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How inositol pyrophosphates control cellular phosphate homeostasis?

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A B S T R A C T

Phosphorus in his phosphate PO_4^{3-} configuration is an essential constituent of all life forms. Phosphate diesters are at the core of nucleic acid structure, while phosphate monoester transmits information under the control of protein kinases and phosphatases. Due to these fundamental roles in biology it is not a surprise that phosphate cellular homeostasis is under tight control. Inositol pyrophosphates are organic molecules with the highest proportion of phosphate groups, and they are capable of regulating many biological processes, possibly by controlling energetic metabolism and adenosine triphosphate (ATP) production. Furthermore, inositol pyrophosphates influence inorganic polyphosphates (polyP) synthesis. The polymer polyP is solely constituted by phosphate groups and beside other known functions, it also plays a role in buffering cellular free phosphate [Pi] levels, an event that is ultimately necessary to generate ATP and inositol pyrophosphate. Although it is not yet clear how inositol pyrophosphates regulate cellular metabolism, understanding how inositol pyrophosphates influence phosphates homeostasis will help to clarify this important link. In this review I will describe the recent literature on this topic, with in the hope of inspiring further research in this fascinating area of biology.

Introduction

Phosphate is one of the essential elements in life, representing about 1% of the human body weight primarily in the form of hydroxyapatites, the major constituent of bone and teeth. Additionally,

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phosphate plays fundamental roles in every cell of all living organisms. Phosphate groups constitute the charged backbone of nucleic acids, such as RNA and DNA. Phosphate transfer reactions are also the principal mean to transfer energy and information between organic molecules. In living organisms, the macromolecule with the highest phosphate content is paradoxically referred to as inorganic polyphosphates (polyP). This polymer contains few to hundreds phosphate residues linked by phosphoanhydride, "high-energy" bonds, similar to the bond found in ATP. Because it does not possess carbons is classified as 'inorganic', even if it is ubiquitous present in every living organism from bacteria to human. Many specific biological functions have been attributed to polyP (Kornberg et al., 1999; Rao et al., 2009) but due to its intrinsic chemical structure, this polymer mainly influences phosphate/cations cellular viability. Between the organic (carbon containing) molecules, diphosphoinositol pentakisphosphte (hereafter call IP_7) has the peculiarity to possess seven phosphates groups, that is, one in excess of the six carbon atoms of the inositol ring. IP_7 belongs to a large family of molecules commonly referred to as inositol pyrophosphates. Because several inositol pyrophosphates have the unique property of possessing more phosphates than carbon atoms (Bennett et al., 2006), it is not surprising that they have been linked to phosphate homeostasis. Unfortunately, the evidences supporting this concept are quite scattered through time and have been obtained using a variety of experimental models; in addition, they often address only single aspects of the complex cellular phosphate homeostasis. Therefore, extensive investigation has not yet been performed to address the connection between inositol pyrophosphates and phosphate metabolism. This assay aims to review the data present in literature with the objective of stimulating the interest in this fascinating area of research.

Background

Before discussing the principal aim of this review it is necessary to introduce the main players of our story, which are inositol pyrophosphate and polyP. I am only briefly introduce inositol pyrophosphate enzymology and functions as several other reviews on this topic have been published recently (Burton et al., 2009; Saiardi, 2012; Shears, 2009).

Two distinct classes of proteins the inositol hexakisphosphates kinases (IP6Ks) and the diphosphoinositol pentakisphosphate kinases (PP-IP5Ks or IP7Ks) are capable of synthesizing inositol pyrophosphates. IP6Ks utilize ATP as a phosphate donor to phosphorylate IP_6 to IP_7, generation the isomer 5PP-IP_5 (Fig. 1A), and inositol pentakisphosphate I(1,3,4,5,6)P_5 to PP-IP_4 (Saiardi et al., 1999, 2000; Losito et al., 2009). Furthermore, at least *in vitro*, IP6Ks generate more complex molecules containing two or more pyrophosphate moieties, or even three-phosphate species (Draskovic et al., 2008; Saiardi et al., 2001). Three IP6K isoforms referred to as IP6K1, 2, 3 exist in mammal; however, there is a single IP6K in the yeast *Saccharomyces cerevisiae* called Kcs1.

