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Zebrafish inositol polyphosphate kinases: new effectors of cilia and developmental signaling

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Introduction

Signal transduction pathways direct changes in cellular programs in response to environmental cues. One such pathway is inositol signaling, which involves phosphatidylinositol (PI) lipids and soluble inositol polyphosphate (IP) messengers (Berridge, 1993). Inositol signaling is a critical intermediate step in a number of receptor-mediated signaling pathways that involve G protein-coupled and cytokine receptors and receptor tyrosine kinases. In response to receptor stimulation, IP production is initiated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by phospholipase C (PLC), producing diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) (Fig. 1) (Irvine, 2003). IP₃ is sequentially phosphorylated by the coordinated actions of specific kinases to produce more highly phosphorylated IP molecules, including inositol tetrakisphosphate (IP₄) isomers,

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- Inositol hexakisphosphate
- Intraflagellar transport

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inositol 1,3,4,5,6-pentakisphosphate (IP₅), inositol hexakisphosphate (IP₆), and inositol pyrophosphate isomers (e.g., PP-IP₄, IP₇) (Fig. 1) (Alcazar-Roman and Wentz, 2008; York, 2006). In addition to acting as the linchpin for the soluble IP production, PIP₂ links the IP pathway to the lipid PI signaling pathway. Perturbation of the PI pathways is linked to multiple pathophysiological states including neurological disorders and cancers of the brain, prostate, and skin (Pendaries *et al.*, 2003).

The pathways for IP production and the cellular IP repertoire are fairly well conserved from yeast to mammals, with the noted diversification and expansion in metazoan enzymatic steps between IP₃ and IP₅. In the yeast *Saccharomyces cerevisiae*, the IP kinase Ipk2 successively phosphorylates I(1,4,5)P₃ on the 6 and then 3 positions producing I(1,3,4,5)P₄ and I(1,3,4,5,6)P₅, which is followed by Ipk1 phosphorylation of the 2 position of I(1,3,4,5,6)P₅ to produce IP₆ (Odom *et al.*, 2000). In humans and other mammals, however, I(1,4,5)P₃ is first phosphorylated by I(1,4,5)P₃ 3-kinase to produce I(1,3,4,5)P₄. This I(1,3,4,5)P₄ isomer is then dephosphorylated by an IP 5-phosphatase to produce I(1,3,4)P₃, for phosphorylation by I(1,3,4)P₃ 5/6-kinase to produce I(1,3,4,6)P₄, the key entry point to the synthesis of all other highly phosphorylated IPs (Verbsky *et al.*, 2005b). The Ipk2 orthologues (IPMK in rat (Saiardi *et al.*, 2001), Ipk2 in mouse (Frederick *et al.*, 2005), and I(1,3,4,6)P₄ 5-kinase (Chang *et al.*, 2002) in human) act on this I(1,3,4,6)P₄ to produce IP₅. The IP₆-kinase Kcs1 in yeast (and mammalian orthologs IHPK1, IHPK2, and IHPK3) convert IP₆ to diphosphoinositol pentakisphosphate (5-PP-IP₅ or IP₇), and IP₅ to PP-IP₄ (Mulugu *et al.*, 2007; Saiardi *et al.*, 2000; Saiardi *et al.*, 1999). These IP₆k enzymes are also involved in the production of bisdiphosphoinositol tetrakisphosphate (PP₂-IP₄ or IP₈). A second class of IP₆/IP₇ kinases that has one isoform in yeast, termed Vip1 and two in humans, acts as a 1/3-kinase contributing to the synthesis of 1/3-PP-IP₅ and 1/3,5-(PP)₂-IP₄ (Choi *et al.*, 2007; Fridy *et al.*, 2007; Lin *et al.*, 2009; Mulugu *et al.*, 2007). Together, the unique activities of these inositol kinases make a versatile repertoire of IPs in a eukaryotic cell. Whereas an extensive literature exists on IP₃ functions, precise cellular functions and roles in vertebrate development and disease are unknown for many of the other IPs. This article provides a summary and analysis of current advances in the IP field in the context of disease and development.

The paradigm and a new perspective

The perturbation of IP synthesis pathways is linked to defects in nutrient homeostasis in fungi (Lee *et al.*, 2007; Mulugu *et al.*, 2007; Odom *et al.*, 2000), apoptosis and insulin secretion in mammalian cells (Illies *et al.*, 2007; Morrison *et al.*, 2001; Nagata *et al.*, 2005), and developmental defects in vertebrates (Frederick *et al.*, 2005; Sarmah *et al.*, 2005; Sarmah *et al.*, 2007; Verbsky *et al.*, 2005a; Wilson *et al.*, 2009). From more than a decade of research, we now know that IPs regulate multiple cellular activities including nonhomologous end joining in DNA repair, chromatin remodeling and transcription, mRNA export, telomere length regulation, exocytosis, RNA editing, translation, Ca²⁺ channels, and ciliary beating and length maintenance (Alcazar-Roman and Wentz, 2008; Bolger *et al.*, 2008; Illies *et al.*, 2007; Irvine and Schell, 2001; Macbeth *et al.*, 2005; Saiardi *et al.*, 2005; Sarmah *et al.*, 2007; Shears, 2004; Shen *et al.*, 2003; Steger *et al.*, 2003; York, 2006; York *et al.*, 1999; York *et al.*, 2005).

