostasis.

Inorganic polyphosphate metabolism is in yeast regulated by PP-IPs, that activate the polyP-synthesizing Vtc4 by binding to its SPX domain ([36] see above). However, it is not completely clear if this regulation goes beyond the yeast experimental model. Vtc4-like proteins have been identified in some parasites of the *Trypanosomatida* order. In *Trypanosoma brucei*, a TbVtc4 conditional knockout is considerably less virulent in mice and shows a 35% decrease in short chain polyP species with no significant changes in the long chain polyP [71]. Therefore another uncharacterized enzyme able to synthesize polyP in *T. brucei* must exist.

Besides the Vtc4-like enzymes, polyphosphate kinase (PPK1), an enzyme of bacterial origin, has been characterized in the social amoeba *Dictyostelium discoideum*. This PPK1 enzyme, unlike Vtc4, lacks a SPX domain and so is not likely directly activated by PP-IPs. Therefore, it remains to be seen if PP-IPs somehow regulate polyP synthesis in *D. discoideum*. A recent report suggests that the IP6K null amoeba (ip6ka), with low level of

PP-IPs [72], has normal levels of polyP during vegetative growth but fails to properly accumulate polyP during development [73]. However, these data are in contrast with our own analysis where we observed normal polyP levels in vegetative cells and normal accumulation throughout development in an ip6ka knockout line (Thomas Livermore, PhD thesis, University College London, 2016). This discrepancy is likely due to the different assays used to quantify polyP: namely the direct visualisation of polyP on gel, or by measuring polyP-induced DAPI (4',6-Diamidino-2-Phenylindole) red fluorescence shift [74]. The latter assay has pitfalls as other phosphate-rich molecules, such as IPs [75] and amorphous calcium-phosphate [76], induce the same type of fluorescence. Thus, unlike in yeast, the direct control of polyP synthesis by PP-IPs is not seen in *D. discoideum*.

However, in mammalian cells, the DAPI-based assay was complemented by other indirect lines of investigation suggesting an interdependence between PP-IPs synthesis and polyP cellular accumulation [77]. Because mammals lack both Vtc4 and PPK1 enzymes, the pathways of polyP synthesis in higher eukaryotes are yet to be discovered, which prevents in depth studies on polyP regulation by PP-IPs and on the effects of polyP synthesis on phosphate homeostasis and primary metabolism.

Studies of *D. discoideum* polyP metabolism revealed a dramatic accumulation, more than one hundred fold, of polyP during development [78]. This polyP accumulation in the spores resembles $IP_6$ build-up in plant seeds [79]. Since $IP_6$ is important in supplying phosphate and cations during plant seed germination [80, 81], polyP could play similar roles during amoeba spore germination. In fact, the ppk1 null amoeba, which has undetectable levels of polyP and is unable to accumulate polyP during development, showed reduced germination efficiency. Analysis of the ppk1 null amoeba has allowed us to further demonstrate the interdependence between polyP and cellular energetics. This strain shows reduced general fitness and low growth rate which can be explained by a substantial decrease in cellular ATP level in vegetative growing cells. Importantly, the absence of an increase in polyP during development in ppk1 mutant cells is balanced by an increase of both ATP and PP-IPs [78]. Thus *D. discoideum* data are supportive of a model in which there is a functional interplay between PP-IPs, ATP and polyP (Figure 4).

**Concluding Remarks**

The recent identification of the SPX domain as a PP-IPs sensor offers unique opportunities to understand how PP-IPs signalling works and how it regulates phosphate

homeostasis. However, further work is required to fully appreciate the importance of such regulatory mechanisms (see Outstanding Questions).

Cellular phosphate homeostasis is obviously closely related to bivalent cation metabolism. Thus studying the metabolism and turnover of phosphate-rich molecules has far-reaching implications, since it will not only affect free phosphate levels but also the physiology of important cations such as magnesium and calcium. The PP-IPs ability to influence phosphate homeostasis could have represented one of the initial features that lead to the evolution of the complex $IP_3$-regulated calcium signalling pathway present in metazoans [82]. Several lines of evidence have suggested that in today's eukaryotic cells there is a reciprocal relationship between PP-IPs, ATP and polyP. Their relative abundance, synthesis and reciprocal regulation are connected, affecting cellular phosphate homeostasis and thus primary metabolism (Figure 4). This hypothesis is based on primary data collected in the yeast and amoeba experimental models, and requires further experimental evidence to be validated in other organisms. However, studying the relationship between IP-IPs, ATP and polyP has given us, and will certainly continue to give us, important insights on the central mechanisms of cell regulation.