The PP-IP5Ks enzymes, synthesize inositol pyrophosphate from IP_6, but not from IP_5, (Losito et al., 2009) generating the isomer 1PP-IP_5. Kinetic studies performed *in vitro* suggested that IP_7, the 5PP-IP_5 isomer generated by IP6Ks, is the primary substrate of this new enzyme, and this finding was confirmed *in vivo* by analysing PP-IP5K null yeast (*vip1*Δ) that accumulate the un-metabolized substrate IP_7 (Azevedo et al., 2009; Onnebo and Saiardi, 2009). Thus PP-IP5K is responsible for IP8, isomer 1,5PP_2-IP_4 synthesis (Fig. 1A). Two PP-IP5K isoforms referred to as PP-IP5Ka and b exist in mammal while a single PP-IP5K called Vip1 is present in *S. cerevisiae*.

Inositol pyrophosphates are hydrolysed by the diphosphoinositol-polyphosphate phosphohydrolases (DIPPs) (Safrany et al., 1998). Four mammalian enzymes DIPP1,2,3,4 have been identified, while only one DIPP protein exists in *S. cerevisiae* called Ddp1. These phosphatases are promiscuous enzymes able to hydrolyse inositol pyrophosphate as well as nucleotide analogues, such as diadenosine hexaphosphate (Ap6A) (Caffrey et al., 2000; Fisher et al., 2002). More recently, it has been shown that DIPPs also degrade polyP (Lonetti et al., 2011). Inositol pyrophosphates control the most disparate biological processes, from telomere length to vesicular trafficking. It is conceivable that all these function can be focused on the fact that inositol pyrophosphates are controlling cellular energy metabolism and consequently, ATP production. We have recently, demonstrated that inositol pyrophosphates control glycolysis and mitochondrial oxidative phosphorylation by both inhibiting the glycolytic flux and increasing mitochondrial activity (Szijgyarto et al., 2011).

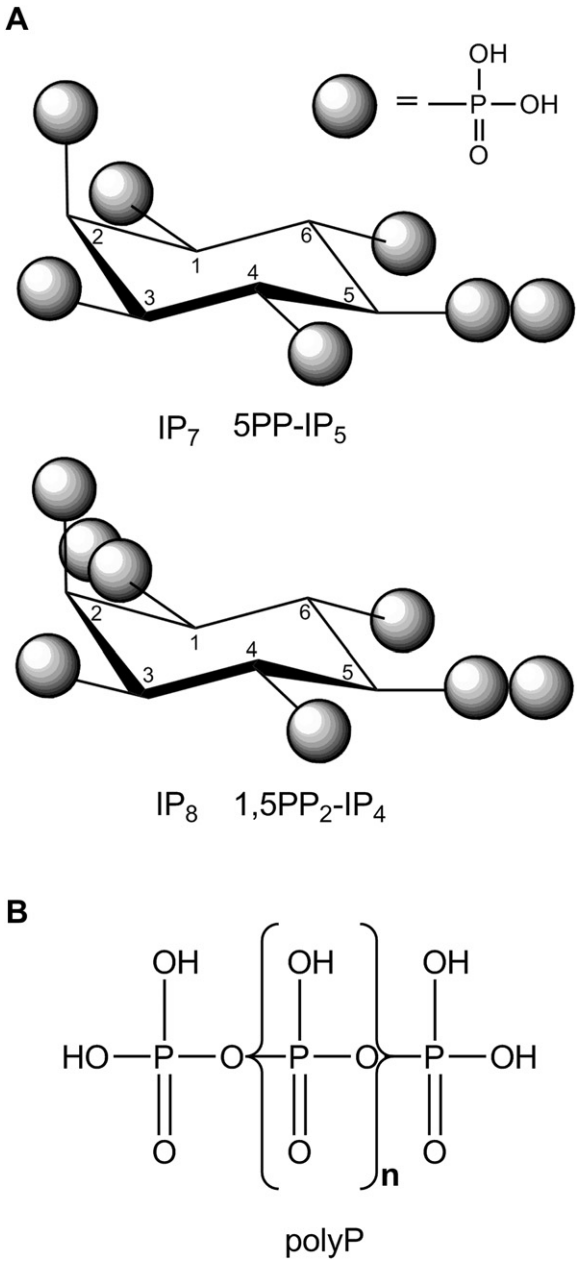

Fig. 1. Inositol pyrophosphate and polyP chemical structures. A) The top molecule depicts the structure of the IP_7 isomer 5PP-IP5 (5-diphosphoinositol pentakisphosphate) generated by the IP6K class of enzymes. On the bottom is depicted the structure of the IP_8 isomer $1,5PP_2$-IP_4 (1,5bis-diphosphoinositol tetrakisphosphate) generate by the sequential action of IP6K and PP-IP5K enzymes. B) polyP molecular structure where '*n*' range from 1 to hundreds. Thus three-phosphate residues constituted the shorter polyP molecule.