Despite the widespread requirement of the IPs, we are only beginning to understand how cells use the IP pathways to elicit selective and diverse responses. Recent reports from different laboratories have pinpointed multiple distinct cellular processes modulated by specific IP binding to target proteins. IP₆ binds to the yeast mRNA export factor Gle1 to activate the DEAD-box ATPase Dbp5 during nuclear mRNA export (Alcazar-Roman *et al.*, 2006). IP₆ binds to the human RNA editing enzyme, ADAR2, and is required for ADAR

protein stability and enzymatic activity (Macbeth *et al.*, 2005). IP₆ is present in the structural core of TIR1 ubiquitin ligase suggesting a role of the IP pathway in the plant hormone auxin signaling (Tan *et al.*, 2007). Arrestin-2 oligomerization and cellular localization are reportedly regulated by IP₆ binding (Milano *et al.*, 2006). On the other hand, IP₇ binds to yeast cyclin-dependent kinase (CDK) inhibitor, Pho81, to inhibit cyclin-CDK activity during nutrient homeostasis (Lee *et al.*, 2008; Lee *et al.*, 2007). IPs are also reported to regulate the activity of the serine/threonine kinase casein kinase 2 (Solyakov *et al.*, 2004). These interesting findings support a paradigm wherein IPs regulate multiple cellular processes through direct IP binding and modulation of target protein activity.

Inositol polyphosphates connect to developmental/extracellular signaling

Several previous studies in yeast, *Dictyostelium*, and mammalian cell systems have identified potential signaling roles for IPs in controlling a number of physiological processes. The *S. cerevisiae* cellular response to phosphate starvation is dependent on the production of 1/3(PP)-IP₅ (or, IP₇) for inhibition of the cyclin/cyclin-dependent kinase (Lee *et al.*, 2008; Lee *et al.*, 2007; Lin *et al.*, 2009). In *Dictyostelium*, the chemoattractant cAMP binding to a G protein-coupled cAMP receptor facilitates localized synthesis of PI(3,4,5)P₃, where it recruits a subset of PH domain-containing proteins and triggers downstream signaling pathways leading to chemotaxis (Funamoto *et al.*, 2002). Interestingly, IP₇ isomers compete with PI(3,4,5)P₃ for binding to PH domain-containing proteins mediating chemotaxis (Luo *et al.*, 2003). On the other hand, IP₇ is required for insulin exocytosis in pancreatic β cells, where IP₇ is maintained at a high concentration allowing rapid adjustment of insulin secretion in response to receptor-stimulus coupling (Illies *et al.*, 2007). This might link to the finding that the IP₆ kinase 1 (IHPK1) gene locus is disrupted in a family with type 2 diabetes mellitus (Kamimura *et al.*, 2004).

Although the functional connections between IP flux and extracellular signaling have not yet been fully defined, several recent studies highlight the existence of such a link. The activation of G α_q , a component of a trimeric G protein required for G-protein coupled receptor (GPCR) activity, leads to stimulation of the IP₃ to IP₈ pathway (Otto *et al.*, 2007). It is possible that such a change in the IP levels brings signaling complexity and specificity in response to environmental signals or agonists of GPCR activity. Interestingly, Wnt3a activation of Frizzled-1 receptor causes a transient increase in IP₅ levels, and depletion of IPMK (also known as Ipk2) or IP₃k inhibits the ability of Wnt3a to stimulate the canonical β -catenin/lymphoid enhancer factor/T cell factor pathway (Gao and Wang, 2007). Upon binding to Frizzled receptors, which are members of the GPCR superfamily, Wnt ligands regulate several critical aspects of early development, including cell fate determination, proliferation, and embryonic patterning (Clevers, 2006). It is significant that the Wnt3a effect involves G α_q activation that triggers IP production and subsequent activation of downstream pathway.

Another interesting potential functional relationship could be between the hedgehog signaling, GPCR activity, and the IP pathway. When expressed in frog melanophores, human Smoothened (Smo) activates G α_i -mediated signaling (DeCamp *et al.*, 2000). Moreover, the G12 family of heterotrimeric G proteins and the small GTPase RhoA are involved in hedgehog signal transduction (Kasai *et al.*, 2004). Hedgehog signaling plays an essential role in embryonic development and patterning, and adult tissue and stem cell functions (Ingham and Placzek, 2006). The pathway is activated when extracellular hedgehog ligands (e.g. Sonic hedgehog) bind to the Patched (Ptc) receptor relieving its inhibitory effect on Smo. This allows Smo to send signals through a series of interacting proteins, including “suppressor of fused”. This results in activation of the Gli family of transcription factors, the key mediators of the cellular response to the signal. The

coexpression of Smo with the G protein α subunit G15 in human cells activates PLC, suggesting a functional coupling between the three (Masdeu *et al.*, 2006). Together, the findings provide compelling evidence for associations between the activation of G proteins, the production of IP₃, and key developmental signaling pathways.

Taking the studies to the zebrafish system

These studies, primarily conducted in yeast and cell culture systems, have allowed significant insights into the role of IP₃ in multiple vital cellular processes and signaling pathways, and lay a foundation for future research on IP signaling in multicellular organisms. Although it is known that IP₃ production is initiated in response to activation of receptor-mediated signaling pathways including GPCRs, and that IP₃ undergoes sequential phosphorylation by multiple kinases, it is not clear how production of these metabolites is precisely regulated. What are the extracellular signals involved in activating IP pathways? How are IP levels regulated within cells? What are IP target proteins during embryonic body organization, organogenesis, and tissue/cell type specific differentiation and physiology? How do cells use IP pathways to elicit selective and diverse responses during development? Addressing these complex questions warrant an integrated approach combining knowledge from studies in multiple models.

