The emerging roles of inositol pyrophosphates in eukaryotic cell physiology

Swarna Gowri Thota¹,² and Rashna Bhandari¹,*

¹Laboratory of Cell Signalling, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500 001, India
²Graduate Studies, Manipal University, Manipal 576 104, India

*Corresponding author (Email, rashna@cdfd.org.in)

Inositol pyrophosphates are water soluble derivatives of inositol that contain pyrophosphate or diphosphate moieties in addition to monophosphates. The best characterised inositol pyrophosphates, are IP₇ (diphosphoinositol pentakisphosphate or PP-IP₅) and IP₈ (bisdiphosphoinositol tetrakisphosphate or (PP)₂-IP₄). These energy-rich small molecules are present in all eukaryotic cells, from yeast to mammals, and are involved in a wide range of cellular functions including apoptosis, vesicle trafficking, DNA repair, osmoregulation, phosphate homeostasis, insulin sensitivity, immune signalling, cell cycle regulation, and ribosome synthesis. Identified more than 20 years ago, there is still only a rudimentary understanding of the mechanisms by which inositol pyrophosphates participate in these myriad pathways governing cell physiology and homeostasis. The unique stereochemical and bioenergetic properties these molecules possess as a consequence of the presence of one or two pyrophosphate moieties in the vicinity of densely packed monophosphates are likely to form the molecular basis for their participation in multiple signalling and metabolic pathways. The aim of this review is to provide first time researchers in this area with an introduction to inositol pyrophosphates and a comprehensive overview on their cellular functions.

1. Structure of inositol and inositol phosphates

Inositol or cyclohexane-1,2,3,4,5,6-hexol is a carbohydrate with the formula C₆H₁₂O₆, and occurs widely in nature. It has nine possible stereoisomers, of which myo-inositol is the most abundant form in living cells (figure 1A). Numbering of carbon atoms on the inositol ring is counterclockwise, as recommended by the Nomenclature Committee of the International Union of Biochemistry (NC-IUB) (1989a, b). Instead of using the Haworth projection (figure 1A), it is easier to understand myo-inositol nomenclature by comparing the thermodynamically stable chair conformation (figure 1B) to a turtle (figure 1C), as suggested by Bernard Agranoff (Agranoff 1986, 2009; Irvine and Schell 2001). The head of the Agranoff turtle is the hydroxyl group at position 2, which is axial (perpendicular to the plane of the ring), and the flippers and tail denote all 5 other hydroxyl groups, which are equatorial (in the plane of the ring). Inositol phosphates are synthesised by replacing the hydroxyl groups with phosphate groups on myo-inositol, and the nomenclature follows Arganoff’s turtle. There are 63 mathematically predicted stereoisomers (including 24 pairs of enantiomers and 15 mesomers) for the inositol ring that is substituted with 1 to 6 monophosphate groups (Barker et al. 2009; Wundenberg and Mayr 2012), and many are found in nature. The six hydroxyl groups on the inositol ring are replaced with monophosphates in inositol hexakisphosphate (IP₆) (figure 1D). It is pertinent to note that the correct IUPAC abbreviation for myo-inositol is ‘Ins’, but it is commonly abbreviated as ‘I’. In this review we have chosen the latter, as it is simpler to vocalize.

Keywords. Bisdiphosphoinositol tetrakisphosphate (IP₈); cell signalling; diphosphoinositol pentakisphosphate (IP₇); inositol hexakisphosphate (IP₆) kinase (IP6K); PP-IP₃ kinase (PPIP5K); pyrophosphorylation

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The inositol pyrophosphates are a sub-family of inositol phosphates in which hydroxyl groups on the myo-inositol ring are substituted with both monophosphate and diphosphate (or pyrophosphate) moieties. Adding diphosphate groups on the ring further increases the number of stereoisomers of inositol phosphates. IP$_7$ (diphosphoinositol pentakisphosphate) consists of an inositol ring substituted with five monophosphates and one diphosphate moiety, and is therefore accurately referred to as PP-IP$_5$ (figure 1E), and IP$_8$ (bisdiphosphoinositol tetrakisphosphate) which contains four monophosphate groups and two diphosphate groups, is (PP)$_2$-IP$_4$ (figure 1F).