Another important molecule to briefly introduce is polyP (Fig. 1B). The interested reader is encouraged to read the following comprehensive reviews (Kornberg et al., 1999; Rao et al., 2009). The polyP polymer likely represents a phosphate buffer that is synthesized and degraded in function of the phosphate needs of the cells. Furthermore, it also functions as a chelator of metal ions, thereby

regulating cellular cation homeostasis. However, polyP also possesses more classical signalling roles. In bacteria for example, it influences pathogenicity (Brown and Kornberg, 2008) and in mammalian cells it has been proposed to regulate fibrinolysis and platelet aggregation (Caen and Wu, 2010). In prokaryotes, polyP synthesis is carried out by a family of conserved polyP kinases (PPKs), whereas degradation is mediated by several polyP phosphatases (Rao et al., 2009). In higher eukaryotes polyP synthesis remains poorly characterized. A homologous of the bacterial PPK1 has been identified in the amoeba *Dictyostelium discoideum*, although it may have been originated through bacterial gene transfer. The same organism also shows an actin-like polyP kinase (DdPPK2) (Gomez-Garcia and Kornberg, 2004). In yeast, Vtc4, a subunit of the vacuolar transporter chaperone complex, functions as a polyP polymerase (Hothorn et al., 2009). Mammalian genomes do not contain genes homologous to those encoding PPK1, or Vtc4. To the contrary, there are several mammalian proteins containing actin-like domains that resemble DdPPK2. However, these peptides are poorly characterized, therefore, the mechanisms of polyP synthesis in higher eukaryotes are still largely unknown (Hooley et al., 2008). PolyP catabolism in budding yeast is controlled at least by three polyP phosphatases: an exo-polyphosphatase named Ppx1, which removes Pi from the end of polyP chains (Wurst et al., 1995), and the endo-polyphosphatase Ppn1, which attacks internal phosphoanhydride bonds, hydrolysing polyP molecules into oligophosphates of smaller size (Shi and Kornberg, 2005). A second endo-polyphosphatase has recently been identified, as the yeast protein Ddp1. Thus the enzyme that degrades inositol pyrophosphate is also capable of metabolising polyP. This metabolic feature is also shared by the mammalian homologous proteins DIPPs (Lonetti et al., 2011). A gene homologous to yeast Ppx1 protein was also identified in mammalian genome and called h-Prune (Tammenkoski et al., 2008).

The facts

In humans alteration of phosphate metabolism is implicated in several pathological states. Higher serum phosphate leads to vascular calcification and cardiovascular complications. Although only very small amount of phosphate circulates in the serum, its concentration is tightly regulated and it is independent from dietary phosphorus intake (de Boer et al., 2009). Therefore, it is not surprising that intense research efforts are aimed to elucidate phosphate uptake and metabolism. IP6K2 was initially cloned while searching for a novel mammalian intestinal phosphate transporter that the group of Murer identified as PiUS (Phosphate inorganic Uptake Stimulator) (Norbis et al., 1997). Once transfected into *Xenopus* oocytes, PiUS stimulated the cellular uptake of radioactive phosphate. Subsequently, two groups discovered that PiUS was capable of converting IP_6 to IP_7 and rename it to IP6K2 (Saiardi et al., 1999; Schell et al., 1999). The ability of inositol pyrophosphate to control the uptake of phosphate is an evolutionary conserved feature; in fact, *kcs1*Δ yeast with undetectable level of IP_7 exhibits a reduced uptake of phosphate from the culture medium (Saiardi et al., 2004).

In mammals, regulation of phosphate homeostasis is not restricted to IP6K2, all three mammalian IP6Ks are likely to play a role. A genome-wide study aimed at identifying genetic variations associated with changes of serum phosphorus concentration identified IP6K3 (Kestenbaum et al., 2010). This human genetic study identified two independent single nucleotide polymorphisms (SNP) at locus 6p21.31, which are localised within the first intron of the IP6K3 gene. Interestingly, this study that analysed more than 16,000 humans identified SNP variant in only seven genes. Three of which, the sodium phosphate cotransporter type IIa, the calcium sensing receptor and the fibroblast growth factor 23, are well known regulators of phosphate homeostasis. These evidences support a role for IP6K3 in controlling serum phosphate levels in humans (Kestenbaum et al., 2010).