Given their multiple cellular roles, several years ago, we hypothesized that IP₃ play critical roles in development and disease of multicellular organisms. To test this, we established a robust model system by combining zebrafish embryology and imaging with cell biological studies in mammalian cell culture systems. Zebrafish provides an excellent embryological model system with its distinct experimental advantages, such as: external fertilization, optical clarity, robustness of embryos, and rapid development. These embryological attributes facilitate high-resolution cell biological analysis in real time, allowing *in vivo* examination of cell movements, shape changes, proliferation, and cell–cell interactions. To begin our studies, we identified the zebrafish orthologs of several known IP kinases (Ipk) by interrogating the zebrafish GenBank database for transcripts orthologous to human IP-kinase sequences (Table 1). As in humans, the IP pathway in zebrafish has multiple Ipk enzymes intervening between IP₃ and IP₅ synthesis (Table 1). However, these pathways again apparently converge on a lone IP₅ 2-kinase, Ipk1 (Sarmah *et al.*, 2005; Verbsky *et al.*, 2002). The conservation of different Ipk at the overall protein sequence level is high (~52–81%) (Table 1), suggesting that the potential functional and/or regulatory diversification is minimal between zebrafish and human. Never the less, the Ipk enzymes have highly conserved putative catalytic site motifs and display functional crossspecies complementation (Ives *et al.*, 2000; Sarmah *et al.*, 2005; Sarmah and Wente, 2009; Verbsky *et al.*, 2002). In order to test IP requirements during organogenesis and development, we systematically depleted the IP components (i.e., IP kinases) by injecting into 1-cell stage embryos with morpholino antisense oligonucleotides (MOs) that impair translation or splicing. By analyzing the resulting phenotypes, we uncovered multiple key developmental events that are controlled by the IP-based signaling mechanisms.

The IP pathway regulates multiple aspects of zebrafish development

IP₆ production is required for left-right axis specification

Our initial studies focused on Ipk1, the lone IP kinase that catalyzes synthesis of IP₆ from IP₅ and the most conserved step in the IP pathway. Our interest on this enzyme also stemmed partly from the fact that IP₆ is one of the most abundant IPs within cells (Bunce *et al.*, 1993; Sarmah and Wente, 2009; York *et al.*, 1999). We observed that *ipk1* is expressed in zebrafish embryos during early developmental stages (Sarmah *et al.*, 2005). The *ipk1* mRNA was maternally deposited, and was ubiquitously distributed throughout blastula

stages of embryogenesis. During gastrulation, *ipk1* was highly expressed in the deep involuted cells that contribute to mesendoderm. At late gastrula and early segmentation stages, axial mesendoderm expressed *ipk1*. Expression was also detected in cells enveloping KV, a structure orthologous to mouse node.

To determine the metabolic effects of Ipk1 depletion on IP production, we coinjected the embryos with the loss-of-function morpholino reagent and 3H-inositol to label the IP pool. We extracted the IPs at 11.5 hr post fertilization and analyzed the composition by HPLC. Interestingly, IP₃ was the predominant steady-state isoform in wild-type embryos with minimal levels of other IPs. In contrast, Ipk1 depleted embryos had marked increases in IP₄ and IP₅ levels with no detectable IP₆. Increased upstream IP levels are an established indicator of inhibited IP₆ production in *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Drosophila melanogaster* S2 cells (Ives *et al.*, 2000; Sarmah and Wentle, 2009; Seeds *et al.*, 2004; York *et al.*, 1999). Thus, the Ipk1 depletion results in a loss of IP₆ production and a general perturbation of the IP synthesis pathway.

The first surprise in our research came when we found that depletion of IP₆ levels in zebrafish embryos causes randomization of the placement of internal organs (Sarmah *et al.*, 2005). The zebrafish embryos with reduced Ipk1 levels failed to place the liver, pancreas, and heart correctly (Fig. 2). These embryos were unable to orient left-right (LR) asymmetry properly, causing inversion of LR anatomy (situs inversus) in ~50% of the population (Fig. 2A). All vertebrates, including humans, are outwardly bilaterally symmetrical. However, they have a conserved LR asymmetry manifested in the position and anatomy of the heart, visceral organs, and brain. To achieve this final body plan, during early vertebrate embryogenesis the left and right sides are distinguished and this information is transmitted to individual organs as they develop. This LR-axis specification is a complex, multi-step process, transforming an early embryonic bilateral symmetry to a LR anatomical asymmetry (Capdevila *et al.*, 2000; Wright, 2001).

To date, all vertebrates exhibit asymmetric expression of homologous genes prior to the situs specific morphogenesis. For example, left lateral plate mesoderm expresses genes encoding intercellular signaling molecules such as nodal, lefty1, and lefty2, and the homeobox transcription factor gene *pitx2*, collectively known as LR-specific genes (Hamada *et al.*, 2002). We found that the expression of LR-specific genes was randomized in Ipk1 depleted embryos. There was also a defect in generating an evolutionarily conserved, transient left-biased Ca²⁺ flux in cells enveloping the Kupffer's vesicle (KV), a transient structure with a ciliated epithelium orthologous to the mouse 'node' (Sarmah *et al.*, 2005). This asymmetric Ca²⁺ transient is reportedly the first observed molecular LR asymmetry in mice (McGrath *et al.*, 2003). In Ipk1 depleted embryos, we did not observe such Ca²⁺ transients. Previous studies have implicated IP₆ as a regulator of Ca²⁺ channel activity (Larsson *et al.*, 1997; Lemtiri-Chlieh *et al.*, 2003; Yang *et al.*, 2001). IPs are also excellent candidates for low-molecular-weight determinants that could move through gap junctions to influence rapid amplification and propagation of Ca²⁺ signals across cell fields in the embryo.

