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Review

Inositol pyrophosphates: metabolism and signaling

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Abstract. Inositol pyrophosphates belong to the diverse family of inositol polyphosphate species that have a range of signaling functions. Since the discovery of inositol pyrophosphates in the early 1990s, enormous progress has been achieved in characterising this class of molecules, linking their biological presence to a wide range of cellular functions, including vesicular trafficking, apoptosis, telomere maintenance and protein phosphorylation. The activity of inositol pyrophosphates appears to be related to their rapid turnover in cells and also to their pyrophosphate groups, which are considered to contain high-energy bonds. Together, these observations suggest that inositol pyrophosphates may represent a class of cellular messengers with basic and not yet fully characterised functions. This review aims at summarising the recent progress of our knowledge of this exciting class of molecules, from inositol pyrophosphate discovery to the description of their physiological functions.

Key words. Inositol pyrophosphates; signaling; kinase; phosphorylation; metabolism; phytic acid.

Introduction

Inositol polyphosphates occur ubiquitously in nature, the best characterised being inositol 1,4,5-trisphosphate (IP_3), a molecule that acts as a second messenger and triggers calcium release from intracellular stores [1, 2]. Inositol hexakisphosphate (IP_6, also known as phytic acid) is by far the most abundant inositol polyphosphate in eukaryotic cells, with a concentration in mammalian cells in the range of 10–60 µM [3, 4] and up to 0.7 mM in slime moulds [5]. IP_6 is known to have housekeeping functions as a phosphate storage molecule and as an antioxidant through its metal-chelating properties [6, 7]. More specific roles attributed to IP_6 as a signaling molecule involve the regulation of vesicular trafficking [7] as well as several nuclear events [7–10]. IP_6 is also the precursor of inositol pyrophosphates, in which the fully phosphorylated IP_6 ring is further phosphorylated to create molecules that contain one or two high-energy pyrophosphate bonds (fig. 1) [11, 12].

The best characterised inositol pyrophosphates are IP_7 and IP_8, which possess one and two pyrophosphate moieties, respectively [11, 12]. IP_7 and IP_8 are present in all eukaryotic cells analysed thus far, from the amoeba, *Dictyostelium discoideum*, to mammalian neurons; moreover, the enzymes responsible for their synthesis are highly conserved throughout evolution. Under most circumstances the concentrations and the relative ratios of IP_6, IP_7 and IP_8 are stable in eukaryotic cells. This stability is, however, a function of the rapid turnover of inositol pyrophosphates. As observed in cultured mammalian cells, the extent of this turnover is such that 20–30% of the large IP_6 reservoir is converted every hour to inositol pyrophosphates [11, 13, 14]. This dynamic turnover has led to the hypothesis that IP_7 and IP_8 may be responsible for many of the signaling-related functions previously attributed to IP_6, and that the turnover of inositol pyrophosphates may constitute a molecular switching activity [15].

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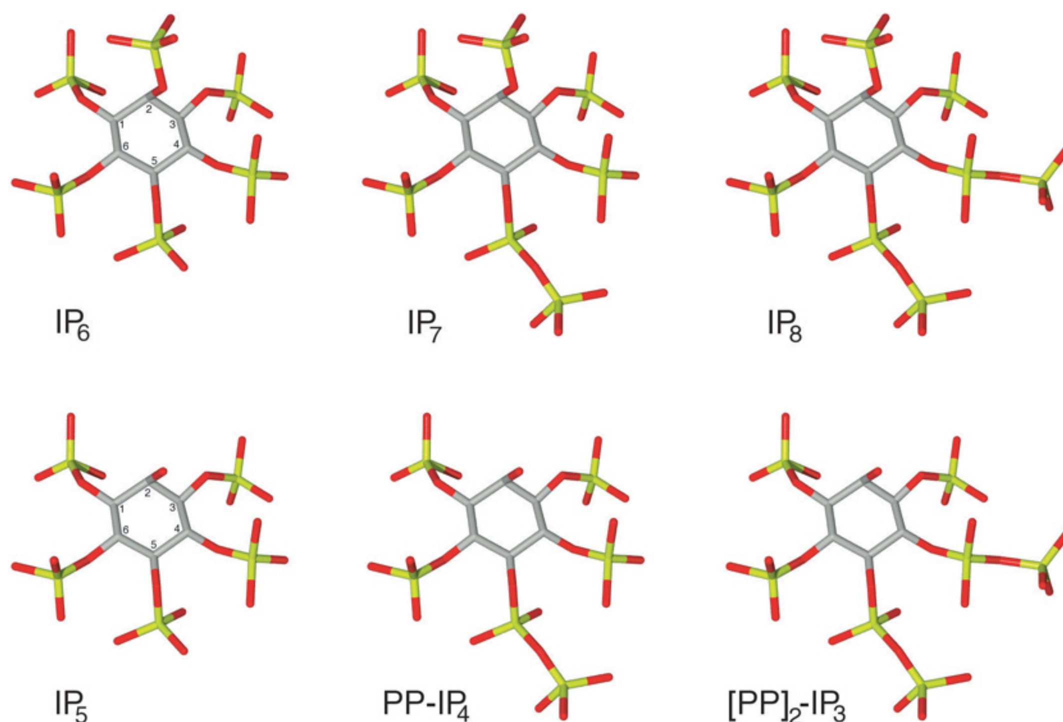

Figure 1. Inositol pyrophosphate chemical structure. The figure shows the structure of phytic acid with its pyrophosphate derivate IP_7 and IP_8, as well as IP_5, with the derivate pyrophosphates PP-IP_4 and $[PP]_2$-IP_3. The pyrophosphate group in IP_7 is attached to C-5, which represents the mammalian form of this molecule. The same is assumed for PP-IP_4. The two forms of IP_7 in *Dictyostelium* are present with the pyrophosphate group attached to C-5 or C-6, with this last isomer being predominant. No mammalian structure is known for the bis-pyrophosphorylate inositol IP_8 and $[PP]_2$-IP_3. Here, we represented the *Dictyostelium* IP_8 resolved structure, where pyrophosphate moieties are present in positions C-5 and C-6. In the figures, carbon, oxygen and phosphate atoms are coloured grey, red and green, respectively. The figures were made using *myo*-inositol as a starting molecule, where the C2 is in axial position, while the remaining carbons are in equatorial positions.

