Review

The emerging roles of inositol pyrophosphates in eukaryotic cell physiology

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Inositol pyrophosphates are water soluble derivatives of inositol that contain pyrophosphate or diphosphate moieties in addition to monophosphates. The best characterised inositol pyrophosphates, are IP_7 (diphosphoinositol pentakisphosphate or PP-IP_5), and IP_8 (bisdiphosphoinositol tetrakisphosphate or $(PP)_2$-IP_4). 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.

[Thota SG and Bhandari R 2015 The emerging roles of inositol pyrophosphates in eukaryotic cell physiology. *J. Biosci.* **40** 593–605] **DOI** 10.1007/s12038-015-9549-x

1. Structure of inositol and inositol phosphates

Inositol or cyclohexane-1,2,3,4,5,6-hexol is a carbohydrate with the formula $C_6H_{12}O_6$, 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 (IP_6) (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 (IP_8); cell signalling; diphosphoinositol pentakisphosphate (IP_7); inositol hexakisphosphate (IP_6) kinase (IP6K); PP-IP_5 kinase (PPIP5K); pyrophosphorylation

Published online: 13 August 2015

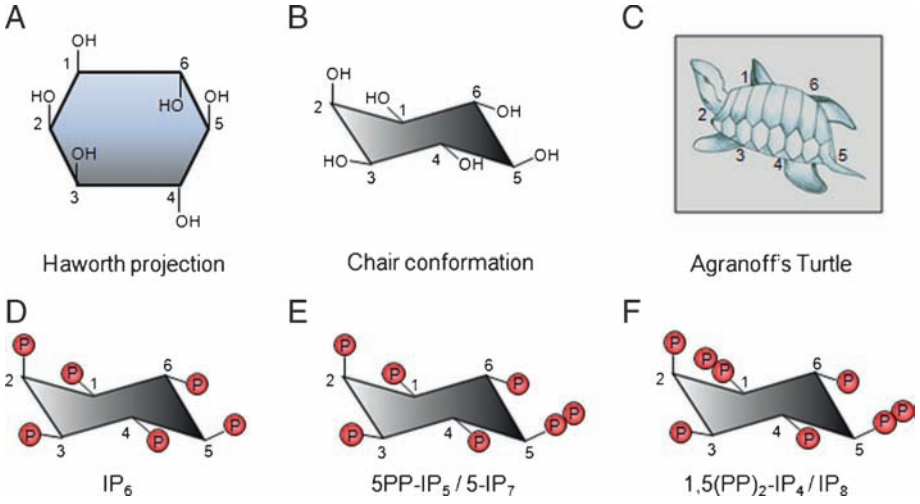

Figure 1. Structure of inositol and inositol pyrophosphates. (**A**) Inositol conformation as suggested by Norman Haworth. (**B** and **C**) Inositol chair conformation (**B**) that is comparable with a turtle (**C**). (**D**, **E** and **F**) Structures of inositol hexakisphosphate (**D**), diphosphoinositol pentakisphosphate (**E**), and bisdiphosphoinositol tetrakisphosphate (**F**).

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, Plc1 (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)P_3, and then phosphorylated by ITPK1 to I(1,3,4,6)P_4. This is acted upon by IPMK to make I(1,3,4,5,6)P_5.

IP_6 is the primary substrate for the synthesis of the major cellular inositol pyrophosphates, IP_7 and IP_8 [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; Saiardi 2012a, b; Shears 2007, 2009, 2015; Shears *et al.* 2011; Thomas and Potter 2014; Tsui and York 2010; Wilson *et al.* 2013; Wundenberg and Mayr 2012)]. IP_7 and IP_8 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 α phosphate at position 5 on IP_6 to make 5PP-I(1,2,3,4,6)P_5 (5-IP_7) (Draskovic *et al.* 2008; Lin *et al.* 2009; Saiardi *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 (Saiardi *et al.* 1999; Saiardi *et al.* 2001; Thomas and Potter 2014). The single *S. cerevisiae* IP_6 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 Symington 1995), and later identified as an orthologue of mammalian IP6Ks (Saiardi *et al.* 1999). Sequence and structure based analyses reveal that IP3K, IPMK, and IP6K belong to the same inositol phosphate kinase superfamily, which possess 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; Saiardi *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 (K_m 1.34–1.7 mM) compared to IP_6 (K_m 0.6–3 μM) and ATP (K_m 0.7–1.4 mM) (Saiardi *et al.* 2000a; Wundenberg *et al.* 2014) and can also function as ATP synthases by donating a phosphate from 5-IP_7 or IP_6 to ADP *in vitro* (Voglmaier *et al.* 1992; Wundenberg *et al.* 2014). Under normal conditions, the physiological concentration of ATP and IP_6 are 1 mM and 10–100 μM respectively, and therefore IP6K drives synthesis of 5-IP_7 from IP_6 in cells. IP6Ks can modulate their enzymatic activity to either phosphorylation or dephosphorylation of IP_6, depending on the ATP/ADP ratio in cells. An increase in the ATP/ADP ratio results in synthesis of 5-IP_7 from IP_6, whereas a reduction in the ATP/ADP ratio results in synthesis of ATP by dephosphorylating IP_6 (Wundenberg *et al.* 2014).