Ipk1 activity is required for ciliary beating and length maintenance

Motile cilia in the mouse node play a critical role in the initial events of the LR body axis specification and in generating counterclockwise fluid flow in the node (Hirokawa *et al.*, 2006; Nonaka *et al.*, 1998; Okada *et al.*, 1999). Similarly, in zebrafish, proper KV morphogenesis, ciliary motility, and directional fluid flow are essential for LR-axis specification (Essner *et al.*, 2005; Kramer-Zucker *et al.*, 2005). In mice, the fluid flow generated by motile cilia has been proposed to bend mechanosensor immotile cilia in the node, triggering a left-sided Ca²⁺ flux (McGrath *et al.*, 2003). Alternatively, cilia-driven

fluid flow might result in a morphogen concentrating toward the nodal left side to influence intracellular signaling (Hirokawa *et al.*, 2006). Nevertheless, both proposed mechanisms are based on a crucial role for nodal ciliary function in LR-axis specification. Because Ca^{2+} flux in cells surrounding the KV was altered and LR asymmetry randomized in Ipk1 depleted embryos (Sarmah *et al.*, 2005), it was critical to investigate ciliary structures and functions in fine detail. Doing so, we observed that the beating of cilia in the KV was substantially altered in Ipk1 depleted embryos. In wild-type embryos, cilia beat with a vortical motion in a counterclockwise orientation. In contrast, in Ipk1 depleted embryos, cilia were immotile (Sarmah *et al.*, 2007). Significantly, the ciliary beating defect in Ipk1 depleted embryos was rescued by coinjection of wild-type *ipk1* mRNA. Moreover, co-injection of mRNA encoding kinase-dead Ipk1 failed to complement this motility defect. This indicates that Ipk1 kinase activity is critical for ciliary beating. We also observed a dose-dependent decrease in the length of cilia in multiple organs, including KV, in Ipk1 depleted embryos. These observations strongly suggest that Ipk1 plays a key role in ciliary motility and length maintenance.

ipk1 is genetically linked to ciliary ift components

Cilia are membranous extensions with microtubule cores extending from basal bodies by intraflagellar transport (IFT) along axonemal microtubules (Praetorius and Spring, 2005; Scholey, 2003). Motile cilia have a characteristic “9+2” axoneme with nine microtubule doublets, two central microtubule singlets, radial spokes linked to the microtubules, and inner and outer dynein arm motors (Praetorius and Spring, 2005). Ciliary beating results from the sliding of outer microtubule doublets relative to one another, powered by the motor activity of axonemal dynein (Ibanez-Tallon *et al.*, 2003). IFT is required for ciliary assembly, motility, and length maintenance (Rosenbaum and Witman, 2002; Scholey, 2003). Blocking IFT results in cilia shortening (Marshall and Rosenbaum, 2001; Rosenbaum and Witman, 2002).

First, we investigated ciliary axonemal structures in multiple organs and found that they were not grossly altered in Ipk1 depleted embryos. Mutations in genes encoding ciliary microtubule-based motor proteins, such as Kif3a (Marszalek *et al.*, 1999; Takeda *et al.*, 1999), Kif3b (Nonaka *et al.*, 1998), Lrd (Supp *et al.*, 1999), and the raft protein complex component Polaris /IFT88 (Murcia *et al.*, 2000) result in LR asymmetry defects. In zebrafish, depletion of either the IFT88 or IFT57 proteins perturb normal LR asymmetry and ciliary length in KV and other organs without altering normal ciliary 9 + 2 organization (Bisgrove *et al.*, 2005; Kramer-Zucker *et al.*, 2005). These phenotypes associated with IFT defects correlated with our observations on the Ipk1 depleted embryos. Thus, we investigated if *ipk1* is genetically linked to *ift* component(s). Strikingly, co-depletion of Ipk1 and intraflagellar transport (IFT) proteins (IFT88 or IFT57) had synergistic perturbations of LR asymmetry suggesting that these act on the same or parallel pathways underlying ciliary functions. We also found that exogenously expressed GFP-Ipk1 is enriched in centrosomes and basal bodies in mammalian cells. We propose that Ipk1 is an important component of the basal body, and that localized IP_6 production is essential for ciliary function and maintenance (Fig. 5). These findings connecting IP production to the motile cilia will impact future studies on cell signaling and ciliary function.