Several good reviews that focus on the pathways that ultimately lead to inositol pyrophosphate synthesis already exist [2, 15–17]. Here we particularly focus on the metabolic and signaling pathways associated with inositol pyrophosphates. As this review is aimed at an audience unfamiliar with inositol pyrophosphates, we have used a simplified nomenclature: IP_7 corresponds to diphosphoinositol pentakisphosphate and IP_8 to bis-diphosphoinositol tetrakisphosphate. An explanation of the scientifically correct chemical nomenclature is presented in table 1 and some of their chemical structures are represented in figure 1.

Table 1. Inositol polyphosphate nomenclature.

Name used	Alternative common names	Scientific nomenclature
IP_5	Ins(1,3,4,5,6)P_5	inositol pentakisphosphate
IP_6	$InsP_6$, Phytic acid	inositol hexakisphosphate
IP_7	$InsP_7$, PP-$InsP_5$	diphosphoinositol pentakisphosphate
IP_8	$InsP_8$, $[PP]_2$-$InsP_4$, 2[PP]-$InsP_4$	bis-diphosphoinositol tetrakisphosphate
PP-IP_4	PP-$InsP_4$	diphosphoinositol tetrakisphosphate
$[PP]_2$-IP_3	$[PP]_2$-$InsP_3$	bis-diphosphoinositol trisphosphate
PP-IP_3	–	diphosphoinositol trisphosphate
$[PP]_2$-IP_2	–	bis-diphosphoinositol bikisphosphate

The left column contains the inositol polyphosphate names used in this review. We simplified the suffix for inositol from 'Ins' to 'I', as done previously. We only refer to the most common of the six possible IP_5 isomers, as shown in the table. Diphospho- represents the scientific correct nomenclature to indicate a pyrophosphate moiety; however, the prefix 'pyro' better translates the 'high energy' characteristic of these molecules.

Inositol pyrophosphate discovery

The name inositol derives from the Greek word *ino* meaning 'sinew', which reflects its role as a vitamin. But we can also offer a different interpretation based on the molecular 'strength' and the metabolic stability of the inositol ring inside cells. Eukaryotic cells readily take up inositol from their extracellular media without any significant degradation. This property of inositol greatly facilitates the labeling and detection, usually with tritiated-inositol

([^{3}H]inositol), of the different inositol polyphosphate species. Inside the cell, the [^{3}H]inositol enters into a complex metabolic system that includes phosphorylation and dephosphorylation reactions and even the formation of lipid-bound inositol molecules. Since a full description of the pathways involved in inositol polyphosphate metabolism is beyond the scope of this review, we direct the readers to the following references [2, 15–17]. After several hours or days, depending on the experimental system, the metabolism of [^{3}H]inositol reaches equilibrium, and all the inositol polyphosphates species are in balance with [^{3}H]inositol. The resulting inositol polyphosphates species are acid extracted and separated by strong anion exchange high-performance liquid chromatography (HPLC). Examples of the [^{3}H]inositol species eluted by anion exchange from mammalian neurons and yeast are shown in figure 2A and B, respectively.

Although researchers in the early 1990s had observed inositol species that we now classify as pyrophosphorylated [18–20], it is generally acknowledged that inositol pyrophosphates were discovered in 1993 by two independent research groups [11, 20] characterising a chromatographic peak that eluted after, and therefore more anionic than, IP_6. Len Stephens and co-workers, following on from their research on the stepwise phosphorylation of inositol in *Dictyostelium* [20], isolated inositol pyrophosphate species [12]. To confirm the pyrophosphate component of these species, they used a combination of ^{1}H-^{31}P nuclear magnetic resonance (NMR) and enzymatic analyses. They also showed that *Dictyostelium* cell lysates utilised ATP to synthesise inositol pyrophosphates [12]. In mammalian cells, Stephen Shears and co-workers, using fluoride anions as metabolic traps to inhibit phosphatases, were also able to detect the ATP-dependent formation of [^{3}H]inositol pyrophosphates [11]. These two studies identified three inositol pyrophosphate species: IP_7, IP_8 and PP-IP_4. At present no inositol pyrophosphates more anionic than IP_8 have been detected under normal physiological conditions. However, lesser phosphorylated forms of inositol pyrophosphates, including species putatively identified as PP-IP_3 and $[PP]_2$-IP_2, have been seen in chromatographic separations from yeast strains defective for enzymes involved in the inositol polyphosphate metabolic pathway [21]. The discovery of these molecules suggests that the inositol pyrophosphate family can be expanded with the identification of other 'lower' phosphorylated inositol pyrophosphates.

The total concentration of inositol pyrophosphate species in cells is in the sub-micromolar range, accounting for less than 5% of the IP_6 concentration. However, slime moulds such as *Dictyostelium discoideum* have IP_7 and IP_8 levels in the 100–200 μM range [22]. So great is their concentration that the IP_7 and IP_8 species have been purified from *Dictyostelium*, and their structures elucidated by ^{1}H-^{31}P 2D NMR spectroscopy [23] and isomeric analysis using stereospecific inositol phosphatases [24]. These structures revealed a single IP_8 isomer present as 5,6-$[PP]_2$-IP_4 and two IP_7 isomers, 5-PP-IP_5 and 6-PP-IP_5 [25], with a relatively higher abundance *in vivo* of the 6-pyrophosphorylated species. It would be wrong, however, to assume that all IP_7 and IP_8 species have the same chemistry. For instance, an IP_8 species from another amoeba, *Polysphondylium pallidum*, has been identified as 1,5-$[PP]_2$-IP_4 [26], and an IP_7 species from *Entamoeba histolytica* as 5-PP-IP_5 [27]. In mammalian cells the structure of only one IP_7 species has been studied and shown to be 5-pyrophosphorylated [25]. While it is well known that *myo*-inositol is the most abundant inositol isomer found in organisms, pyrophosphate derivatives of other isomers exist. For instance, *Entamoeba histolytica* possesses two *neo*-inositol pyrophosphate species, 2-PP-*neo*-IP_5 and 2,5-$[PP]_2$-*neo*-IP_4 [27]. Thus, despite the great challenges in purifying the quantities of inositol pyrophosphate species necessary for structural analyses, these results indicate that the inositol pyrophosphates are structurally diverse and may also suggest that organisms possess multiple inositol pyrophosphate species with varied functions.