### 2. Synthesis of inositol phosphates and pyrophosphates

In all eukaryotic cells, inositol is available in the cytosol as free inositol or its phosphorylated derivatives, and in the membrane as inositol head groups in phosphatidylinositol (PI) lipids. Inositol phosphate metabolism is well conserved from yeast to humans (Irvine and Schell 2001). In most eukaryotes, the inositol lipid phosphatidylinositol (4,5) bisphosphate, also known as PI(4,5)P$_2$, predominantly located on the plasma membrane (Varnai and Balla 2006) is the precursor for the synthesis of inositol phosphates and inositol pyrophosphates. The budding yeast, S. cerevisiae, possesses a simple metabolic pathway with a single enzyme for each step of inositol phosphate synthesis, whereas mammals have a larger repertoire of inositol phosphates and multiple isoforms of inositol phosphate synthesizing enzymes (figure 2). The inositol head group on phosphoinositide is accessible to the enzyme phosphoinositide-specific phospholipase-C (PLC) (Yang et al. 2013), which cleaves PI(4,5)P$_2$ to generate inositol (1,4,5) trisphosphate (IP$_3$) and diacylglycerol (DAG) (figure 2). In mammalian cells, IP$_3$ released into the cytoplasm activates downstream effectors such as calcium channels on the endoplasmic reticulum leading to an increase in Ca$^{2+}$ in the cytosol (Berridge et al. 2003). The single phosphoinositidase in yeast, Pcl1 (Flick and Thorner 1993; Yoko-o et al. 1993) cleaves PI(4,5)P$_2$ to generate IP$_3$ (figure 2), but there is no calcium signalling activated by IP$_3$ in yeast. IP$_3$ is phosphorylated at positions 6 and 3 on the ring to form inositol (1,4,5,6) tetrakisphosphate (IP$_4$) and inositol (1,3,4,5,6) pentakisphosphate (IP$_5$) by a nuclear enzyme called Ipk2 (also called Arg82 or ArgRIII) in yeast (Odom et al. 2000; Saiardi et al. 2000b; Saiardi et al. 1999). In mammals and Drosophila, an additional enzyme family which is absent in yeast, called inositol-trisphosphate 3-kinases (IP3Ks or ITPKs) (Dewaste et al. 2000; Takazawa et al. 1990; Takazawa et al. 1991), convert I(1,4,5)P$_3$ to I(1,3,4,5)P$_4$, and the orthologue of Ipk2 called inositol polyphosphate multikinase (IPMK) (Saiardi et al. 1999) converts this form of IP$_4$ to IP$_5$ (figure 2). The yeast enzyme Ipk1 (York et al. 1999), predominantly found at the nuclear envelope, synthesizes inositol (1,2,3,4,5,6) hexakisphosphate (IP$_6$) from IP$_5$ by substituting the hydroxyl group at position 2 on the inositol ring, and in mammals the orthologue of Ipk1, inositol pentakisphosphate 2-kinase (IPPK) (Verbsky et al. 2002) generates IP$_6$. Mammals and plants possess an additional inositol phosphate kinase, called inositol (1,3,4) trisphosphate 5/6-kinase (ITPK1, not to be confused with ITPKs) (Wilson and Majerus 1996), which provides an alternate route to the generation of IP$_5$ (Verbsky et al. 2005). In this pathway, I(1,3,4,5)P$_4$ generated by IP3K is first dephosphorylated by a
5 phosphatase (Hansen et al. 1987) to I(1,3,4,6)P₄, and then phosphorylated by ITPK1 to I(1,3,4,6)P₄. This is acted upon by IPMK to make I(1,3,4,5,6)P₅.