## Glossary

**EXS domain**: Protein region named after the ERD1/XPR1/SYG1 proteins that is predicted to contain several transmembrane helices forming a channel to transport phosphate.

**Inositol hexakisphosphate kinases (IP6Ks/Kcs1)**: Enzymes primarily responsible for the conversion of $IP_6$ (inositol hexakisphosphate) to the isomer 5PP-$IP_5$ of $IP_7$ (diphosphoinositol pentakisphosphate). In mammalian cells there are three IP6K enzymes, IP6K1-2-3, whereas in yeast there is a single enzyme called Kcs1.

**Inorganic polyphosphate (polyP)**: A linear polymer of four to hundreds of phosphate residues linked by high-energy phosphoanhydride bonds, ubiquitously present in living organisms.

**Inositol polyphosphates (IPs)**: Refers to the family of water soluble inositol phosphates, from inositol monophosphate (IP) to the fully phosphorylated inositol hexakisphosphate ($IP_6$). In the context of this review we exclude from this nomenclature the inositol pyrophosphates.

**Inositol pyrophosphates (PP-IPs)**: High-energy molecules comprising seven ($IP_7$) or eight ($IP_8$) phosphate groups attached to the six-carbon *myo*-inositol ring, therefore possessing one or two pyrophosphate moieties. The correct nomenclature is diphosphoinositol-pentakisphosphate for $IP_7$, also called PP-$IP_5$; and diphosphoinositol-tetrakisphosphate for $IP_8$, also called $(PP)_2$-$IP_4$. These molecules are present in all eukaryotic cells and with signalling functions that are starting to be understood.

**SPX domain**: A ~180-~400 amino acid long domain named after the SYG1/Pho81/XPR1 proteins. This domain is mostly found at the amino terminus of proteins primarily involved in the regulation of phosphate metabolism.

**Xpr1**: Multi-pass membrane protein with an N-terminal SPX domain and a C-terminal EXS-domain. Originally identified as the cell-surface receptor for xenotropic and polytropic murine leukemia retroviruses (X- and P-MLV), hence the name Xenotropic and Polytropic Retrovirus Receptor 1, it was later found to be involved in phosphate homeostasis by mediating phosphate export from the cell.

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**Box 1: PHO regulon of *Saccharomyces cerevisiae***

In this yeast the transcription factor Pho4 plays a key role in the PHO regulon. In phosphate-rich conditions, Pho4 is phosphorylated by the cyclin-CDK (cyclin dependent kinase) complex Pho80-Pho85, that is in turn regulated by the CDK inhibitor Pho81. Phosphorylated Pho4 localises to the cytoplasm. Conversely, under phosphate starvation Pho4 is dephosphorylated and translocates to the nucleus, where it induces the transcription of a set of genes, encoding transporters and phosphatases, responsible for acquiring phosphate from the environment.

**Box 2: Inositol polyphosphates**

Inositol polyphosphates (IPs) are highly phosphorylated molecules containing a 6-carbon inositol ring that can be sequentially phosphorylated (Figure 3A). In *S. cerevisiae* the

synthesis of these molecules starts with the hydrolysis of the membrane phosphoinositide $PI(4,5)P_2$ ($PIP_2$) by Plc1, generating $I(1,4,5)P_3$ ($IP_3$). $IP_3$ is converted to $IP_4$ by two enzymes, the $IP_3$-3Kinase ($IP_3$-3K) or by the inositol polyphosphate multikinase Arg82, which also converts $IP_4$ to $IP_5$. The inositol pentakisphosphate kinase Ipk1 phosphorylates $IP_5$ to the fully phosphorylated and most abundant of the inositol species, $IP_6$ (inositol hexakisphosphate or phytic acid). Despite being fully phosphorylated, $IP_6$ can still be metabolized to generate the inositol pyrophosphate (PP-IP) $IP_7$, a molecule with one pyrophosphate moiety, by inositol hexakisphosphate kinase Kcs1. $IP_8$ is the most phosphorylated of the inositol species described to date and is generated through the phosphorylation of $IP_7$ by the diphosphoinositol pentakisphosphate kinase Vip1 (PPIP5Ks).