ipk1 has a global ciliary role

We speculated that a cilia-specific function for Ipk1 would affect the development and proper function of other ciliated organs besides the KV. Dysfunctional cilia have been implicated in polycystic kidney disease (Drummond, 2005; Rosenbaum and Witman, 2002), and disruption of zebrafish ciliary structure or motility results in luminal expansion of kidney tubules and pronephric cyst formation (Kramer-Zucker *et al.*, 2005). To investigate if

there was a defect in kidney (pronephros) structure, we performed histological studies on sections of wild-type and *Ipk1* depleted embryos (Fig. 3). The pronephros, which consists of a glomerulus, pronephric tubules, and paired pronephric ducts, is the primary blood filtration and osmoregulatory organ in free-swimming zebrafish larvae (Drummond, 2005). Interestingly, we observed that the pronephric tubules and ducts in *Ipk1* depleted embryos were dilated compared to uninjected embryos (B.S. and S.R.W., unpublished). The tubular perimeter in the *Ipk1* depleted embryos was 2-fold dilated compared to uninjected embryos. An expanded pronephric tubule lumen serves as an early marker for cyst formation in zebrafish (Drummond, 2005). We also found that the majority (61.6%, $n=120$) of the *Ipk1* depleted embryos had a distinctive ventrally curved body axis (Fig. 3), and a significant fraction developed pronephric cyst (20% of the embryos) (B.S. and S.R.W., unpublished). These are also common features of embryos deficient in *IFT88/polaris* and *IFT57/hippi* (Kramer-Zucker *et al.*, 2005), and many zebrafish cystic kidney mutants (Drummond, 2005).

On the other hand, cilia in the spinal canal function to maintain normal cerebrospinal fluid distribution. Impaired ciliary function could cause fluid build-up and distension of the brain ventricles or hydrocephalus (Kramer-Zucker *et al.*, 2005). This was indeed observed in the *Ipk1* depleted embryos, with hydrocephalus and pericardial edema found in 35% of the embryos (Fig. 3) (B.S. and S.R.W., unpublished). Taken together, these findings support the hypothesis that *Ipk1* has a conserved ciliary role in multiple organs and its deficiency results in diverse pathologies associated with defective cilia.

Interestingly, the loss of *Ipk1* in mice is lethal, and the embryos die before day 8.5 postcoitum (Verbsky *et al.*, 2005a). Mouse embryos express *ipk1* in the notochord, the ventricular layer of the neural tube, the myotome of the somites, and in the yolk sac. In adult mice, *ipk1* expression is detected in the hippocampus, cortex, Purkinje layer of the cerebellum in the brain, cardiomyocytes, and testes. The failure to develop yolk sac is suggested to cause the early lethality of *ipk1* null mice. Interestingly, IP_5 and $PP-InsP_4$ also accumulated in the heterozygous mouse embryonic fibroblasts compared to wild-type cells. This correlates with our own findings that IP levels change as a consequence of *Ipk1* knockdown in zebrafish embryos.

IP pathway is linked to craniofacial development

We have also started testing other components of the IP pathway that act upstream or downstream of *Ipk1*. IP_6 kinases (ortholog of yeast Kcs1) catalyze synthesis of a number of IPs both upstream and downstream of *Ipk1* (Mulugu *et al.*, 2007; Saiardi *et al.*, 2000; Saiardi *et al.*, 1999) (Fig. 1). When IP_6K2 was depleted in embryos, the ciliary motility in the KV cilia was not affected and the LR morphogenesis was normal. This indicates a distinct role for IP_6 in motile cilia. Strikingly, we found that zebrafish embryos depleted of IP_6K2 develop deformed craniofacial structures with severe reduction of cartilage elements (Fig. 4). These craniofacial defects were not observed in *Ipk1* depleted embryos. The majority of head skeleton is derived from cranial neural crest cells (NCCs). The neural crest is a migratory cell population that detaches from the embryonic neural epithelium along the dorsal neural tube and populates various regions of the body differentiating into a broad range of cell types including neurons and glia of the peripheral nervous system, cartilage and bones of the face, and melanocytes (Knecht and Bronner-Fraser, 2002). Cranial NCCs arise from fore- and hindbrain regions and give rise to the frontonasal skeleton and pharyngeal arches (Santagati and Rijli, 2003).

We investigated three key stages in early NCC development: initial induction/specification, maintenance, and migration. In the IP_6K2 depleted embryos, NCCs were properly specified but defective in maintenance and migration. In the depleted embryos, NCCs from mid- and hindbrain regions migrated aberrantly toward the optic vesicle and ventral-side, respectively.

Additionally, streams of NCCs that normally migrate ventrally from the neural tube into the trunk were also abnormal and reduced. Notably, in addition to craniofacial defects, IP₆k2 depleted embryos were defective in somite and slow muscle development. In contrast to chevron-shaped somites in wild-type embryos, IP₆k2 depleted embryos developed U-shaped somites, reminiscent of reduced hedgehog signaling. Hedgehog signaling also provides positional cues for cranial NCC migration (Tapadia *et al.*, 2005; Wada *et al.*, 2005).

As the craniofacial defect is uniquely present in IP₆k2 depleted embryos and absent in Ipk1 depleted embryos, we speculated that IP₆k2 production of PP-IP₄ from IP₅ is critical for craniofacial development. Moreover, as the phenotypes displayed by IP₆k2 depleted embryos are hallmarks of disrupted hedgehog signaling in zebrafish (Barresi *et al.*, 2000; Blagden *et al.*, 1997; Eberhart *et al.*, 2006; Stickney *et al.*, 2000; Wada *et al.*, 2005), we hypothesized that efficient hedgehog signal transduction requires IP₆k2 function. To investigate if the IP₆k2 depleted embryos lacked a hedgehog signaling response during their development, we examined the expression of two key hedgehog target genes, *gli1* and *patched1*, both of which are also genes encoding essential components of the Hedgehog signal transduction pathway. Strikingly, we observed that the expression of both the genes was significantly down-regulated in IP₆k2 depleted embryos. A role for this IP kinase in development and tissue patterning was not previously known and its link to the hedgehog pathway might impact future studies of both hedgehog and inositol signaling.