Work carried out in yeast substantiates this connection. In *S. cerevisiae*, phosphorus homeostasis is under the control of the PHO pathway that in turn regulates the expression of a set of genes responsible for phosphate metabolism. A classical model of the PHO pathway contemplates that the phosphorylation status of the transcription factor Pho4 regulates its nuclear-cytoplasmic shuttling. The cyclin–cyclin-dependent kinase complex Pho80–Pho85 in association with the cyclin-dependent kinase inhibitor Pho81, controls the phosphorylation status of Pho4. Under conditions of high phosphate levels, Pho80–Pho85 phosphorylation of Pho4 determines its exit from the nucleus inhibiting transcription of the PHO genes.

A genome-wide *S. cerevisiae* screen designed to identify secreted acid-phosphatase activity encoded by Pho5, identified Plc1, Arg82 and Kcs1 (the metabolic pathway that leads to IP_7 synthesis) as responsible for the constitutive expression of Pho5 (Auesukaree et al., 2005). This report confirms previous *arg82*Δ and *kcs1*Δ yeast strains' microarray analysis in which the de-repressed expression of genes regulated by phosphate was identified (El Alami et al., 2003). Thus in the yeast mutants for phospholipase C *plc1*Δ, inositol polyphosphates multi kinase *arg82*Δ, or inositol hexakisphosphate kinase *kcs1*Δ, Pho5 is constitutively expressed while in wild type yeast Pho5 expression is repressed in phosphate rich medium. Interestingly, this screen failed to identify Vip1 as regulator of Pho5 expression. Another systematic screens carried out in *Schizosaccharomyces pombe* failed to report any link between the soluble inositol polyphosphate pathways and the expression of acid phosphatases, it is unclear whether these mutants were tested (Henry et al., 2011). However, the *S. pombe* Vip1 homologous mutant (*asp1*Δ) was analysed; and showed to have a normal induction of the acidic phosphatase in low phosphate condition (Henry et al., 2011). The fact that two genome-wide screens failed to identify Vip1 as a regulator of the PHO pathways contrasts with the model proposed by the Oshea's group, suggesting a direct regulation by Vip1-generated IP_7 of the Pho81–Pho80–Pho85 complex (Lee et al., 2007). According to this model, selective binding of the IP_7 isoform 1PP-IP_5 augmented the inhibitory activity of Pho81, thereby increasing Pho4 transcriptional efficacy. This group also demonstrates an increase of IP_7 levels under low phosphate conditions (Lee et al., 2007). This finding is in contrast with the observation that lowering phosphate medium concentration dramatically reduces the levels of ATP required for IP_7 synthesis (Boer et al., 2010). In fact, in a recent report, exposure of WT yeast to phosphate-free medium for 20 min resulted in a decrease of intracellular inositol pyrophosphate levels by 80%, without affecting IP_6 levels (Lonetti et al., 2011). Nevertheless, because homologous of Pho4 and Pho81 are absent in *S. pombe* and Pho80 and Pho85 in this yeast are not involved in regulating the PHO pathway (Tanaka and Okayama, 2000), IP_7-dependent regulation of Pho81-Pho80–Pho85 cannot be considered a universal mechanism. Furthermore, the PHO pathway seems to be absent in mammals where IP_7 exerts a tight control on phosphorus homeostasis (see above).

An additional genome-wide study implicated *S. cerevisiae* IP6K gene Kcs1, but not the PP-IP5K gene Vip1 in controlling phosphate response (Nishizawa et al., 2008). The comprehensive mapping of Pho4 and RNA polymerase II subunit Rpo21 binding using a tiling array revealed that in low phosphate conditions, Pho4 promotes transcription of antisense and intragenic RNAs in the Kcs1 locus without affecting transcription of Vip1. The Pho4-regulated Kcs1 antisense transcript might be responsible for the formation of the intragenic shorter Kcs1 mRNA (Nishizawa et al., 2008). The truncated Kcs1 enzyme generated under these conditions may possess different and yet unknown enzymatic properties.