## Figures Legend

**Figure 1. Architecture of proteins containing SPX domain of yeast, plant and human.**
The domain architecture of SPX domain (PF03105)-containing proteins are depicted; these were determined using the Pfam protein families database website (http://pfam.xfam.org) [83]. In some cases, manual analysis of the sequence was performed to identify the full extent of the SPX regions. This was necessary when Pfam analysis revealed a partial or fragmented SPX region, since the SPX domain is defined by three regions of homology intercalated by non-homologous sequences (see Figure 2). When more than one protein possesses the indicated domain organization, the structure of the first protein is portrayed. This analysis revealed that the always N-terminus localised SPX domain can be associated with: the VTC domain (PF09359) that, in Vtc4, is catalytically responsible for polyP synthesis; the DUF202 domain (PF02656) that defines the putative membrane region; the ankyrin repeat, ANK (PF00023), a domain usually involved in protein–protein interactions; the CitMHS domain (PF03600) of the citrate transporter, similar to the sodium:sulfate symporter, representing the transmembrane region of the phosphate transporter; the GDPD domain (PF03009) from the glycerophosphoryl diester phosphodiesterase family, involved in lipid metabolism; the EXS domain (PF03124), a region containing several predicted transmembrane helixes that likely form a phosphate channel; the Really Interesting New Gene or RING domain (PF00097), representing a zinc finger-type structural domain; and the major facilitator superfamily MFS domain (CL0015), representing one of the best characterised families of membrane transporters.

**Figure 2. SPX domain organization.**
SPX domain sequence alignments from four *S. cerevisiae* proteins (Vtc2, Pho90, Pho91, Syg1), four *A. thaliana* proteins (SPX1, PHO1, NLA, SPX-MSF1), and one *H. sapiens* (Xpr1). These alignments include three homology regions (green boxes) as defined by the SPX domain Pfam entry PF03105, and an additional C-terminus amino acid sequence, as defined in the construct prepared to crystallise Vtc2 [35]. This analysis was performed using the Cobalt program (constraint-based multiple alignment tool) from the NCBI web page (https://www.ncbi.nlm.nih.gov/tools/cobalt/). The amino acid columns in red indicate highly conserved amino acid positions (Cobalt conservation setting, 3 bits). The yellow boxes define the six helical regions as identified in the resolved Vtc2 structure [35]. While the first SPX

homology region is defined by two alpha helices, homologous domains two and three substantially overlap with only alpha helix three and four, respectively. The position of the three amino acids defining the Phosphate Binding Cluster, one tyrosine (Y) and one lysine (K), localised on the second alpha helix, and one lysine localised in alpha helix four are highlighted in bold and by an asterisk (*). Similarly highlighted in bold and by the hash symbol (#) are the three lysine residues on the fourth alpha helix that define the Lysine-Binding Cluster. The resolved SPX domain crystal structures revealed that these two clusters, which together define the inositol phosphate binding region, are in the vicinity of each other as represented in the topology diagram of the Vtc2 structure (left).

**Figure 3. Inositol pyrophosphate biosynthetic pathway and $IP_7$ and polyP structures**

A simplified PP-IPs biosynthetic pathway is depicted in (A). The yeast enzymes are represented in blue while the respective mammalian enzymes are listed in red. In *S. cerevisiae* the synthesis of PP-IPs begins with the hydrolysis of the membrane phosphoinositide $PI(4,5)P_2$ ($PIP_2$) by Plc1 (homologous to mammalian Phospholipase C; PLC), generating $I(1,4,5)P_3$ ($IP_3$). This is converted to $IP_4$ by the $IP_3$-3Kinase ($IP_3$-3K) or by the inositol polyphosphate multikinase Arg82 (IPMK in mammals), which also converts $IP_4$ to $IP_5$. This is subsequently metabolised by the inositol pentakisphosphate kinase Ipk1 (homologous to IP5K). The fully phosphorylated $IP_6$ is metabolized by inositol hexakisphosphate kinase Kcs1 (homologous to IP6Ks) to generate $IP_7$ that can be further phosphorylated to $IP_8$ by the diphosphoinositol pentakisphosphate kinase Vip1 (PPIP5Ks). Additionally, inositol pentakisphosphate, $IP_5$, can be metabolised by the IP6K enzymes to the inositol pyrophosphate PP-$IP_4$. The structure of the prototypical inositol pyrophosphate $IP_7$ is represented in (B). This particular isomer 5PP-$IP_5$ is synthesized by the IP6Ks [84] while the PPIP5Ks are able to generate a pyrophosphate at position one [85]. In (C) the structure of inorganic polyphosphate (polyP) is represented, where ‘n’ can have a value of two to several hundred. The carbon is represented with white, the phosphorus with red, and the oxygen with green circles respectively.