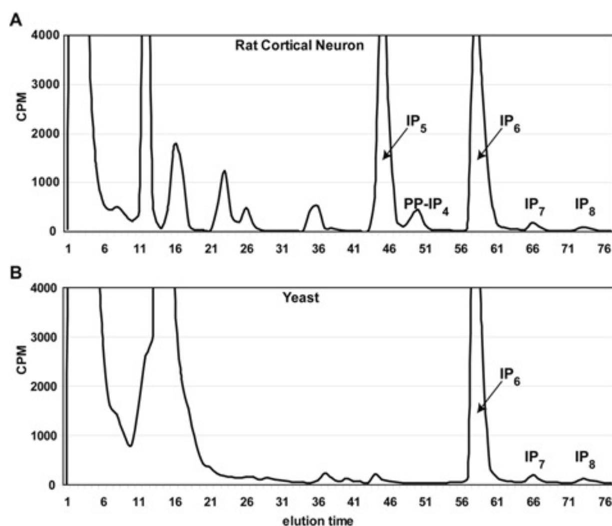

Figure 2. The HPLC analysis of [^{3}H]inositol-labeled cells. (*A*) Primary rat cortical neurons were labeled for 5 days and subsequently the soluble inositols were acid extracted and analysed by strong anion HPLC as previously described [82]. (*B*) Yeast *Saccharomyces cerevisiae* were labeled overnight to steady state with [^{3}H]inositol. After breaking the cell with glass beads, the soluble inositols were extracted, and HPLC was separated [60]. The elution of inositol polyphosphate standards is indicated.

Metabolism of inositol pyrophosphates

Anabolism

Simultaneous with the discovery of inositol pyrophosphates, it was observed that they are rapidly metabolised

in cells. Shears and co-workers studying inositol pyrophosphate metabolism in a variety of mammalian cells found that about 20% of the IP_5 pool and 50% of the IP_6 pool are converted within 1 h to the pyrophosphate derivatives $PP\text{-}IP_4$ and IP_7, respectively [11]. Similarly, in primary hepatocytes the pool of IP_7 is turned over 10 times every 40 min, compared with 1/10th of the pools of IP_6 [13]. In mammalian cells, IP_8 levels are sensitive to a variety of pharmacological agents [28]. A single study on the variations of inositol polyphosphates with respect to the cell cycle in WRK-1 mammalian cell has shown a twofold difference in IP_7 levels between the G1 and S phases of the cell cycle [29]. Yet perhaps the most dramatic modulation of inositol pyrophosphate levels occurs during the life cycle of *Dictyostelium* and *Polysphondylium*; here, inositol pyrophosphate concentrations increase considerably as the organisms reach the stationary phase, but disappear with sporulation [22], cyclic AMP (cAMP) signaling has been implicated in these changes [30].

The first enzyme able to synthesise inositol pyrophosphates was purified to homogeneity from rat brain in Solomon Snyder's laboratory [31]. Inositol hexakisphosphate kinase (IP6K) is able to convert IP_6 plus ATP to IP_7 and ADP; ATP cannot be substituted by other nucleotides such as CTP or GTP [31]. The same laboratory later cloned the enzyme and initially characterised one yeast and two mammalian proteins with inositol hexakisphosphate kinase activity, called KCS1 (Kinase C Suppressor 1, also known as yIP6K), IP6K1 and IP6K2, respectively [32]. Independently, a second group while characterising an enzyme with sequence homology to $I(1,4,5)P_3$-3Ks (IP_3-3K) – a family of enzymes that converts $I(1,4,5)P_3$ to $I(1,3,4,5)P_4$ – showed that IP6K2 had previously been identified as a protein called PiUS (Phosphate inorganic Uptake Stimulator), which is involved in the uptake of inorganic phosphate [33]. Subsequently, a third mammalian gene, IP6K3, was cloned [34]. All the mammalian IP6Ks phosphorylate *in vitro* IP_6 to IP_7, IP_5 to $PP\text{-}IP_4$ and $[PP]_2\text{-}IP_3$ [34, 35].

The high conservation of IP6Ks throughout evolution (fig. 3) has facilitated the identification of IP6K enzymes from distant organisms, including *Dictyostelium discoideum* [30]. The cloning of IP6Ks also helped to identify an evolutionarily conserved family of inositol polyphosphate kinases known as inositol polyphosphate multi-kinases (IPMKs) [32]. Characterization of yeast IPMK, also known as Arg82, ArgRIII and IPK2, demonstrated that this enzyme phosphorylated a broad range of substrates to yield a variety of inositol polyposphate reaction products [15, 32, 36, 37]. Subsequently, the characterisation of IPMKs from other organisms has confirmed that substrate ambiguity is a general property of these enzymes [36–40], and that some IPMK proteins are able to synthesise inositol pyrophosphate species [38, 41, 42]. The use of recently discovered plant metabolites as specific inhibitors of IP_3-3K and IPMK [43] will clarify the role of these proteins in regulating cell functions.

Altogether, the IP6Ks, IMPKs and IP_3-3Ks belong to an inositol polyphosphate kinase superfamily, the IPKs (PFAM accession number PF03770), that evolved from a common ancestor (reviewed in [15, 17]). Phylogenetic analysis of their sequences predicts that IP6Ks arose first, followed by IPMKs, and lastly IP_3-3Ks (fig. 3). Experimental analysis of IPK enzymes from evolutionary distant organisms will be necessary to confirm this theoretical prediction. At present the structural information for this superfamily is limited to the crystal structures of the catalytic domains of a rat and human IP_3-3KA isoform [44, 45]. An alignment of this IP_3-3KA structure with the sequences of IP6Ks and IMPKs shows that these three families differ mostly with respect to their inositol binding domains. In IP_3-3KA, the inositol binding domain consists of a four α-helices, rich in basic residues and spans a region of 60 residues, while in both IP6Ks and IMPKs, the inositol binding domain is much shorter, spanning about 30 residues [44]. This structural information combined with the phylogenetic analysis of the IPK superfamily clearly supports the hypothesis that IP_3-3Ks are a recent evolutionary addition [33] that may have evolved in metazoans about the same time as IP_3 receptors [46]. While this raises intriguing questions regarding the evolution of inositol pyrophosphates as an event that preceded the development of inositol polyposphate signaling (fig. 3), it is worth noting that structurally the catalytic domains of IP_3-Ks minus their relatively small inositol binding domains are highly similar to the protein kinase superfamily [44]. Despite the low sequence homology between protein kinases and the IPK superfamilies, deeper sequence analysis may reveal that the protein kinase superfamily and the IPK superfamily share a common ancestor, as suggested [44]; otherwise they provide an extraordinary example of convergent evolution. As will be discussed in more detail later, inositol pyrophosphates have also been implicated in the phosphorylation of proteins. This raises the possibility of an evolutionary adaptation of the substrate specificity of a protein kinase for the synthesis of IP_7, which in turn is able to drive protein phosphorylation.