Taken together, these studies underline the fundamental role played by inositol pyrophosphates in controlling yeast and mammalian phosphate homeostasis. It is therefore likely that an evolutionary conserved mechanism exists that explains this connection. Thus, beside yeast-specific transcriptional control, probably exist a metabolic link between inositol pyrophosphates and cellular phosphate homeostasis. An important observation arose from two reports demonstrating that yeast not possessing inositol pyrophosphates, have low or undetectable levels of polyP polymer, which is the main intracellular phosphate storage molecule. One study used ^{31}P NMR to determine yeast polyP levels (Auesukaree et al., 2005), while the second study biochemically extracted and resolved polyP by gel electrophoresis (Lonetti et al., 2011). A similar analysis is reported in Fig. 2; yeast strains without inositol pyrophosphates lack the metachromatic toluidine blue smear typical of polyP. Moreover, the dynamic turnover of the high-energy inositol pyrophosphates is linked to polyP biosynthesis. The decrease in levels and re-synthesis of both molecules occur in parallel during the phosphate overplus assay, an assay where phosphates are initially withdrawn and then resupplied to the medium. The same report also demonstrated that Ddp1, the enzyme that hydrolyses the pyrophosphate moiety of inositol pyrophosphate, in yeast, as well as the human homologous DIPPs, are inorganic polyphosphate endo-polyphosphatases (Lonetti et al., 2011). These data support a evolutionary conserved, metabolic link between inositol pyrophosphate and the polyP polymer. Because polyP is buffering phosphates and cations, understanding how inositol pyrophosphates regulate polyP metabolism will likely address the question of how inositol pyrophosphates control cellular phosphate homeostasis.

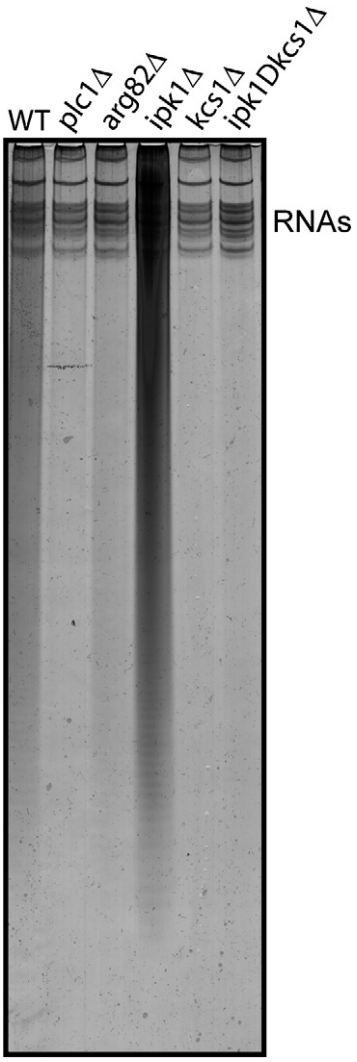

Fig. 2. Absence of polyP in mutant yeast devoid of inositol pyrophosphates extracted polyP was resolved on polyacrylamide gel and visualized with toluidine blue. This analysis revealed a striking correlation between the lack of inositol pyrophosphates and the absence of the polyP polymer. While wild type (WT) yeast and *ipk1*Δ strain (IP_5-2K null) possess inositol pyrophosphates the following yeast do not, *plc1*Δ (phospholipase C null), *arg82*Δ (inositol polyphosphate multi kinase null), *kcs1*Δ (IP6K null), and *kcs1*Δ*ipk1*Δ (double IP6K and IP_5-2K null) (Azevedo et al., 2009; Onnebo & Saiardi, 2009).

The hypothesis

Although, inositol pyrophosphate may have acquired unique organism-specific functions, the conserved ability of this class of molecules to regulate phosphate metabolism suggests an evolutionary ancient role. In this last paragraph, I will formulate few hypotheses that I hope will stimulate further research aimed at elucidating the biological link between phosphate, inositol pyrophosphates and polyP.