**Figure 4. Proposed model for PP-IPs, ATP and polyP interplay.**

The three phosphate rich molecules ATP, polyP and PP-IPs may regulate their relative abundances through signalling events (black arrows). One such event is the ability of PP-IPs

to stimulate yeast polyP synthesis by binding the SPX domain of Vtc4 [35]. The polymeric polyP can itself participate in signalling events regulating ATP and PP-IPs through its protein chaperone ability [69], and/or by driving lysine polyphosphorylation [70]. The ability of PP-IPs to regulate ATP levels [61] could represent another regulatory event. Between these molecules, aside from these signalling actions, there are metabolic interconnections (coloured dashed arrows). Free phosphate ($P_i$) is obviously at the centre of such flux of phosphate groups. Thus, to fully appreciate phosphate homeostasis and metabolism regulation we must understand the PP-IPs, ATP and polyP relationships more deeply.

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***Saccharomyces cerevisiae***

Vtc2; Vtc3; Vtc4


Vtc5


Pho81


Pho87; Pho90; Pho91


Gde1


Syg1


***Arabidopsis thaliana***

SPX1-4


NLA; NLA2

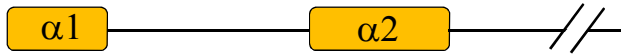

SPX-MSF1-3


PHO; PHO1-10


***Homo sapiens***

Xpr1

Homology region 1

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Yeast Vtc2      1    -MLFGVKLANEV---YPPWKG-SYINYEGLKKFLKEDSVKDG- [ 6]    43
Yeast Pho90     1    -MRFSHFLKYNA---VPEWQN-HYMDYSELKNLIYTLQTDELQ [90]    128
Yeast Pho91     1    -MKFSHSLQFNS---VPEWST-KYLAYSQLKKLIYSLQKDKLY [24]    62
Yeast Syg1      1    -MKFADHLTESA---IPEWRD-KYIDYKLGKKKLRRYKEKLDA [50]    84
Human XPR1      1    -MKFAEHLSAHI---TPEWRK-QYIQYEAFKDMLYSAQD---- [13]    47
Plant SPX1      1    -MKFGKSLSNQIEGTLPEWQD-KFLSYKELKKRLKLIGS---- [15]    52
Plant PHO1      1    MVKFSKELEAQL---IPEWKE-AFVNYCLLKKQIKKIKTSRKP [68]    107
Plant NLA       1    -MKFCKKYEEYM-GGQKEKKNLPGVGFKKLKKILKRCRRNHVP [13]    52
Plant SPX-MSF1  1    MVAFGKKLKERS---IQEWQG-YYINYKLMKKKVKQYSR---- [ 7]    42
                                        *   *
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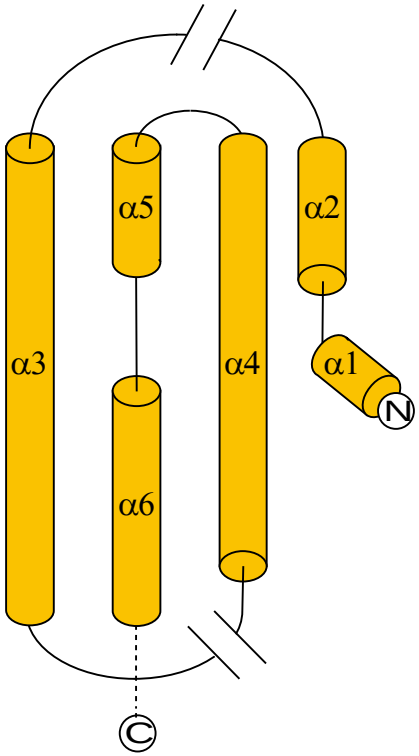