Recent reports suggest that other enzymes that do not belong to the IPK family also synthesise inositol pyrophosphates. York's group reported the presence of large amounts of IP_7 in yeast null for *kcs1Δ* and *ddp1Δ*, an enzyme that degrades inositol pyrophosphate (see below). The enzymatic activity responsible for this IP_7 synthesis has been named inositol diphosphoryl synthase 1 (IDS1) [47]. The same group has recently reported the existence of a second inositol pyrophosphate synthase gene product called IPS1 [21]. However, it can't be excluded that IDS1 and IPS1 represent the same gene product [21]. Additionally, Shear and co-workers using yeast lacking the

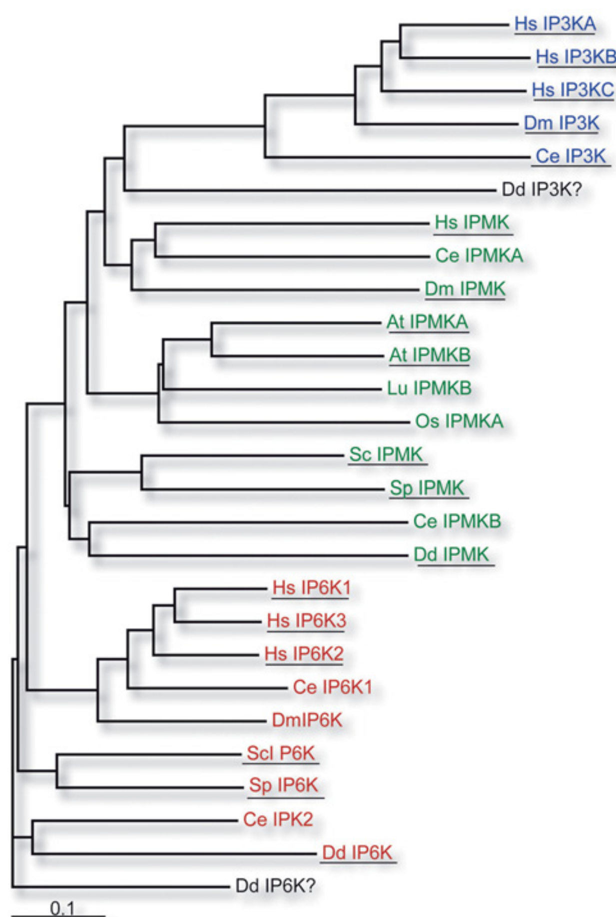

Figure 3. Phylogenetic tree of IPK proteins family members. Phylogram in which the branch lengths are proportional to the amount of inferred evolutionary change was generated with CLUSTAL W [104], using identity matrix and window size 1 from the European Bioinformatic Institute (EBI) website. The tree was generated utilising the most conserved domain of this protein family. We utilised 62 aa surrounding the inositol binding region PxxxDxKxG [17, 32, 105, 106], specifically from 14 aa before to 40 aa after. The IP6K proteins are represented in red, the IPMK enzymes are indicated in green and the IP_3-3K members are shown in blue. The black proteins represent two enzymes putatively classified as IP_3-3K and IP6K, but due to the particular divergent sequence and localisation on the tree, an analysis of their enzymatic properties is needed to confirm these classifications. Proteins that have been biochemically tested to be a specific member of each sub-family of enzyme are underlined. The following abbreviations are used: At, *Arabidopsis thaliana*; Ce, *Caenorhabditis elegans*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Hs, *Homo sapiens*; Lu, *Linus utilissimum*; Os, *Oryza sativa*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

IP6K activity (*kcs1*Δ) identified a novel IP_7-Kinase activity, which is able to specifically convert IP_7 to IP_8 [48]. This situation resembles the mammalian system, where the enzyme responsible for IP_8 synthesis, IP_7-Kinase, has been purified but not cloned [49].

Catabolism

At present, almost all the research on the catabolism of inositol pyrophosphates has focused on the MutT/Nudt/Nudix superfamily of phosphohydrolases. Shears's group originally purified and cloned an enzyme with inositol pyrophosphatase activity from rat brain and named this enzyme diphosphoinositol polyphosphate phosphohydrolase (DIPP) [50]. This led to the identification of a family of inositol pyrophosphatases which possess a conserved sequence, known as the MutT or Nudix motif, first identified in the *E. Coli* MutT protein involved in DNA repair [51]. The importance of the MutT motif for the inositol pyrophosphatase activity of these enzymes has been confirmed by site-directed mutagenesis [50, 52]. Each phosphohydrolase belonging to this superfamily has varied substrate specificity. In addition to recognising inositol pyrophosphate substrates, this family of phosphatases commonly degrade diadenosine polyphosphates (Ap_xA), molecules whose cellular roles are still uncertain, and sugar pyrophosphates, including phosphoribosylpyrophosphate [53], a substrate that regulates glycolysis and the synthesis of many molecules, including nucleotides. The overlapping specificity of the Nudix hydrolases has led to the speculation that there may be co-regulation of these substrates [51].

Studies in yeast strains altered by the deletion or addition of Nudix hydrolases have shown that the concentrations of inositol pyrophosphates and dideadenosine substrates do vary, but not necessarily in an easily explainable way. For instance, disruption of Aps1, both an inositol pyrophosphohydrolase and Ap5A/Ap6A hydrolase, in *Schizosaccharomyces pombe*, increased IP_7 concentrations threefold without affecting diadenosine polyphosphate concentrations [54]. Overexpression of Aps1, although resulting in 60% greater enzymatic activity in cell extracts, had no effect on inositol pyrophosphate levels but caused large increases in Ap5A concentrations [54]. The substrate ambiguity of the Nudix phosphohydrolases suggests that there may be some co-regulation between inositol pyrophosphates and nucleotide synthesis.

Inositol pyrophosphate functions

Inositol pyrophosphates are known to influence a variety of cellular functions. For simplicity, we review the physiological aspects affected by inositol pyrophosphates by dividing them with respect to the organism studied. However, when a specific function has been studied in different organisms we will discuss them together.

Inositol pyrophosphate in plants

In plants the inositol pyrophosphate precursor, phytic acid, is the major source of phosphorus in developing

seeds. Furthermore, because phytic acid is deposited in protein bodies as a mixed salt of mineral cations, it also represents the major mineral storage for K^+, Mg^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+} [6]. This property makes phytic acid the number one enemy of cattle producers because the chelation of mineral cations reduces their dietary availability and also contributes to the eutrophication of surface waters.