IP₆ is the primary substrate for the synthesis of the major cellular inositol pyrophosphates, IPγ and IP₇ [for other reviews on these molecules see (Azevedo et al. 2011; Barker and Berggren 2013; Barker et al. 2009; Bennett et al. 2006; Burton et al. 2009; Chakraborty et al. 2011; Koldobskiy and Snyder 2014; Sairadi 2012a, b; Shears 2007, 2009, 2015; Shears et al. 2014; Thomas and Potter 2014; Tsui and York 2010; Wilson et al. 2013; Wundenberg and Mayr 2012)]. IP₇ and IP₈ were discovered independently by Georg Mayr in collaboration with Len Stephens in Dictyostelium discoideum (Mayr et al. 1992; Stephens et al. 1993), and by Stephen Shears’ group in pancreatoma cells (Menniti et al. 1993). Inositol hexakisphosphate kinase (IP6K) adds a β phosphate on the pre-existing σ phosphate at position 5 on IP₆ to make SPP-I(1,2,3,4,6)P₅ (5-IP₇) (Draskovic et al. 2008; Lin et al. 2009; Sairadi et al. 1999; Voglmaier et al. 1996) (figure 2). IP6K has three mammalian paralogs, IP6K1/2/3, which are expressed in different tissues and sub-cellular compartments (Sairadi et al. 1999; Sairadi et al. 2001; Thomas and Potter 2014). The single S. cerevisiae IP₆ kinase, Kcs1 (kinase C suppressor 1), was initially discovered as a suppressor of hyper-recombination caused by a mutation in protein kinase-C (Huang and Smynging 1995), and later identified as an orthologue of mammalian IP6Ks (Sairadi et al. 1999). Sequence and structure based analyses reveal that IP3K, IPMK, and IP6K belong to the same inositol phosphate kinase superfamily, which possesses N- and C-terminal lobes with an ATP binding cleft similar to protein kinases, and an additional inositol phosphate binding domain (Bennett et al. 2006; Gonzalez et al. 2004; Holmes and Jogl 2006; Miller and Hurley 2004; Sairadi et al. 1999; Wang et al. 2014a). A recent crystal structure of the Entamoeba histolytica IP6K suggests that this superfamily is likely to have evolved from an ancestor with hybrid IP3K-IP6K enzyme activity (Wang et al. 2014a). IP6Ks show low affinity towards ADP (Kₘ 1.34–1.7 mM) compared to IP₆ (Kₘ 0.6–3 μM) and ATP (Kₘ 0.7–1.4 mM) (Sairadi et al. 2000a; Wundenberg et al. 2014) and can also function as ATP synthases by donating a phosphate from 5-IP₇ or IP₆ to ADP in vitro (Voglmaier et al. 1992; Wundenberg et al. 2014). Under normal conditions, the physiological concentration of ATP and IP₆ is 1 mM and 10–100 μM respectively, and therefore IP6K drives synthesis of 5-IP₇ from IP₆ in cells. IP6Ks can modulate their enzymatic activity to either phosphorylation or dephosphorylation of IP₆, depending on the ATP/ADP ratio in cells. An increase in the ATP/ADP ratio results in synthesis of 5-IP₇ from IP₆, whereas a reduction in the ATP/ADP ratio results in synthesis of ATP by dephosphorylating IP₆ (Wundenberg et al. 2014).

In S. cerevisiae, the IP₆ kinase Vip1 synthesizes IP₈ [1,5(PP)₂-I(2,3,4,6)P₄] by adding a phosphate at position 1 on 5-IP₇ (figure 2) (Lin et al. 2009; Mulugu et al. 2007; Wang et al. 2012). In mammals there are two isoforms of Vip1, also known as PIP5Ks, that synthesize IP₈ from 5-IP₇ (Choi et al. 2007; Fridy et al. 2007; Tsui and York 2010; Wang et al. 2012). Vip1 can also function as an IP₆ kinase and synthesize 1IPP-I(2,3,4,5,6)P₆ (1-IP₈) from IP₆, which can then be converted to IP₇ by the action of Kcs1 (figure 2) (Lin et al. 2009; Mulugu et al. 2007; Tsui and York 2010; Wang et al. 2012). Vip1 also possesses a C-terminal histidine acid phosphatase domain (Gokhale et al. 2011; Mulugu et al. 2007). In Asp1, the Vip1 homologue in Schizosacharomyces pombe, the phosphatase activity of this domain has been shown to reduce the synthesis of 1-IP₇ from IP₆, suggesting that the N-terminal kinase activity of Vip1 may be autoregulated by the C-terminal domain (Pohlmann et al. 2014). Recent structural analysis has revealed that this enzyme possesses a unique substrate binding site on its surface which serves to capture the substrate from the bulk phase and direct it towards the catalytic site (Wang et al. 2014b). Vip1 paralogs have also recently been identified in Arabidopsis (Desai et al. 2014; Laha et al. 2015), and shown to be primarily responsible for the synthesis of IP₇ from IP₆. It was shown that an enzyme responsible for the synthesis of IP₇ from IP₆ exists in plants, but is likely to be distinct from mammalian and yeast IP6Ks (Laha et al. 2015).

In budding yeast, the deletion of Kcs1 leads to undetectable levels of both IP₇ and IP₈, whereas deletion of Vip1 causes an increase in 5-IP₇ levels (Onnebo and Sairadi 2009). This observation suggests that in yeast, the predominant pathway for IP₇ synthesis from IP₆ is via 5-IP₇, and that 5-IP₇ is more abundant compared with 1-IP₇. In yeast and in mammals there are other minor inositol pyrophosphates, 5PP-IP₄, 1PP-IP₄ and 1,5(PP)₂-IP₃, synthesized from IP₃ (figure 2) (Draskovic et al. 2008; Wundenberg and Mayr 2012). In yeast, these minor inositol pyrophosphates are only detectable in the absence of Ipk1, the IP₆ kinase which synthesizes IP₇ (Sairadi et al. 2002). IP₈ is the preferred substrate over IP₇ for the synthesis of inositol pyrophosphates due to its high physiological concentration and higher affinity of the IP6Ks for IP₈ (Wundenberg and Mayr 2012). All mammalian IP6Ks can add a γ phosphate to 5-IP₇ to make 5PPP-IP₃, a triphospho- group containing inositol phosphate (Draskovic et al. 2008), whose in vivo occurrence has not yet been unequivocally demonstrated.