Homology region 2


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Yeast Vtc2      44   -RWDDSDESKFVEELDKELEKVYGFQLKKYNNLMERLSHLEKQTDTEAAIK---------          93
Yeast Pho90     129  ENLQLDTYDTFVGDLTAEKQKVDDFY--KRTEAKFYDKFDALVKDLKKIGVIEYDIDDDT [48]    235
Yeast Pho91     63   SPDDQFYISKFVAALNQELKKIDKFYISQETGLIANYNELKDDVMELENTNKATQLFNQQ [81]    203
Yeast Syg1      85   LQREFVADFIEDISFQLSKCNEFYLWLLKECDKKFEVLQSQLHYYSLQKNYERDNLNR-- [104]   250
Human XPR1      48   KRYFAKFEEKFFQTCEKELAKINTFYSEKLAEAQRRFATLQNELQSSLDAQKESTGVTTL [17]    124
Plant SPX1      53   SVGISKEEINFIQLLEDELEKFNNFFVEKEEEYIIRLKEFRDRIAKAKDSMEK-------          105
Plant PHO1      108  LFSEEDEVKVFFARLDEELNKVNQFHKPKETEFLERGEILKKQLETLAELKQILSDRKKR [104]   281
Plant NLA       53   SRECPVCDGTFFPELLKEMEDVVGWF--NEHAQKLLELHLASGFTKCLTWLRGN------          106
Plant SPX-MSF1  43   --ERRHVLKDFSRMLDNQIEKIALFMLEQQGLLASRLQTLRGSHDALQEQPDIS------          94
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Homology region 3