Forms of inositol more anionic than phytic acid have been reported in duckweed, *Spirodela polyrrhiza* [55], and barley [56, 57]. During turion formation in duckweed, low levels of one, and sometimes two, peaks more anionic than IP_6, possibly IP_7 and IP_8, have been observed. In this organism, the plant hormone abscisic acid leads to a dramatic increase in IP_6, while IP_7 and IP_8 levels remain unaltered or under the detection limit [55]. Studies in mature and germinating barley seeds have reported the presence of a species more anionic than IP_6, likely to be IP_7 [56, 57]. It has been suggested that in developing barley seeds, the low level of inositol pyrophosphates is a consequence of their rapid turnover rather than a reduced rate of synthesis [57].

Analysis of the completed *Arabidopsis thaliana* genome has revealed that it only possesses two members of the IPK superfamily (AtIPK2a and AtIPK2b), and although these proteins are able to metabolize several inositol polyphosphates and can be classified as inositol polyphosphate multi-kinase subfamily members (fig. 3), they do not synthesise inositol pyrophosphates [39, 40]. Therefore, and due to the lack of structural analyses of the putative IP_7 and IP_8 species present in plants, it will be essential to identify the kinase(s) that could be responsible for the conversion IP_6 to IP_7 and IP_8.

Inositol pyrophosphate signaling in yeast

Most of the early information about the function of inositol pyrophosphates has been obtained from genetic studies in the yeast *Saccharomyces cervisiae*. For example, a study by Huang and Symington on the mechanism underlying homologous recombination, which is the major mode of repairing double-stranded DNA breaks in yeast, identified a mutant with defects in protein kinase C1 (*pkc*1-4) [58]. This mutant had an increased recombination rate, which was completely reversed by a second mutation in a gene designated Kinase C Suppressor 1 (KCS1) [58]. Several years later we identified the protein encoded by KCS1 as the yeast homologue of mammalian IP6K (yIP6K) [32]. Subsequently, it was shown that IP_7 synthesised by KCS1 and not the KCS1 protein itself is required for controlling DNA recombination in *pkc*1-4 yeast [59].

The cloning of the different enzymes involved in inositol pyrophosphate biosynthetic pathways has helped to elucidate the role of this pathway in different organisms, with the yeast *Saccharomyces cerevisiae* selected as main experimental model in several laboratories. Yeast with the IP6K gene deleted (*kcs1*Δ) has been widely used to study inositol pyrophosphates and their kinases. It has been demonstrated by different groups that the enzymatic activity of KCS1 is important for inositol pyrophosphate synthesis, as *kcs1*Δ strains have absent or close to zero steady-state levels of IP_7 and IP_8 [35, 60]. The *kcs1*Δ cells are twice as large as wild-type, grow slower than wild-type and are growth impaired at 37 °C. Furthermore, *kcs1*Δ cells are also hypersensitive to salt stress but unaffected by osmotic challenge [61]. Complementation of *kcs1*Δ with wild-type KCS1 resulted largely in restoration of IP_7 and IP_8 levels and rescued several defects, whereas transformation with a catalytic inactive form of kcs1 did not rescue the mutant phenotypes. Interestingly, *arg82*Δ, which synthesises the precursor for inositol pyrophosphates, shows similar phenotypes as *kcs1*Δ strain. Overexpression of KCS1, in *arg82*Δ cells, partially overcame this metabolic block and rescued some phenotypic defects [61]. However, although the IP_7 level it is not rescued, there is production of some unusual inositol pyrophosphate species, such as PP-IP_2 [21], suggesting overlapping functions for the different inositol pyrophosphates species in cells.

The completion of the sequence of *Saccharomyces cervisae* genome enabled analysis of protein function and interactions on a genomic scale. In one such genome-wide screen aimed at examining putative protein-protein interactions, Uetz et al. identified KCS1 as an interacting partner of Bmh2p (yeast 14-3-3). Bmh2p is involved in cell differentiation, signal transduction, vesicular trafficking and chemotaxis [62]. A study in yeast based on a high-throughput mass spectrometric method to identify protein complexes also identified Bmh1p (yeast 14-3-3) as a binding partner of KCS1 [63]. Interestingly, two independent studies have found that the two 14-3-3 yeast proteins interact with KCS1. In yet another large-scale study in yeast, four independent approaches were applied to analyse uncharacterised proteins essential for viability but with unknown functions. Results from this study revealed that KCS1 interacts with a component of ubiquitin-protein ligase complex Cdc23p, the ubiquitin-conjugating enzyme Qri8p, and two uncharacterized proteins, Ydr131cp and Ydr267cp [64].

Recently, two independent studies proposed new roles for inositol pyrophosphates in the regulation of telomere length and in cell death [47, 65]. Telomeres consist of long, repetitive, protective DNA sequences at the end of chromosomes that prevent DNA degradation. Telomere removal results in activation of the DNA damage response pathway and to subsequent cell cycle arrest, senescence or cell death [66, 67]. In yeast, the two phosphatidylinositol 3-kinase-related kinase (PIKK) members, Mec1 and Tel1, are important for telomere mainte-

nance [68, 69]. We observed that *kcs1*Δ yeast are resistant to the lethal action of two known PIKK inhibitors, caffeine and wortmannin [65]. To find out whether inositol pyrophosphates influence signaling by members of the PI3K-related protein family, the telomere lengths in different yeast mutants were investigated. Telomere length is decreased in the *tel1*Δ strain [70]. In contrast, *kcs1*Δ showed increased telomere length. If KCS1 acts by generating inositol pyrophosphates that in turn antagonise Tel1 activity, then yeast with elevated levels of inositol pyrophosphates should have shorter telomeres. Indeed, *ipk1*Δ yeast, which have perturbed inositol phosphate metabolism but still form high levels of inositol pyrophosphates, have reduced telomere lengths. Furthermore, complementation of *kcs1*Δ and *ipk1*Δ yeast with the respective genes leads to the reverse of the phenotypes. Thus far, the regulation of telomere length appears to be the most important specific function of KCS1 in yeast nuclei. Several studies have proposed a role for inositol polyphosphates in controlling messenger RNA (mRNA) nuclear export, chromatin remodelling, transcriptional regulation, DNA repair and very recently even mRNA editing [8–10, 36, 71]. These studies conclude that yeast IPMK, synthesising IP_4, IP_5 [36] or the lipid $PI(3,4,5)P_3$ [72], play a key role in these regulatory processes, while the ability of KCS1 to synthesise inositol pyrophosphate is not required. These findings suggest that the inositol pyrophosphates have functions distinct to those of other inositol polyphosphates.