Inositol pyrophosphates are hydrolysed by the enzyme DIPP (diphosphoinositool polyphosphate phosphohydrolase), which exists as five isoforms in mammals, and a single isoform called Ddp1 (diadenosine and diphosphoinositol phosphohydrolase) in yeast (figure 2) (Caffrey et al. 2000; Kilari et al. 2013; Safrany et al. 1998; Safrany et al. 1999). DIPPs hydrolyse diphosphate groups on IP₇ and IP₈ to form IP₆, and on PP-IP₄ and (PP)₂-IP₃ to form IP₅ (figure 2).
These hydrolases prefer 1-IP$_7$ over 5-IP$_7$ as a substrate (Kilari et al. 2013; Lonetti et al. 2011), which could contribute to the presence of higher amounts of 5-IP$_7$ compared to 1-IP$_7$ in cells.

The levels of inositol pyrophosphates are tightly regulated as a result of constant synthesis by IP$_6$ and IP$_7$ kinases and hydrolysis by DIPPs. Early work by the Shears’ laboratory indicated a high turnover rate for these molecules, estimating that 50% of the IP$_6$ pool and 20% of the IP$_7$ pool are converted into inositol pyrophosphate derivatives every hour in mammalian cells (Menniti et al. 1993), and that the IP$_7$ pool can turn over ten times every 40 min (Glennon and Shears 1993). In yeast, mammals and plants, the steady state levels of the major inositol pyrophosphates, IP$_7$ and IP$_8$, have been found to be very low, with 5-IP$_7$, the most abundant inositol pyrophosphate, occurring at only 1-5% of the level of its precursor, IP$_6$ (Laha et al. 2015; Onnebo and Saiardi 2009; Wundenberg and Mayr 2012). Most of these studies have relied on HPLC based separation and measurement of inositol polyphosphates (Azevedo and Saiardi 2006). A new method developed by the Saiardi laboratory, which involves pull down of inositol phosphates with titanium oxide beads and analysis by PAGE, suggests that IP$_7$ levels may be higher in some cell lines, reaching approximately 20% of IP$_6$ levels in HCT116 cells (Wilson et al. 2015). The estimated physiological concentration of IP$_7$ ranges from 0.5-5 μM, most of which is 5-IP$_7$ in yeast and mammals (Albert et al. 1997; Barker et al. 2004; Illies et al. 2007; Ingram et al. 2003; Lin et al. 2009; Onnebo and Saiardi 2009; Wundenberg and Mayr 2012), whereas 1-IP$_7$ contributes minor levels (Wundenberg and Mayr 2012). Biological levels of IP$_8$ can vary significantly, from being undetectable in some cells, to up to 10-20% of IP$_7$ levels in others, (Choi et al. 2008; Choi et al. 2005; Glennon and Shears 1993; Onnebo and Saiardi 2009; Shears et al. 2013; Wilson et al. 2015; Wundenberg and Mayr 2012). In contrast to other organisms, slime moulds have high amounts of inositol pyrophosphates, with a recent estimate based on

Figure 2. Pathway of synthesis of inositol pyrophosphates. Synthesis of inositol pyrophosphates, starting with the generation of I(1,4,5)P$_3$ from P(4,5)P$_2$. The pathway that occurs in both yeast and mammals is depicted in black arrows and the pathway that is exclusive to mammals is shown in blue arrows. Yeast enzymes are represented in green; mammalian enzymes are represented in blue; the undetermined inositol pyrophosphate structure is indicated with a question mark.
3. Signalling and metabolic functions of inositol pyrophosphates

The low levels of cellular inositol pyrophosphates are similar to steady state levels of IP$_3$, which is considered a classical second messenger (Streb et al. 1983), and so it is believed that like IP$_3$, inositol pyrophosphates can participate in cell signalling. However, unlike IP$_3$, levels of inositol pyrophosphates have not been shown to respond dramatically to different external stimuli. Instead it has been seen that cellular ATP levels govern inositol pyrophosphates (see below). Nevertheless, several laboratories have conducted over-expression, knockdown and knockout based analyses of the inositol pyrophosphate synthesizing enzymes, to assign a wide variety of cell and organism level functions to these molecules. At the molecular level however, it is believed that inositol pyrophosphates can act by two mechanisms:

(A) Binding: Inositol phosphates and inositol pyrophosphates are stereospecific in nature. They can bind to specific domains on individual proteins and allosterically regulate protein function. 1-IP$_7$ has been shown to interact with a cyclin-dependent kinase inhibitor in budding yeast to regulate phosphate metabolism (Lee et al. 2008). IP$_7$ synthesized by mammalian IP6K2 can bind and activate the protein kinase CK2, to trigger a signalling pathway promoting apoptosis (Rao et al. 2014a). Several studies have shown that 5-IP$_7$ can compete with the lipid inositolite PI(3,4,5)P$_3$ to specifically bind pleckstrin homology (PH) domains and inhibit downstream PI$_{3,4,5}$P$_3$-PH-domain interaction dependent signalling (Chakraborty et al. 2010; Luo et al. 2003; Prasad et al. 2011; Rao et al. 2014a; Wu et al. 2013). Interestingly, different PH domains show stronger binding to IP$_7$ compared with 1-IP$_7$, although the degree of difference has been shown to vary (Chakraborty et al. 2010; Gokhale et al. 2013; Luo et al. 2003). This appears counterintuitive, given that IP$_4$ is considered a mimic for the head-group of PI(3,4,5)P$_3$, and the molecular underpinnings for this specificity are yet to be determined. Some PH domains tested in a recent study show a much higher affinity for IP$_6$ and 5-IP$_7$ compared with 1-IP$_7$ and IP$_8$ (Gokhale et al. 2013), underlining the stereospecific nature of protein binding as a mechanism for inositol pyrophosphate function.

(B) Pyrophosphorylation: Pyro is the Greek word for fire or heat, and as suggested by their name, inositol pyrophosphates, are high energy molecules that release energy upon hydrolysis of the pyrophosphate bond. Based on bond energy calculations, the energy of hydrolysis of C-O-P on the inositol ring is 3.4 kcal/mol whereas the bond energy of P-O-P in IP$_7$ is 6.6 kcal/mol, which is higher than the P-O-P bond in ADP (6.4 kcal/mol) (Stephens et al. 1993; Wundenberg and Mayr 2012). Phosphoanhydride bonds in inositol pyrophosphates are more susceptible to hydrolysis due to the steric interference generated by the surrounding negatively charged phosphate groups, and therefore the energy of their hydrolysis might be higher than the calculated energies (Hand and Honek 2007; Laussmann et al. 1996). Inositol pyrophosphates can therefore transfer the β-phosphate of the P-O-P bond on the inositol ring to pre-phosphorylated serine residues on proteins, in presence of divalent cations like Mg$^{2+}$ as a co-factor (Bhandari et al. 2007). Mass spectrometry analysis of IP$_7$ substrate proteins has revealed that the pyrophosphorylatable serine residues are present in acidic motifs and are potential substrates for casein kinase 2 (Bhandari et al. 2007; Sasiardi et al. 2004). Thus, inositol pyrophosphates donate their β-phosphate to prephosphorylated serine residues and bring about protein pyrophosphorylation (Bhandari et al. 2007). Unlike protein binding, which is specific to either 1- or 5-IP$_7$ (Gokhale et al. 2013; Lee et al. 2008), serine pyrophosphorylation can be brought about by all inositol pyrophosphates (Bhandari et al. 2007). However, until we gain a deeper understanding of the phosphotransfer process from inositol pyrophosphates to phosphoserine, we cannot rule out the possibility of stereoselectivity in the inositol pyrophosphate donor depending on the sequence context of the acceptor phosphoserine. The pyrophosphates on serine residues in proteins are acid labile but they show strong resistance to protein phosphatases (Bhandari et al. 2007). Longer incubations of synthetic pyrophosphopeptides with alkaline phosphatases can remove the β-phosphate group on pyrophosphoserine containing peptides (Yates and Fiedler 2015), suggesting that protein pyrophosphorylation may be a reversible process participating in many cellular signalling pathways.

Inositol pyrophosphates and the enzymes responsible for their synthesis participate in many physiological pathways from yeast to mammals. Given below is a short summary of the role played by inositol pyrophosphates in each physiological function described for these molecules so far (figure 3).

Growth and stress response: kcs1Δ yeast that have decreased levels of IP$_3$ and IP$_8$ display slow growth at 30°C, temperature sensitivity at 37°C and increased cell volume, suggesting that inositol pyrophosphates are essential for normal cell growth in yeast (Dubois et al. 2002).
Resistance to salt stress and cell wall integrity also require IP$_7$ (Dubois et al. 2002). In mammalian cells, IP$_5$ levels rise sharply in response to hyperosmotic or thermal stresses (Choi et al. 2005; Pesesse et al. 2004), and PPIP5K1 is activated in response to a hyperosmotic challenge (Choi et al. 2007). Treatment of budding yeast with hydrogen peroxide leads to a reduction in levels of IP$_7$ and IP$_8$ via oxidation of a cysteine residue in Kcs1 and direct inhibition of its enzyme activity (Onnebo and Saiardi 2009). Conversely, kcs1$\Delta$ and vip1$\Delta$ yeast, which lack IP$_8$, show resistance to cell death caused by treatment with hydrogen peroxide via increased activation of the Rad53 pathway (Onnebo and Saiardi 2009).