Developmental roles for other IP kinases

The cellular level of IP₆ is tightly connected with the upstream and downstream components of the IP metabolic pathway. We hypothesized that these IPs have distinct or overlapping roles with IP₆ that are critical for embryonic development and organogenesis including LR morphogenesis. We have recently identified genes encoding zebrafish homologues of I(1,3,4)P3 5/6-kinase, Ipk2, and Vip-1 & 2 kinases that synthesize IP₄, IP₅, IP₇ and IP₈ isomers, respectively (Fig. 1, Table 1). We have started testing roles of these kinases in ciliary function, LR asymmetry, and other aspects of zebrafish development. We anticipated that the knockdown of genes that encode enzymes upstream of Ipk1 (such as IP₃ 5/6-kinase and Ipk2) might phenocopy the *ipk1* knockdown phenotype. However, there could be additional effects with graded severity reflecting their positions in the pathway. Additionally, knockdown of downstream components might not produce phenotypes similar to that of *ipk1* knockdown if it is related to a IP₆ specific function.

By injecting embryos with a splicing block morpholino oligonucleotide to deplete *ipk2* mRNA levels, we reproduced the *ipk1* knockdown phenotypes in left-right asymmetry. On the other hand, we found that IP₃ 5/6-kinase was essential for embryonic development as the embryos injected with *ip3-5/6-k* translation blocking morpholino oligonucleotides died prior to completing segmentation period. In mice, *ipk2* is reported to be essential (Frederick *et al.*, 2005). The *ipk2* null embryos die around embryonic day 9.5 with multiple morphological defects, including small body size, no visible somites, and abnormal folding of the neural tube. Recently, a study showed that mice embryos homozygous for a hypomorphic *itpk1* allele encoding I(1,3,4)P3-5/6-kinase are viable but display neural tube and axial skeletal defects, and growth retardation (Wilson *et al.*, 2009). The homozygous mutant embryos have lower levels of the protein than their wild-type and heterozygous littermates, potentially resulting in a less severe phenotype. Together, these discoveries pinpoint multiple essential roles for the IP-kinases during embryonic development and patterning.

IPs potentially constitute a cilia-based cellular signaling mechanism

Cilia act as cellular antennas to sense developmental (extracellular) signaling molecules (Eggenchwiler and Anderson, 2007; Singla and Reiter, 2006). These primary cilia, which

could also be mechano- and/or chemosensory in nature, coordinate numerous signaling pathways including hedgehog and Wnt signaling that regulate embryonic development and patterning, and tissue repair and maintenance in adults (Gerdes *et al.*, 2009). On the other hand, motile cilia that occur on cells of respiratory epithelium, female reproductive tract, brain ependyma, and nodal (KV) epithelium generate flows of mucus or fluid critical for normal health and development. Consequently, defects in ciliary structure and functions lead to severe developmental diseases and disorders, commonly referred to as ciliopathies. This includes polycystic kidney disease, *situs inversus*, Kartagener syndrome, and Bardet-Biedl syndrome (Marshall, 2008).

Several recent studies have implicated the PI pathway in cilia biogenesis and functions. The depletion of PI(4,5)P₂ in developing *Drosophila* male germ cells inhibits sperm flagellar biogenesis due to inherent defects in microtubule organization and basal body docking to the nuclear envelope (Wei *et al.*, 2008). Other PIs (e.g., PI(3)P) have also been suggested to have role in ciliary biogenesis. The BBS5, a component of the BBSome required for ciliogenesis, binds specifically with PI(3)P (Nachury *et al.*, 2007). PIs are known to be important for vesicular transport, remodeling of actin cytoskeleton, and signal transduction (Di Paolo and De Camilli, 2006). Mutations in the gene encoding inositol polyphosphate-5-phosphatase E (INPP5E) were identified in individuals with Joubert syndrome, a ciliopathy characterized by multiple defects including retinal dystrophy, fibrosis and polydactyly. INPP5E hydrolyzes the 5-phosphate of PI(3,4,5)P₃ and PI(4,5)P₂ and the mutations abolish enzyme activity resulting in altered PI levels. Interestingly, INPP5E localizes to cilia and the mutations perturb ciliary maintenance (Bielas *et al.*, 2009). It is noteworthy that PIs (e.g., PI(4,5)P₂) immediately upstream of the soluble IP pathway are required for ciliary biogenesis and/or function.

In zebrafish, we showed that the production of IP₆ is critical for the generation of asymmetric Ca²⁺ flux in the KV and establishing LR asymmetry (Sarmah *et al.*, 2005). Specifically, IP₆ production is required for ciliary beating and length maintenance in zebrafish KV. Motile cilia are critical for establishing the LR body axis as well as generating asymmetric Ca²⁺ flux in mouse node (Hirokawa *et al.*, 2006; McGrath *et al.*, 2003; Nonaka *et al.*, 1998; Okada *et al.*, 1999). Thus, IP₆ production required for normal ciliary movement might be essential for the induction of the Ca²⁺ flux. However, it remains possible that IP₆ production also independently contributes to Ca²⁺ signaling by amplifying and propagating the signal across cell fields independent of its cilia-specific role. Nevertheless, IPs, intracellular Ca²⁺ flux, and cilia together form a key signaling mechanism that mediates LR-axis specification. We speculate that IP₆ contributes to IFT by acting as a cofactor for an IFT, cilia, or basal body protein and allowing for their optimal activity. IFT is essential for ciliary assembly, motility, and maintenance (Rosenbaum and Witman, 2002; Scholey, 2003). As such, in the absence of IP₆ production, we speculate that IFT is compromised resulting in immotile and shortened cilia.