Evidence that inositol polyphosphates are involved in vesicle trafficking has been mounting over the last 15 years. In 1991 it was demonstrated that the binding of IP_6 to the clathrin complex AP2 [73, 74] contributes to the assembly of clathrin-coated vesicles which are involved in receptor-mediated endocytosis at the plasma membrane [75]. Subsequently, different groups identified several proteins involved in vesicular trafficking as binding partners of IP_6 and IP_7 *in vitro* [76–78]. Phenotypical characterisation of *kcs1*Δ yeast strains has revealed an altered vacuolar morphology with several smaller fragmented vacuoles [35] and the accumulation of membranous vesicular structures derived from the plasma membrane [60]. These vesicular structures were identified by electron microscopy as stacked cisternae reminiscent of the 'class E' multilamellar endosomal compartment [60]. Immunogold localisation, with antibodies against proteins associated with the endosomal membranes, confirmed that these membranes were aberrant endosomal intermediates [60]. Further evidence for the involvement of inositol pyrophosphates in vesicular trafficking arose with the observation that in *kcs1*Δ yeast the endocytic pathway is affected in several ways. The endosomes show slower transit, and instead of fusing to the vacuole, they form large multilamellar intermediates; also, ligand-dependent internalisation is slower and processing is abnormal [60]. Interestingly, *kcs1*Δ strains possess almost normal levels of IP_6, indicating a specific role only for inositol pyrophosphate in controlling vesicular trafficking. Two models have been proposed to explain these results. It is possible that, in the absence of IP_7 and IP_8, epsin N-terminal homology (ENTH) domain-containing proteins bind more efficiently to $PI(4,5)P_2$, increasing endocytosis [79]. Alternatively, interconversion between IP_6 and IP_7 might affect the conformation of clathrin-associated proteins. A more detailed description of the hypothesised inositol pyrophosphate molecular mechanism of action will be described in the next section.

Work on mammalian cells reinforces the connection between inositol pyrophosphate and vesicular trafficking. GRAB was identified in a yeast two-hybrid screen as an IP6K1 binding protein [80]. GRAB is a physiological guanine nucleotide exchange factor (GEF) for Rab3A that is involved in synaptical vesicle exocytosis. As a result of competition between IP6K1 and Rab3A for binding to GRAB, the ability of GRAB to promote GDP release from Rab3A was reduced in cells overexpressing IP6K1. This ability was shown to be IP_7 independent, since IP6K2, which shows a similar capacity of synthesizing IP_7 and does not bind to GRAB, is unable to block binding of GRAB to Rab3A [80]. Importantly, the catalytically inactive form of IP6K1 is still able to block the binding of GRAB to Rab3A [80], which weakens the authors' suggestion that the IP6K blockage of GRAB binding to Rab3A is linked to inositol pyrophosphate turnover.

Inositol polyphosphate functions in dictyostelium

Historically, the unicellular free-living amoeba *Dictyostelium discoideum* has been one of the most popular experimental models to study inositol polyphosphate signaling. Unlike any other organism studied to date, this amoeba contains very high amounts of IP_7 and IP_8, facilitating the discovery and first structural characterisation of the inositol pyrophosphates [12, 20]. In the vegetative state, inositol pyrophosphate levels are similar to the ratios observed in mammalian and yeast cells, corresponding to only 2–5% of the cellular IP_6 levels. However, IP_7 and IP_8 levels dramatically increase during chemotaxis and aggregation, events that are both dependent on cAMP signaling [30], reaching amounts comparable to IP_6 [22].

Screening of the *Dictyostelium* genome reveals the existence of at least four genes belonging to the IPK superfamily. One of these genes, Dictybase (http://dictybase.org/) gene name *I6KA,* was identified as an IP6K [30]. Although the kinase activity of this protein has not been tested *in vitro*, disruption of *I6KA* by homologous recombination dramatically decreases IP_7 and IP_8 levels, which could be restored to wild-type levels upon complementa-

tion. Phenotypically, the growth of the mutant cells was normal, as was the shape and size of their fruiting body. However, starvation-induced aggregation occurred more rapidly in the mutant than in wild-type amoeba, especially at the earliest stages. Moreover, mutant cells possess an increased sensitivity to chemotactants, responding more rapidly to low concentrations of cAMP gradients than wild-type cells. These results suggest an alteration of chemotactic pathways in the null mutants. The authors suggest that this phenotype could be a result of IP_7 ability to compete with $PI(3,4,5)P_3$ for the pleckstrin homology (PH) domain binding of CRAC (Cytosolic Regulator of Adenylyl Cyclase) [30]. Membrane translocation of CRAC mediated by the PH domain appears to be an essential step for directional sensing, since either decreasing the amount of $PI(3,4,5)P_3$, by disrupting PI3K genes, or abnormally increasing $PI(3,4,5)P_3$ levels by disruption of PTEN diminishes the ability of cells to orientate and to move in response to chemotactic gradients [81]. The absence of IP_7 in the null mutant would free the CRAC-PH domain to bind to $PI(3,4,5)P_3$, and its translocation to the membrane would thus increase.

Dictyostelium and other slime moulds have for many years been excellent experimental models, but we are still very much in the dark in regards to the most basic question: Why do they have special needs for enormous levels of inositol pyrophosphates?

Inositol pyrophosphate signaling in mammalian cells

The first study to demonstrate a close link between the turnover of inositol pyrophosphate and signal transduction events was conducted by Safrany and Shears [82]. By utilising DDT_1MF-2 smooth muscle cells as a model to determine how fluoride anions lower IP_8 formation, these authors showed that the metabolism of IP_8 is regulated by β_2-adrenergic receptors, but that IP_8 levels are not affected either by a reduction in ATP levels or by activation of phospholipase C [82]. Instead, the main mechanism by which fluoride modifies IP_8 levels is through augmented cyclic nucleotide levels, either by the phosphodiester inhibitor IBMX or after application of Bt2cAMP, Bt2cGMP or isoproterenol, which acts through the β_2-adrenergic receptor [82]. This effect was specific, as isoproterenol did not cause a drop in the levels of any other inositol pyrophosphate. The effect on IP_8 metabolism was unchanged by Protein Kinase A and G (PKA and PKG) inhibitors, suggesting that cAMP regulates IP_8 turnover independent of PKA or PKG signaling. Furthermore, changes in calcium concentrations did not affect IP_8 levels, confirming that cyclic nucleotide-mediated depletion in IP_8 levels is not caused by any significant rise in calcium entry [82]. Unfortunately, this interesting signaling pathway has not been further investigated.