Inositol pyrophosphates regulate the entry of phosphate into the cells (Norbis et al., 1997), suggesting that they could affect phosphate uptake either directly (by stimulating a transporter, for example) or a indirectly by helping 'fixing' free phosphates in organic molecules. The cytosolic

concentration of free phosphate [Pi] cannot fluctuate widely. Therefore, cellular entry of phosphates and its utilization may well be coupled. For example, the synthesis of polyP may be linked to phosphate entry in the cell. Inositol pyrophosphate control of energy metabolism (Szijgyarto et al., 2011) affects not only ATP levels but it can also alter the entire cellular balance of adenine nucleotides. Given that phosphate transfer reactions mainly use ATP as a vehicle for the phosphate groups, inositol pyrophosphate could affect phosphate metabolism by regulating the adenylate cellular pool. Moreover, it is tempting to speculate the existence of a feedback mechanism that coordinates the metabolic balance between ATP, phosphate and inositol pyrophosphates.

Inositol pyrophosphates could either contribute to the regulation of polyP synthesis, play a role in polyP degradation, or both. The yeast polyP polymerase has been identified with the subunit four (Vtc4) of the vacuolar membrane transporter chaperone (VTC) complex (Hothorn et al., 2009). Interestingly, pyrophosphates (Pi–Pi) dramatically accelerate the polyP polymerase reaction. It would therefore be interesting to determine whether the pyrophosphate moiety of IP_7 can stimulate polyP vacuolar synthesis in a similar fashion. Similarly, it would be interesting to analyse the effect of inositol pyrophosphates on controlling the activity of the actin-like DdIPK2 enzyme. It should be noted however, that the existence even in yeast or *Dictyostelium* of other enzymes able to synthesize different polyP pools cannot be excluded. Thus, we will be able to validate and fully appreciate the role played by inositol pyrophosphates on polyP synthesis only after the identification of higher eukaryotes polyP-synthesizing peptide/s.

An evolutionary conserved function for IP_7 in regulating the synthesis of polyP would entail a 'primer' role for the eukaryotic polyP kinases/synthetases. The pyrophosphate moiety of IP_7 may work as a docking and starting point for the enzyme synthesizing polyP. This event will trigger the synthesis of polyP molecules with a fully phosphorylated inositol ring attached to one extremity. The ubiquitous presence of very active exophosphatases requires that the polyP edges are protected. Therefore, although IP_7 may not be used as primer in the synthesis of polyP itself the attachment of IP_7 to the polymer ends, it might still function as the "cap" structure, similarly to what is observed for mRNA, thereby protecting it from degradation. The fact that yeast exophosphatase Ppx1 is unable to degrade IP_7 (Lonetti et al., 2011) suggests that IP_6 at the edge of polyP can protect this polymer from the action of exophosphatases.

Nudix hydrolyses, such as yeast DDP1 and mammalian DIPPs are remarkable enzymes that metabolise three different substrate polyP, inositol pyrophosphates and adenine dimer. Controlling both cellular levels of the two most phosphates rich molecules such as polyP and inositol pyrophosphates, and the levels of adenosine, these enzymes are ideal candidates to regulate energy metabolism and ATP synthesis. Nudix hydrolases are considered cellular surveillance agents, that are capable of regulating cellular homeostasis (McLennan, 2006). Although DDP1 are known to metabolise adenine dimers, IP_7 and polyP, further research will be needed to fully appreciate the multifaceted activity of these hydrolyses. Little is known about the mechanisms by which these enzymes are regulated and the specificity of the substrates. The yeast Ddp1 enzyme preferentially metabolises polyP *in vitro* (Lonetti et al., 2011) and IP_7 *in vivo* (Ingram et al., 2003), but to date, a comparative study of the three substrates has not been performed *in vivo*. Further investigations are required to define the role of DIPPs in controlling cellular energy production and phosphate homeostasis under physiological conditions.

The most abundant form of organic phosphate on earth is IP_6, or phytic acid, a molecule that is highly abundant in plant seeds from which was originally characterised. In plant seeds, IP_6 represents a phosphate storage molecule that it is hydrolysed during germination, releasing phosphates and cations. It will be an astonishing twist of event if inositol pyrophosphates were controlling the levels of their own precursor IP_6 (Raboy, 2003), although due to the evolutionary conserved ability of inositol pyrophosphate to control phosphate homeostasis we should not be entirely surprised.

The analysis of yeast mutant that does not synthesize inositol pyrophosphates has revealed a striking correlation between the lack of inositol pyrophosphates and the absence of polyP (Auesukaree et al., 2005; Lonetti et al., 2011). In this regard, future investigation of the metabolic and functional interconnection between these two classes of highly phosphorylated molecules will certainly reward us with exciting discoveries.

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