**DNA repair**: The absence of Kcs1 leads to a defect in DNA recombination, suggesting that Kcs1 products, either 5-IP$_7$ or 5PP-IP$_4$, play a role in the regulation of DNA recombination (Huang and Symington 1995; Luo et al. 2002). In mammalian cells the absence of IP6K1 leads to a defect in homologous recombination repair (Jadav et al. 2013). Yeast Tel1, a protein that belongs to the phosphoinositide-3-kinase related protein kinase (PIKK) family, is involved in telomere length maintenance, and the action of this kinase is negatively correlated with the levels of PP-IP$_4$ (Resnick et al. 2005; York et al. 2005). kcs1$\Delta$ yeast show reduced survival compared with wild type cells in response to treatment with the DNA double strand break inducer phleomycin (Onnebo and Saiardi 2009). In response to UV stress, mammalian IP6K1 synthesizes IP$_7$ that further activates the E3 ubiquitin ligase CRL4, which leads to protein degradation, thereby regulating nucleotide excision DNA repair and apoptosis (Rao et al. 2014b).

**Vesicular trafficking**: Yeast that lack inositol pyrophosphates contain abnormally small vesicles and small fragmented vacuoles, and are impaired in endocytic membrane trafficking (Dubois et al. 2002; Saiardi et al. 2000a; Saiardi et al. 2002).
IP$_7$ has been shown to bind a coatomer protein involved in intercisternal Golgi vesicle transport (Ali et al. 1995; Fleischer et al. 1994) and the clathrin assembly protein AP180 (Ye et al. 1995), and inhibit the functions of these proteins, but a subsequent review pointed out that these assays were conducted under inappropriate salt conditions (Shears 2001). Ip6k1 knockout mice display reduced plasma insulin levels (Bhandari et al. 2008) whereas overexpression of IP6K1 in pancreatic β cells increases the exocytosis of insulin (Illies et al. 2007). In mammalian cells, pyrophosphorylation of AP3β1 (a subunit of a clathrin associated adaptor protein complex) impairs its interaction with Kif3A (a kinesin motor protein) thereby inhibiting the release of HIV-1 like viral particles (Azevedo et al. 2009).

**Cell cycle and cell death:** In the yeast cell cycle, progression through G phase after release from pheromone induced cell cycle arrest requires IP$_7$ (Banfic et al. 2013). In mammalian cells, IP$_7$ levels fluctuate during different phases of the cell cycle, but the functional significance of this is unknown (Barker et al. 2004). Overexpression of mammalian IP6K2 leads to increased susceptibility to cell death by apoptosis (Morrison et al. 2002; Morrison et al. 2001; Nagata et al. 2005) and binding of Hsp90 to IP6K2 blocks this activity (Chakraborty et al. 2008). IP6K2 knockout mice show increased susceptibility to tumourigenesis as a consequence of reduced apoptosis (Morrison et al. 2009). Deletion of IP6K2 in mammalian cells impairs p53-mediated apoptosis (Koldobskiy et al. 2010) and binding of 5-IP$_7$ to CK2 enhances p53-mediated cell death (Rao et al. 2014a).

**Akt signalling:** IP$_7$ acts as a physiological inhibitor of the Akt signalling cascade by binding to the PH domain of Akt, preventing its interaction with PI(3,4,5)P$_3$ and its subsequent phosphorylation and activation by PDK1. IP$_7$ therefore reduces Akt membrane translocation and insulin-stimulated glucose uptake (Chakraborty et al. 2010). Thus, deletion of Ip6k1 in mice results in increased insulin sensitivity and resistance to weight gain when fed a high-fat diet (Chakraborty et al. 2010). Inositol pyrophosphates regulate aging of bone marrow derived mesenchymal stem cells, with older cells showing more IP$_7$ levels and reduced Akt signalling compared with young cells (Zhang et al. 2014). In human neutrophils, 5-IP$_7$ inhibits Akt-PI(3,4,5)P$_3$ interaction mediated functions such as superoxide production and phagocytosis (Prasad et al. 2011). IP$_7$ has also been shown to bind other PH domains. IP$_7$ binding to PH domains modulates cyclic AMP induced aggregation in D. discoideum, and amoebae lacking IP6K show rapid aggregation and increased cyclic AMP sensitivity (Luo et al. 2003). PH domain binding appears to be isomeric specific, with the PH domains of Akt, SIN1 and GRP1 showing approximately 30 fold higher affinity for 5-IP$_7$ compared with 1-IP$_7$ or IP$_8$ (Gokhale et al. 2013).