Results are now emerging that environmental stress, including heat shock and hyperosmotic stress, raises IP_8 levels in mammalian cells. Two recent studies suggest that, unlike in yeast, osmotic stress affects inositol pyrophosphates in mammalian cells [28, 83]. After induction of osmotic challenge by sorbitol, there was a rapid accumulation of IP_8, as a consequence of ERK1/2 and p38MAPa/B kinase activation [28, 83]. Similarly, it was demonstrated that thermal stress in mammalian cells causes an ERK/MEK-dependent increase in IP_8 [48]. In contrast, in *Saccharomyces cerevisiae*, inositol pyrophosphates are not significantly altered by osmotic imbalance, heat stress or salt toxicity [48]. However, this is in partial disagreement with our own unpublished observations in which both IP_7 and IP_8 levels show a threefold increase after heat shock in *Saccharomyces cerevisiae* [M.B. and A.S., unpublished observation].

Several recent studies have linked inositol pyrophosphates to mammalian cell death. IP6K2 was first identified as a positive regulator of apoptosis through a technical knockdown approach [84]. In ovarian carcinoma cells, IP6K2 deletions conferred protection against interferon alpha (IFN-α)-induced cell death, whereas over-expression of full-length IP6K2 enhanced the degree of apoptosis induced by both IFN-α and γ-irradiation. In contrast, expression of a dominant negative IP6K2 abrogated cell death induced by these agents. IP6K2 has been further implicated in apoptosis, since its protein levels are elevated following IFN-α treatment and γ-irradiation in NIH-OVCAR-3 cells [84, 85]. Subsequently, the same authors have demonstrated that, in addition to the expression of the Apo2L/TRAIL ligand that initiates apoptosis through death-receptor signaling, nuclear localisation of IP6K2 is also required for IFN-α-induced apoptosis [86]. These data demonstrate a function for IP6K2 as a mediator of growth control and apoptosis and suggest a role of inositol pyrophosphates as regulators of the Apo2L/TRAIL cell death pathway.

These findings were extended to show that the apoptotic function of IP6K2 is not restricted to a specific cell line or apoptotic pathway [87]. Transfection with IP6K2 also increased cell death in HEK293, HeLa, PC12, Jurkat T and HL60 cells exposed to different cell stressors such as hydrogen peroxide, staurosporine, etoposide and hypoxic conditions, while the kinase-dead IP6K2 prevented cell death in all instances. Moreover, the cytotoxicity induced by the different cell stressors was associated with a rise in IP_6-kinase activity and in IP_7 formation, further reinforcing a function of inositol pyrophosphates in cell death. Interestingly, cytotoxic stimulation triggered IP6K2 translocation from the nuclei to the damaged mitochondria while no alterations were observed in the intracellular localisation of IP6K1 or IP6K3 [87]. The authors were also able to demonstrate that all three isoforms of IP6Ks induce cell death in untreated cells, but only deletion of

IP6K2 decrease stressors induced cell death, suggesting that the regulation of apoptosis is selective for IP6K2.

Recently, it was demonstrated that there is a correlation between the transforming activity of β-catenin and the upregulation of IP6K2 in chicken embryo fibroblasts (CEFs), which suggests that IP6K2 is a direct target of the lymphoid-enhancer binding factor (LEF-1) in these cells [88]. This is an interesting finding, because oncogenic transformation by β-catenin is believed to result from translocation of this protein from the cytoplasm to the nucleus where it combines with LEF to form transcriptional regulatory complexes that activate specific target genes [89]. In addition, nuclear accumulation of β-catenin and upregulation of LEF target genes are found in many cancers [88, 90].

Determining the mechanisms by which inositol pyrophosphates trigger apoptosis is an important task for researchers in this area. Drug resistance of cancer cells to many conventional therapeutics remains a problem, and inositol pyrophosphates have the potential to become a therapeutic tool to sensitise cancer cells for subsequent treatments.

Molecular mechanisms of action

Binding

Binding of IP_3, the best characterised of the inositol polyphosphates, to its receptor leads to an alteration of the tridimensional structure of the channel, which in turn allows Ca^{++} efflux [91]. A similar ligand binding mechanism, which modifies the functions of specific proteins, has been hypothesised for inositol pyrophosphates. *In vitro*, IP_7 was shown to bind, with high affinity, to several proteins, including proteins important in controlling vesicular trafficking such as AP3/AP180 [60, 78], the Golgi coatomer [76, 77] and the clathrin-assembly adaptors AP2 [73, 74]. However, these *in vitro* binding experiments, performed with this family of high negatively charged 'ligands', should be viewed with caution, as previously reviewed [7]. Many of these studies were performed either in the absence of bivalent cations, which are physiologically important in coordinating the negative charge of the inositol ring [92], or without the appropriate controls. Furthermore, IP_6 often binds to a specific protein with an affinity only 10 times lower than IP_7, and because IP_6 is usually a hundred times more abundant than IP_7, it seems likely that IP_6 may represent the significant physiological binding partner. In *Dictyostelium*, the reality is different, since the CRAC pleckstrin homology domain was shown to bind to IP_7 with 10 times higher affinity than IP_6. In this amoeba, during cell aggregation, IP_6 and IP_7 levels are comparable, suggesting that IP_7 can compete for the PH domain of CRAC. So far, the ability of IP_7 to physiologically bind to the PH domain seems restricted to the *Dictyostelium* CRAC. A recent study in which the researchers investigated the inositol polyphosphate binding characteristics of the PH domain of phosphoinositide-dependent protein kinase-1 (PDK1) revealed that IP_6 is a plausible cytosolic binding partner for this protein [93]. PDK1 was also found to bind to IP_7, with, however, a significantly lower affinity than IP_6. Because in mammalian cells the IP_7 concentration is much lower than IP_6, it is unlikely that IP_7 is a physiological ligand for the PH domain of PDK1 [93].

Phosphorylation

Inositol pyrophosphates have attracted much attention because of their rapid turnover and the substantial free-energy change upon hydrolysis of β-phosphate of the diphosphate moiety. It has been theoretically calculated that the energy of hydrolysis present in the pyrophosphate moiety of IP_7 and IP_8, due to the release of strict electrostatic and steric constraints, is similar to the high-energy phosphate bond found in adenosine triphosphate (ATP) [12, 24], leading to the idea that inositol pyrophosphate could be important for driving phosphotransferase reactions.