In *S. cerevisiae*, the IP_7 kinase Vip1 synthesizes IP_8 [1,5$(PP)_2$–I(2,3,4,6)P_4] by adding a phosphate at position 1 on 5-IP_7 (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 PPIP5Ks, that synthesize IP_8 from 5-IP_7 (Choi *et al.* 2007; Fridy *et al.* 2007; Tsui and York 2010; Wang *et al.* 2012). Vip1 can also function as an IP_6 kinase and synthesize 1PP-I(2,3,4,5,6)P_5 (1-IP_7) from IP_6, which can then be converted to IP_8 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 *Schizosaccharomyces pombe*, the phosphatase activity of this domain has been shown to reduce the synthesis of 1-IP_7 from IP_6, 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 IP_8 from IP_7. It was shown that an enzyme responsible for the synthesis of IP_7 from IP_6 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 IP_7 and IP_8, whereas deletion of Vip1 causes an increase in 5-IP_7 levels (Onnebo and Saiardi 2009). This observation suggests that in yeast, the predominant pathway for IP_8 synthesis from IP_6 is via 5-IP_7, and that 5-IP_7 is more abundant compared with 1-IP_7. In yeast and in mammals there are other minor inositol pyrophosphates, 5PP-IP_4, 1PP-IP_4 and 1,5$(PP)_2$-IP_3, synthesized from IP_5 (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 IP_5 kinase which synthesises IP_6 (Saiardi *et al.* 2002). IP_6 is the preferred substrate over IP_5 for the synthesis of inositol pyrophosphates due to its high physiological concentration and higher affinity of the IP6Ks for IP_6 (Wundenberg and Mayr 2012). All mammalian IP6Ks can add a γ phosphate to 5-IP_7 to make 5PPP-IP_5, 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 (diphosphoinositol 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 IP_7 and IP_8 to form IP_6, and on PP-IP_4 and $(PP)_2$-IP_3 to form IP_5 (figure 2).