**Phosphate metabolism:** In yeast, 1-IP$_7$, the product of Vip1, binds to Pho80-Pho85-Pho81, a cyclin - cyclin dependent kinase (CDK) – CDK inhibitor complex, and inhibits its kinase activity under phosphate starved conditions to enable Pho4-dependent transcription of phosphate metabolism genes Pho84 (high affinity phosphate uptake transporter) and Pho5 (acid phosphatase involved in P$_i$ mobilization) (Lee et al. 2008). In vip1Δ yeast, Pho4 remains inactive during phosphate starvation (Lee et al. 2007). Conversely, Kcs1 mutant yeast constitutively express Pho5, and overexpression of Kcs1 leads to suppression of Pho5 expression under low phosphate conditions (Auesukaree et al. 2005). There are conflicting reports on the effect phosphate starvation in yeast has on the levels of total IP$_7$, with one report showing that total IP$_7$ levels rise upon phosphate starvation (Lee et al. 2007) and a contrasting report showing that total IP$_7$ goes down upon phosphate starvation (Lonetti et al. 2011; Saiardi 2012b). Deletion of Kcs1 causes reduced uptake of inorganic phosphate in yeast (Saiardi et al. 2004), and in vertebrates, phosphate uptake is increased upon over-expression of IP6K2 (Norbis et al. 1997).

Polyphosphates are long chains of inorganic phosphate moieties linked by phosphodiester bonds, and have a size range of 60-100 units per chain in eukaryotes. Yeast that lack Kcs1 are deprived of polyphosphates whereas yeast lacking Vip1 have normal levels of polyphosphates (Auesukaree et al. 2005; Lonetti et al. 2011). Adding back active but not inactive mouse IP6K1 restored polyphosphate levels in kcs1Δ yeast, suggesting that 5-IP$_7$ is required to maintain normal levels of polyphosphate in yeast (Lonetti et al. 2011). Ip6k1 knockout mice do not have platelet polyphosphates and show defects in blood clotting (Ghosh et al. 2013). Addition of polyphosphates but not IP$_7$ could rescue clotting in sera derived from Ip6k1 knockout mice (Ghosh et al. 2013). These studies indicate that a metabolic link exists between 5-IP$_7$ and polyphosphates, which is conserved in yeast and mammals, but the molecular basis of this link is yet to be uncovered.

**Energy homeostasis:** The low affinity of inositol hexakisphosphate kinases towards ATP (K$_m$ ~1 mM) (Saiardi et al. 1999; Volgmaier et al. 1996; Wundenberg and Mayr 2012), lies in the same range as cellular ATP levels, reflecting the fact that 5-IP$_7$ levels can be influenced by changes in intracellular ATP concentration (Choi et al. 2008; Nagel et al. 2010). Yeast lacking 5-IP$_7$ and mouse embryonic fibroblasts lacking IP6K1 display dysfunctional mitochondria, leading to a reduction in ATP synthesis through the electron transport chain (Szejgiyarto et al. 2011).

In yeast, inositol pyrophosphates regulate the transcription of major glycolytic pathway genes by pyrophosphorylating and reducing the activity of the transcription factor Gcr1. Therefore, the loss of 5-IP$_7$ in kcs1Δ yeast leads to increased...
transcription of glycolytic enzymes, thereby increasing ATP synthesis via glycolysis (Sziggyarto et al. 2011). Put together, these observations suggest that inositol pyrophosphates are influenced by cellular ATP levels and act in a feedback mechanism to maintain the levels of ATP by controlling the glycolytic vs mitochondrial metabolic ratio (Sziggyarto et al. 2011).

Ribosome biogenesis: Yeast nucleolar proteins Nsr1 and Srp40 are targets of pyrophosphorylation by inositol pyrophosphates (Bhandari et al. 2007; Saiardi et al. 2004). These proteins are involved in the localisation of box C/D snoRNA, which in turn are required for ribose methylation in rRNA (Verheggen et al. 2001). It was shown that deletion of Kcs1 in budding yeast is able rescue the cold sensitivity of an rrs1 mutant, which shows a defect in assembly of the 60S ribosome subunit (Horigome et al. 2014). kcs1Δ yeast grown at 16°C showed reduced levels of polysomes assembled on mRNA compared to 80S monosomes (Horigome et al. 2014). A recent study by our group revealed that rRNA Pol I, the enzyme responsible for synthesis of rRNA is pyrophosphorylated by IP$_7$, and that rRNA synthesis and ribosome levels are reduced in the absence of inositol pyrophosphates (Thota et al. 2015). This observation suggested that rRNA Pol I pyrophosphorylation by 5-IP$_7$ is required for its activity. Cellular ATP levels determine not only the synthesis of IP$_7$, but also the synthesis of ribosomes, as this process accounts for 80% of a cell’s ATP consumption. We speculate that IP$_7$ acts as a mediator molecule, signalling the availability of ATP to RNA Pol I to pursue rRNA synthesis.