In addition to documenting specific roles for IP₆ production in zebrafish development and ciliary functions, we also uncovered a new role for IP₆k2 in craniofacial development and neural crest cell migration, and in hedgehog signal transduction. Interestingly, primary cilia are essential for transduction of the Hedgehog signal in mammals (Oro, 2007). Many of the essential hedgehog signaling components, such as Gli2, Gli3, Smo and Ptc, localize to primary cilia. It is speculated that regulated movement of key proteins into and out of the cilium generates a switch by which cells can turn this powerful signaling on and off during development and tissue homeostasis (Christensen and Ott, 2007; Rohatgi *et al.*, 2007). A protein similar to IP₆-kinase is found in the *Chlamydomonas* flagellar proteome (Pazour *et al.*, 2005). We propose that in IP₆k2 depleted NCCs, hedgehog signal transduction is perturbed due to a potential defect in their sensory cilia.

Based on our findings and multiple connections documented by other studies discussed above, we propose a model wherein IPs constitute a novel cilia-based signaling mechanism (Fig. 5). Within the cilia, we speculate that the pathway is activated downstream of a GPCR (based on the distinct connection of GPCR with the induction of the IP pathway (Otto *et al.*, 2007)). A number of GPCRs have been identified in the cilia of many cell types including neurons (Reviewed in (Berbari *et al.*, 2009)). Recent studies have linked IPs with components of the Wnt and hedgehog signaling pathways (Gao and Wang, 2007; Masdeu *et al.*, 2006). Primary cilia have key regulatory roles in both of these signaling pathways (reviewed in (Berbari *et al.*, 2009)). And lastly, lipid PIs, upstream to the IP metabolic pathway have distinct roles in cilia biogenesis and/or function (Bielas *et al.*, 2009; Nachury *et al.*, 2007; Wei *et al.*, 2008). As presented in Fig. 5, the IP metabolic pathway, potentially activated in response to extracellular stimuli, would mediate both ciliary functions and cilia-based signaling mechanism(s). We predict that such an IP-based regulation involves modulation of target protein activity through IP binding.

Multiple outstanding questions still remain: 1) What are the mechanism(s) through which Ipk1 activity regulates ciliary function? Here we propose that Ipk1 and/or IP₆ contribute to IFT by acting as a cofactor for an IFT, cilia, or basal body protein and allowing for its optimal activity. 2) How does IP₆k2 activity modulate NCC development and migration, and hedgehog signal transduction? We predict that IP₆k2 is required for adequate functioning of the hedgehog pathway components (e.g., Smo, Gli factors) or the ciliary switch for regulating hedgehog signal transduction. A common theme emerging from these findings is that an IP-based ciliary signaling mechanism might underlie the establishment of LR asymmetry and craniofacial development. Motile cilia (e.g., those in KV) require IP₆ for their motility, whereas, primary, non-motile cilia (e.g., those present in hedgehog responsive cells) might require PP-IP₄ to function as a sensory organelle.

Summary

We described here our recent findings that Ipk1 catalyzed production of IP₆ regulates LR-axis specification (Sarmah *et al.*, 2005) and that IP₆ is an essential effector of ciliary beating and length maintenance in zebrafish (Sarmah *et al.*, 2007). We have also uncovered a novel role for the IP kinase IP₆k2 in craniofacial development, neural crest cell migration, and hedgehog signal transduction (B.S. and S.R.W., unpublished). Together, these findings place IP production as a key mediator for cellular signaling mechanisms that regulate vital cellular and developmental processes. How these and other IPs are integrated with cell-cell signaling networks during complex processes, such as, tissue morphogenesis and maintenance of cell fate and function? We propose that with its enormous resource and unique set of structural, functional, and sensory attributes, cilium provide a platform for executing IP-based signaling functions. Given the evolutionary conservation of the IP repertoire and pathways, the developmental and molecular events uncovered in our studies in the zebrafish system could be applicable in other vertebrates including humans. This unbiased approach of systematic identification of IP functions in cilia and development will aid in understanding of multiple disease pathologies including ciliopathies and dysmorphic syndromes.

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Fig. 1. The pathways for synthesis of soluble inositol polyphosphates (IPs) in yeast and vertebrates
 IP production is initiated with the synthesis of IP₃ through PLC catalyzed hydrolysis of PI(4,5)P₂. IP₃ undergoes sequential modifications by specific kinases and phosphatases to produce more highly phosphorylated IP molecules, including inositol tetrakisphosphate (IP₄) isomers, inositol 1,3,4,5,6-pentakisphosphate (IP₅), inositol hexakisphosphate (IP₆), and inositol pyrophosphate isomers (e.g., PP-IP₄, IP₇, and IP₈). The pathways intervening between IP₃ and IP₅ are diversified across species. In yeast, IP₃ is sequentially phosphorylated to IP₅ by the dual-specificity kinase Ipk2, whereas, three specific kinases and a phosphatase are involved in the production of IP₅ from IP₃ in vertebrates (see text for details). Black arrows indicate PLC-driven hydrolysis and IP kinase-driven phosphorylation, and grey arrows indicate dephosphorylation reactions.