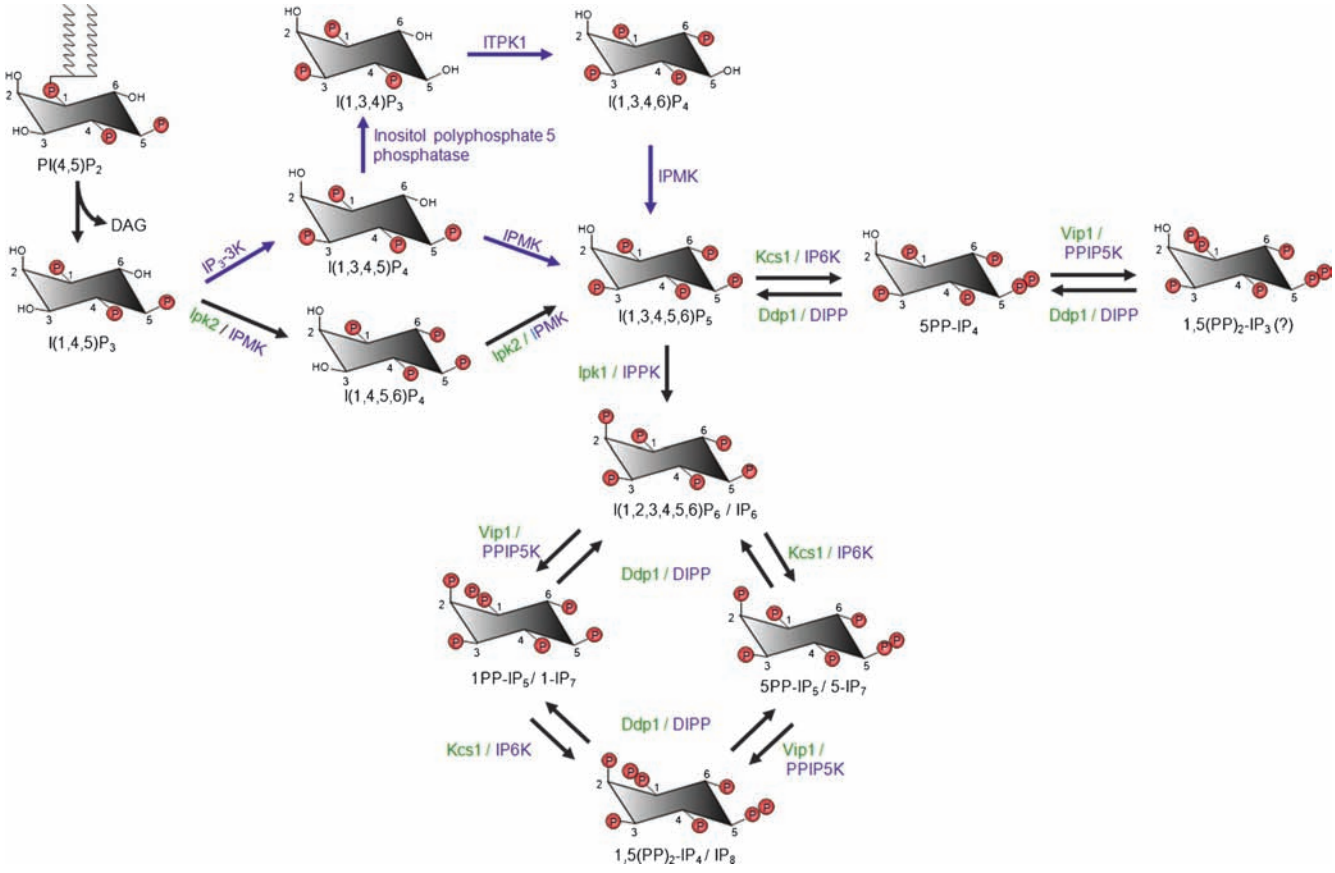

Figure 2. Pathway of synthesis of inositol pyrophosphates. Synthesis of inositol pyrophosphates, starting with the generation of $I(1,4,5)P_3$ from $PI(4,5)P_2$. The pathway that occurs in both yeast and mammals is depicted in black arrows and the pathway that is exclusive to mammals is shown in blue arrows. Yeast enzymes are represented in green; mammalian enzymes are represented in blue; the undetermined inositol pyrophosphate structure is indicated with a question mark.

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_5 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

PAGE analysis showing steady state levels of 60 μM IP_7 and 180 μM IP_8 in *Dictyostelium discoideum* (Pisani *et al.* 2014).

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 inositide PI(3,4,5)P_3 to specifically bind pleckstrin homology (PH) domains and inhibit downstream PIP_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 IP_6, 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; Saiardi *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: kcs1*Δ yeast that have decreased levels of IP_7 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).