Interestingly, several decades ago, Morton and Raison, working with wheat endosperm proteoplasts, proposed that phytate or phytate derivatives might act as a substrate in phosphotransfer reactions possibly by maintaining the optimum ATP concentration for the synthetic functions [94]. At the time, this idea was disregarded, since the standard free energy of IP_6 hydrolysis is insufficient for ADP-to-ATP conversion. In light of what is known today about IP_7 functions, the idea was very original.

Recently, the pyrophosphate constituent of IP_7 has been shown to act as a phosphate donor to proteins, in a non-enzymatic and temperature-dependent reaction [95]. Interestingly, protein phosphorylation by IP_7 is eukaryotic specific, since no phosphorylation was detected in *E. coli*. Three potential IP_7 substrates were initially identified in yeast; NSR1, a nucleolar protein involved in ribosome assembly and export; YGR130c, a 98-KDa protein of unknown function; and SRP40, a second nucleolar protein that functions as a ribosomal chaperone [95]. These three proteins show unique sequence similarity, as all possess stretches of serine residues surrounded by acidic residues. Phosphorylation mediated by IP_7 was shown to be resistant to acid, but sensitivity to alkali treatments indicated specificity to serine/threonine. However, the sequence characteristics of the identified substrates made it experimentally difficult to identify specific phosphorylated residues. So far, these efforts were limited to deletion analysis and site-directed mutagenesis on specific serine and aspartate/glutamate residues, demonstrating the importance of these amino acids for IP_7 phosphorylation. However, these re-

sults must be taken with caution, as no structural data has determined the effect of the mutations on the protein conformation [95]. The observation that the surrounding amino acids also influence the chance of a specific protein to be phosphorylated by IP_7 suggests, as proposed by York and Hunter, that substrate conformation assists in the reaction [47].

Recent experiments conducted in Snyder's laboratory indicate that IP_7-mediated phosphorylation requires an initial modification by a factor present in eukaryotic cells but absent in bacteria [R. Bhandari, S. Snyder personal communication]. Proteins that are phosphorylated by IP_7 in eukaryotic cells are no longer phosphorylated if expressed in bacteria, suggesting that substrate priming, through a post-translation modification, is an essential requirement for IP_7-driven phosphorylation [96]. The IP_7 phosphorylation consensus site revealed the presence of several serine residues, and preliminary results are consistent with the hypothesis that an initial ATP kinase-dependent phosphorylation of a serine residue is necessary to trigger the subsequent non-enzymatic IP_7-dependent phosphorylation of the remaining serines [R. Bhandari, S. Snyder personal communication]. However, an even more fascinating hypothesis is formally possible, namely that IP_7 may phosphorylate existing phosphoserine residues, generating pyrophosphorylation modifications of serine.

The importance of this novel phosphorylation mechanism is also demonstrated under physiological conditions, since lack of IP6K activity in *kcs1*Δ resulted in a 60% decrease in NSR1 phosphorylation *in vivo* [95]. The proteins best characterised as IP_7 targets are yeast NSR1 and SRP40, and mammalian Nopp140 and TCOF1, all of which are localised in the nucleolus. The major function of the nucleolus is to synthesise ribosomal RNA (rRNA) which is transcribed as a large pre-rRNA precursor and later cleaved to generate rRNAs 5.8S, 18S and 28S. The rRNAs undergo post-transcriptional modifications and are later assembled, with ribosomal proteins, into ribosome subunits [97]. Interestingly, yeast cells lacking the enzyme that synthesises inositol pyrophosphates, *kcs1*Δ*,* are hypersensitive to antibiotics that disrupt ribosomal function, including the aminoglycosides geneticin and hygromycin B [A.S. unpublished results]. This suggests that IP_7-dependent phosphorylation of nucleolar proteins physiologically affects ribosomal biogenesis. This study has raised several questions, some of which are being addressed in our laboratory. How is the phosphate transfer activated? Is this a phosphorylation or a pyrophosphorylation? What are the total numbers of substrates phosphorylated by IP_7? Do the other inositol pyrophosphates, IP_8, PP-InsP_4 and $[PP]_2$-IP_4, have the capacity to phosphorylate proteins? Most important of all: What is the functional relevance of protein phosphorylation by IP_7?

Perspectives: do inositol pyrophosphates link basic metabolism to signaling?

The discovery that PiUS, a protein named after its role as a stimulator of inorganic phosphate uptake [98], was an IP6K, suggested that inositol pyrophosphates might have a role in cellular phosphate metabolism. Subsequently, it was shown that yeast deficient in *kcs1*Δ exhibit a considerably reduced inorganic phosphate uptake [95]. When compared with wild-type yeast, the *kcs1*Δ mutant also shows an increased expression of mRNAs coding for a number of genes important for regulating cellular phosphate metabolism, collectively known as PHO genes [99]. Indeed, microarray analyses suggest that the effects of either *arg82*Δ or *kcs1*Δ deficiency in yeast are limited to the upregulation of certain PHO genes and to the downregulation of a number of genes involved in nitrogen catabolism. Two significant PHO genes include PHO84, a membrane protein involved in phosphate transport, and PHO5, an acid phosphatase. Both enzymes are considered to be important for responding to low concentrations of cellular phosphate and were shown to be upregulated in inositol pyrophosphate-deficient yeast [99].

Recently, a screening of yeast deletion mutants for alteration in phosphate metabolism based on PHO5 activity revealed that KCS1 [100, 101] and other proteins important for inositol pyrophosphate biosynthesis such as phospholipase C and ARG82 are negative regulators of the PHO pathway [101]. Moreover, *arg82*Δ and *kcs1*Δ null strains have reduced intracellular polyphosphate levels, suggesting that inositol pyrophosphates are necessary for inorganic polyphosphate synthesis and maintenance [101]. Together, these observations strongly implicate inositol pyrophosphates as sensors of the phosphate intracellular status. However, the mechanism by which inositol pyrophosphates are able to transduce the cellular phosphate status in signaling events is unknown. One possibility is that the hyper-phosphorylation of specific proteins driven by IP_7 may be important in these events. Ribosome-mediated protein synthesis is central to cell functioning, and it is influenced by many nutrient-sensing pathways [102, 103]. In this case, phosphorylation of proteins, involved in ribosomal biogenesis, by IP_7 may represent the signaling mechanism. Specific investigation is required to fully address this hypothesis.

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