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Yeast Vtc2      94   ALDADAFQRVLEELLSESTELDNFKRLNFTGFAKIVKKHDKLYPKYPSVKSLLEVR--    ------LKELPSHS   EEYSPLLYR[5]NILRSNFNTASEP  184
Yeast Pho90     236  SQKKSLLKKSIVNLYIDLCQLKSFIELNRIGFAKITKKSDKVLHLNTRTELIESEQFF[4]AFQAETIELLNSKI   SQLVTFYAR[5]H------------  326
Yeast Pho91     204  TQHRLSLKKRLISIYTQLSELKDFIELNQTGFSKICKKFDKSLNTNLKQNYLNYIKFH[2]VFNPATINRIQHHI   --TETILTY[2]--LNKGTRRP---  294
Yeast Syg1      251  TQARNLLSNAIIEYYLYLQLVKSFRDINVTGFRKMVKKFDKTCHTRELTTFMSYARTH[4]KHADANVQLVAQKM   QQ--ITSS-   QPTPTSELS----  341
Human XPR1      125  HRNIKDLKLAFSEFYLSLILLQNYQNLNFTGFRKILKKHDKILETSRGADWRVAHVEV   -APFYTCKKINQLI   SETEAVVT-   NELEDGDRQKAM-  215
Plant SPX1      106  ---MIKIRKEIVDFHGEMVLLENYSALNYTGLVKILKKYDKRTGDLMRLPFIQKVLQ-   -QPFYTTDLLFKLV   KESEAMLD-   QIFPANETESEII  193
Plant PHO1      282  QSAEKKIRSAFVELYRGLGLLKTYSSLNMIAFTKIMKKFDKVAGQNASSTYLKVVK--   RSQFISSDKVVRLM   DEVESIFT-   KHFANNDRKKAMk  372
Plant NLA       107  -----SRKKDHHGLIQEGKDLVNYALINAVAIRKILKKYDKIHESRQGQAFKTQVQKM[4]LQSPWLCELMAFHI[6]KESGATITS   ------------  192
Plant SPX-MSF1  95   --HMSYLKEEYRAVGQDLLKLLFFVEMNAIGIRKILKKFDKRFGYRFTNYYVKTRAN-   -HPYSELQQVFRHV   G-LGAVVGA[5]HELQNNQGSYLSI  188
                                                  #  #*  #
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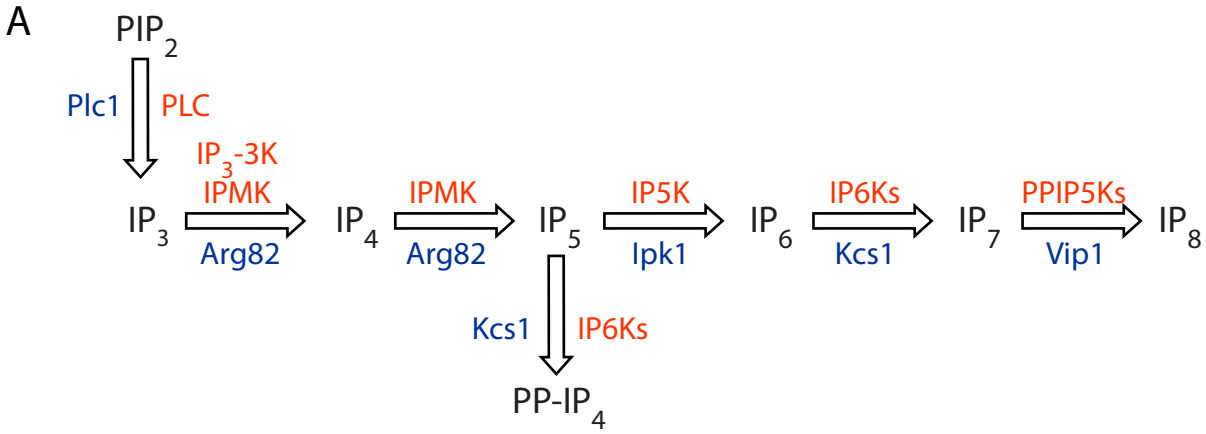
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IPMK
IP3
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IPMK
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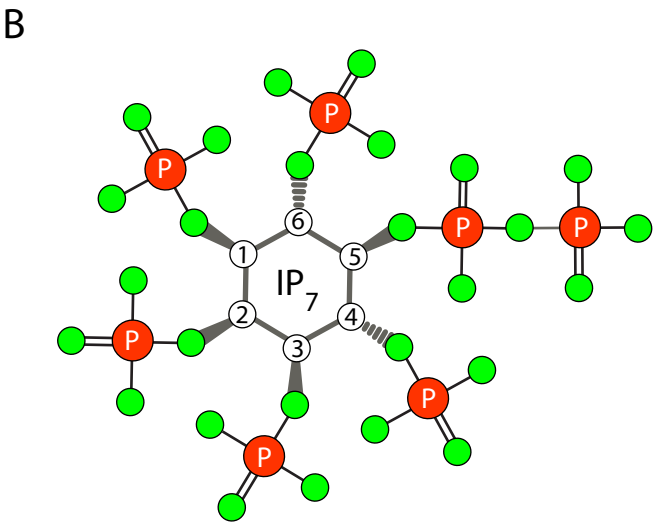
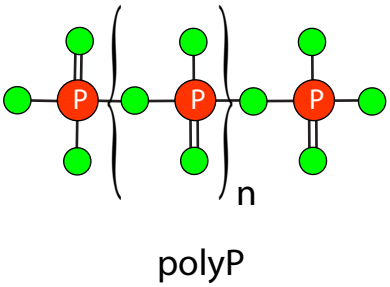
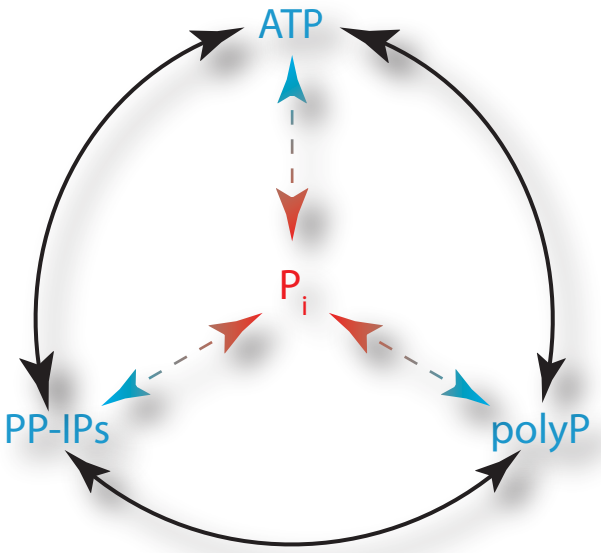
IP8
Kcs1
IP6Ks
PP-IP4


B
P
1
2
3
4
5
6
IP7


C
P
n
polyP


ATP
Pi
PP-IPs
polyP