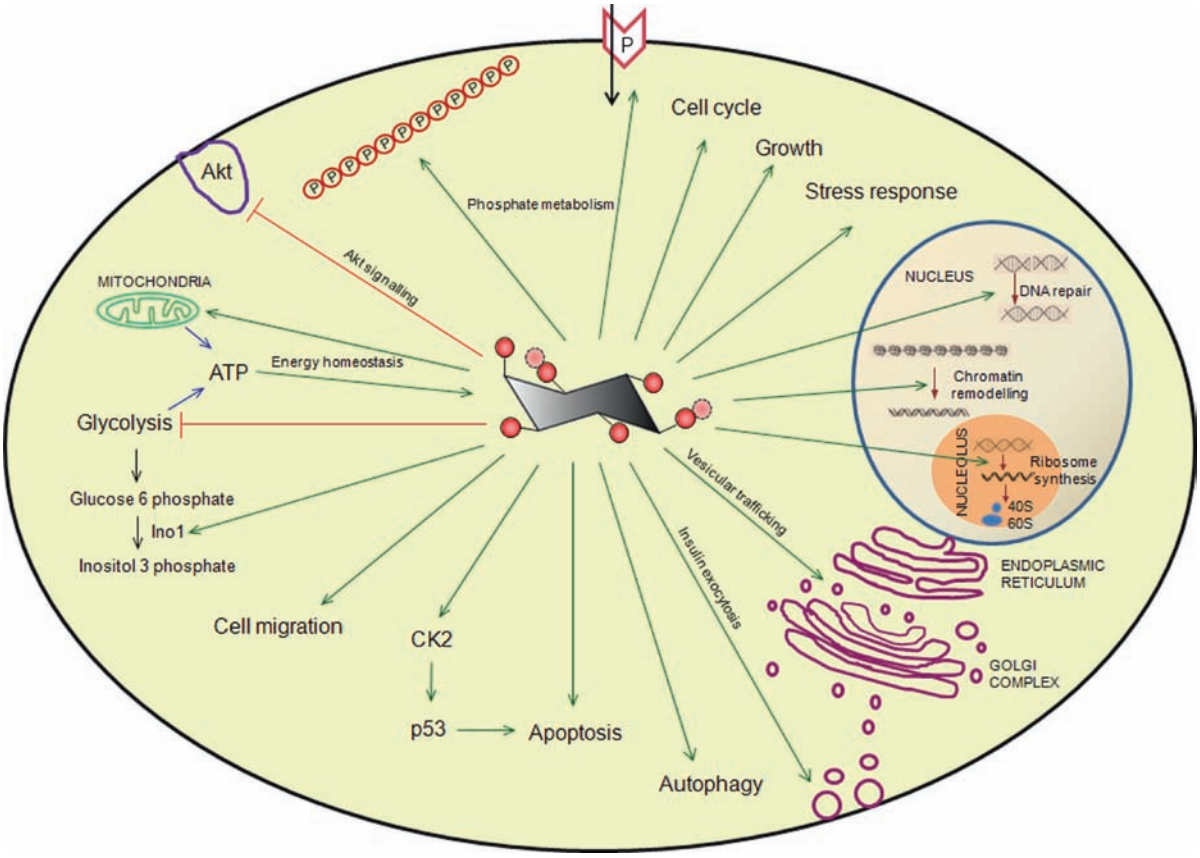

Figure 3. Diagrammatic representation of the cellular functions inositol pyrophosphates. The inositol pyrophosphate in the centre of a eukaryotic cell represents 5-IP_7, 1-IP_7 or IP_8. Red lines depict negative regulation and green arrows represent positive regulation of signalling or metabolic pathways by inositol pyrophosphates. ATP positively influences the intracellular levels of inositol pyrophosphates.

Resistance to salt stress and cell wall integrity also require IP_7 (Dubois *et al.* 2002). In mammalian cells, IP_8 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*Δ and *vip1*Δ 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*Δ 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 S 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 isomer 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 *vip1*Δ 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 *kcs1*Δ 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; Voglmaier *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 (Szijgyarto *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 *kcs1*Δ yeast leads to increased

transcription of glycolytic enzymes, thereby increasing ATP synthesis via glycolysis (Szijgyarto *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 (Szijgyarto *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). *kcs1*Δ 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 RNA 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 RNA 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 synthesized 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 ensue 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 synthesize and degrade inositol pyrophosphates, and link this information to the protein targets through which inositol pyrophosphates influence individual pathways governing cell physiology.

Acknowledgements

We thank Manasa Chanduri for invaluable feedback during the writing of this review. We thank Shubhra Ganguli and Pallavi Chinnu Varghese for artwork. The Bhandari Lab is supported by the Wellcome Trust/Department of Biotechnology India Alliance (WT/DBT IA; 500020/Z/09/Z) and Centre for DNA Fingerprinting and Diagnostics core funds. SGT is a recipient of a research fellowship from the Department of Biotechnology toward the pursuit of a Ph.D. degree of the Manipal University. RB is supported by a senior fellowship from the WT/DBT IA.

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MS received 11 May 2015; accepted 06 July 2015

Corresponding editor: Veena K Parnaik