Fig. 2. The production of IP₆ is required for left-right (LR) axis specification and normal placement of body organs

(A) Normal asymmetry of the heart tube placement is randomized in *Ipk1* depleted zebrafish embryos. Live fluorescent and DIC overlay images at 28 hours post fertilization (hpf) of uninjected embryos (left) and embryos injected with the *ipk1* morpholino (*ipk1*^{MO}) (right) that were expressing *Tg(cmlc2-EGFP)* (Huang *et al.*, 2003) for EGFP throughout the myocardium. Nearly 50% of the *ipk1*^{MO} injected embryos display reversed (right-sided) heart tube placement in contrast to its consistent left-sided placement in the uninjected embryos. (B) Model displaying connections between *Ipk1*/IP₆ production, cilia, and left-biased Ca²⁺ signaling during LR-axis specification. LR-axis specification emerges from three positional cues: anterior (a) - posterior (p) axis, dorsal-ventral axis, and chirality of the nodal (KV) ciliary beating. Once the LR-axis is specified, the LR information is relayed through the action of a set of long-range signaling molecules (e.g., nodal, lefty1, and lefty2) and induction of the homeobox transcription factor gene *pitx2* culminating in situs-specific morphogenesis. The left-biased Ca²⁺ signaling induced by ciliary beating acts as the connector between cilia and LR signaling molecules. Lefty1 functions as a midline barrier restricting LR signals to the left. Shown here are KV cilia (in red) detected by anti-acetylated tubulin immunohistochemistry superimposed on a live fluorescent image displaying left-biased Ca²⁺ flux (in green as revealed by flash-pericam Ca²⁺ indicator protein). Our studies reveal that *Ipk1* activity is essential for KV ciliary beating. Thus, loss

of *Ipk1* might directly impact the LR cascade downstream of cilia. On the other hand, *ipk1* is expressed in cells enveloping KV and it might mediate expansion of asymmetric Ca^{2+} flux, initiated by motile KV cilia, across cell fields. This could drive asymmetric expression of signaling molecules. Bright-field low magnification image (ventral view) of a 12 hpf embryo showing the KV is presented on the bottom-right.

Fig. 3. Ipk1 depleted zebrafish embryos display curved body axis and dilation of pronephric tubules

Disruption of *ipk1* function by *ipk1*^{MO} injection results in ventrally curved body axis at 72 hpf (B) compared to uninjected embryos (A). *ipk1*^{MO} embryos also develop pronephric cyst, hydrocephalus, and pericardial edema at 5 dpf (not shown). Histological cross sections of an uninjected embryo (C) and an *ipk1*^{MO} embryo at 72 hpf (D) show the midline fused glomerulus, pronephric tubules (red arrowhead), and paired pronephric ducts (yellow arrowhead) on either side. *ipk1*^{MO} embryos show dilation of pronephric tubules (red arrowhead) and pronephric ducts (yellow arrowhead).

Fig. 4. The IP kinase IP₆k2 is required for the normal development of craniofacial skeleton (A,B) Live images of uninjected (A) and *ip6k2*^{MO} injected zebrafish embryos at 5 days post fertilization (dpf). (C,D) Images of alcian blue stained head skeletons of uninjected (C) and *ip6k2*^{MO} injected (D) embryos at 5 dpf. The flat-mount (ventral) images display normal pharyngeal skeleton and parts of the neuroocranium of the uninjected embryos (C). In the IP₆k2 depleted embryos, most of the cartilage elements of the pharyngeal skeleton are not formed and have a shortened neuroocranium (D). e, eye; m, Meckel's cartilage; pq, palatoquadrate; ch, ceratohyal; cb 3–7, ceratobranchials arches 3 to 7; eth, ethmoid plate; nc, notochord.

Fig. 5. IPs might act as effectors of a versatile cilia-based signaling network

Presented here is a model showing that IPs, after synthesized in response to an activated GPCR, transduce extracellular signals to cilia and/or cell anterior. In primary cilia, PP-IP₄ plays an as “yet undefined” role during hedgehog signaling response, controlling critical developmental and morphogenetic events (e.g., craniofacial and somite developments). On the other hand, IP₆ acts as an effector of ciliary motility and/or IFT. The IP₆ role(s) in motile cilia are essentially required for key developmental processes (e.g., LR-axis specification) as well as maintenance and functions of organs (e.g., kidney).

Table 1

IP kinase (Ipk) homologs in yeast *S. cerevisiae*, zebrafish, and human

IP-kinase	Yeast <i>S. cerevisiae</i>	Human	Zebrafish	Identity *	Similarity *
IP ₃ /6-k	None	1	1 (IP ₃ /6-k)	63.28%	85.99%
Ipk2	1	1	1 (Ipk2)	52.16%	75.24%
Ipk1	1	1	1 (Ipk1)	55.60% (Ipk1)	82.48% (Ipk1)
IP ₆ -k	1 (Kcs1)	3	2 (IP ₆ k1, IP ₆ k2)	72.33% (IP ₆ k1)	89.34% (IP ₆ k1)
				61.33% (IP ₆ k2)	81.20% (IP ₆ k2)
IP ₆ /IP ₇ -k	1	2	2 (Vip1, Vip2)	66.43% (Vip1)	83.32% (Vip1)
				81.26% (Vip2)	91.32% (Vip2)

* Predicted amino acid sequences of zebrafish and human IP kinases were analyzed by ClustalW program (Thompson *et al.*, 1994) and % protein identity (identical residues) and similarity (Identical, conserved, and semiconserved residues) were determined.