Chromatin remodelling: In yeast, inositol pyrophosphates function parallel to the TORC pathway to regulate the class I histone deacetylase, Rpd3L, thus regulating chromatin remodelling in response to stress or starvation signals (Worley et al. 2013). In mammals, IP6K1 is associated with chromatin and binds the histone demethylase JMJD2C (Burton et al. 2013). IP$_7$ synthesised by IP6K1 indirectly inhibits the activity of JMJD2C, leading to specific alterations in histone methylation and acetylation in cells lacking IP6K1 (Burton et al. 2013).

Other functions of inositol pyrophosphates: In mammals, the antiviral immune response pathway is positively regulated by 1-IP$_7$, which enhances the production of type 1 interferon by increasing the phosphorylation and activation of the transcription factor IRF3. A non-hydrolysable analogue of IP$_7$ was not able to recapitulate this effect, suggesting that IP$_7$ may act by pyrophosphorylation of IRF3 (Pulloor et al. 2014). Yeast that lack Kcs1 show decreased expression of Ino1, the inositol-3-P synthase which catalyses the rate limiting step in inositol synthesis from glucose-6-P. This suggests that inositol pyrophosphates regulate their own metabolism by regulating the levels of inositol (Ye et al. 2013). Yeast and mammals with reduced IP$_7$ levels display defects in autophagy (Nagata et al. 2010; Taylor et al. 2012). Ip6k1 knockout male mice are sterile and display a severe reduction in mature spermatozoa in the epididymis, suggesting that this enzyme or its product 5-IP$_7$ is essential for spermatogenesis (Bhandari et al. 2008). IP$_7$ synthesised by mammalian IP6K2 sequesters the protein kinase LKB1 leading to enhanced cell migration and tumour metastasis (Rao et al. 2015).

4. A perspective on the future of inositol pyrophosphate research

Inositol pyrophosphates display pleiotropic effects in cells by regulating various biological processes as a consequence of binding to or pyrophosphorylating many different proteins. Since the discovery of inositol pyrophosphates in 1993, research has been focused mostly on their physiological implications rather than the regulation of their synthesis. The synthesis of inositol pyrophosphates depends on availability of ATP (Nagel et al. 2010; figure 3), and it is therefore speculated that IP$_7$ can act as a ‘metabolic messenger’ or ‘energy biosensor’ to co-ordinate between energy flux and signalling pathways (Shears 2009, 2015; Wilson et al. 2013). For example, in yeast, absence of IP$_7$ results in a reduction in ribosome synthesis, a process that consumes 80% of the cell’s energy (Thota et al. 2015). Inositol pyrophosphates also regulate the HDAC Rpd3L, thereby affecting gene expression in phosphate starvation, glycolysis, ribosome biogenesis and environmental stress response pathways (Worley et al. 2013). Inositol pyrophosphate levels influence the levels of polyphosphate (Auesukaree et al. 2005; Ghosh et al. 2013; Lonetti et al. 2011), a macromolecule which also depends on ATP for its synthesis. Progression through the cell cycle from G1 to S phase is accompanied by a gradual increase in the levels of inositol pyrophosphates (Banfic et al. 2013), and it is known that transition through the G1/S checkpoint is dependent on the energy status of the cell (Finkel and Hwang 2009). Thus, inositol pyrophosphates could act on many cellular pathways simply by transducing ATP levels. On the other hand, it is also likely that the enzyme activity and localisation of inositol pyrophosphate kinases and hydrolases is regulated by different signals to influence inositol pyrophosphate levels and subcellular localisation (Shears 2015; Thomas and Potter 2014). The low cellular concentration of IP$_7$ and IP$_8$ suggests that their synthesis should occur in close proximity to the proteins that will be pyrophosphorylated or provide docking sites for their binding. This would ensure only when inositol pyrophosphate kinases are localised to the target proteins and this localisation may in turn depend on upstream regulators. Therefore, future studies should focus
on determining the regulation of expression, activity, subcellular localisation and interacting partners of the enzymes that synthesise and degrade inositol pyrophosphates, and link this information to the protein targets through which inositol pyrophosphates influence individual pathways governing cell physiology.

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