Chapter 5

ROLE OF PHOSPHATIDYLINOSITOL 5-PHOSPHATE 4-KINASE α IN ZEBRAFISH DEVELOPMENT

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ABSTRACT

Phosphatidylinositol 5-phosphate 4-kinases (PIP4Ks) phosphorylate phosphatidylinositol 5-phosphate (PI5P) to generate phosphatidylinositol 4,5-bisphosphate; their most likely function is the regulation of the levels of PI5P, a putative signalling intermediate. There are three mammalian PIP4Ks isoforms (α, β and γ), but their physiological roles remain poorly understood. In the present study, we identified the zebrafish orthologue (zPIP4Kα) of the high-activity human PIP4K α isoform and analyzed its role in embryonic development. RT-PCR analysis and whole-mount in situ hybridization experiments showed that zPIP4Kα is maternally expressed. At later embryonic stages, high PIP4Kα expression was detected in the head and the pectoral fins. Knockdown of zPIP4Kα by antisense morpholino oligonucleotides led to severe morphological abnormalities, including midbody winding defects at 48 hpf. The abnormal phenotype could be rescued, at least in large part, by injection of human PIP4Kα mRNA. Our results reveal a key role for PIP4Kα and its activity in vertebrate tissue homeostasis and organ development.

Key words: Zebrafish; Development; Phosphoinositides; Phosphatidylinositol-phosphate kinase; Signalling

INTRODUCTION

Phosphoinositides are a minor but important class of membrane phospholipids that play a key role in a vast array of cellular processes. Through reversible interaction with specific phosphoinositide-binding domains, phosphoinositides can regulate protein localization and enzymatic activities, thereby regulating such diverse cellular processes as cytoskeletal dynamics, migration, vesicle trafficking, gene transcription and proliferation (Di Paolo *et al.*, 2006). Phosphatidylinositol (PI) can be phosphorylated at the 3', 4' and 5' position of the inositol head group by distinct lipid kinases, thereby generating seven different phosphoinositide species (Irvine, 2005). Among these, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is arguably the most versatile player in diverse signalling pathways, functioning not only as an intermediate in the IP3/diacylglycerol pathway and as substrate of PI 3-kinases, but also as a docking phospholipid and regulator of ion channel activity (Divecha, 2010; Doughman *et al.*, 2003; van den Bout *et al.*, 2009). Two distinct families of lipid kinases, the phosphatidyl inositol 5-phosphate 4 kinases (PIP4Ks) and phosphatidyl inositol 4-phosphate 5-kinases (PIP5Ks), catalyze the formation of PI(4,5)P2 through phosphorylation of PI5P and PI4P on the 4' and 5' position, respectively (Figure 1A). The PIP5Ks are well characterized and likely are the major contributors to the synthesis of PI(4,5)P2 (van den Bout *et al.*, 2009; Volpicelli-Daley *et al.*, 2010). The less well understood PIP4Ks are encoded by three distinct genes (α, β and γ), with the α isoform having much higher catalytic activity than the β and γ isoforms (Bultsma *et al.*, 2010; Clarke *et al.*, 2008; Wang *et al.*, 2010). Since cellular PI4P levels are much more abundant than those of PI5P (Sarkes *et al.*, 2010), the PIP4Ks are probably less important than the PIP5Ks in regulating the bulk of PI(4,5)P2. Thus, PIP4Ks are thought primarily to regulate the levels of their substrate PI5P (Bultsma *et al.*, 2010; Jones *et al.*, 2006; Keune *et al.*, 2012; Sarkes *et al.*, 2010) or to regulate a specific pool of PI(4,5)P2 (Morris *et al.*, 2000). Importantly, PI(5)P has the characteristics of a signaling intermediate. In particular, through interaction with specific protein domains, PI5P has been implicated in modulating chromatin structure and gene transcription (Alvarez-Venegas *et al.*, 2006; Gozani *et al.*, 2003; Ndamukong *et al.*, 2010), membrane trafficking (Lecompte *et al.*, 2008; Ramel *et al.*, 2011), cell migration (Oppelt *et al.*, 2012) and the regulation of oxidative stress responses (Jones *et al.*, 2012; Keune *et al.*, 2012).

The three known isoforms of PIP4K differ not only in catalytic efficiency but also in their subcellular localization and relative expression levels. PIP4Kα localizes to the cytosol, PIP4Kβ is found in the cytoplasm and in the nucleus (Ciruela *et al.*, 2000) and PIP4Kγ is localized to an as yet undefined intracellular membrane compartment (Clarke *et al.*, 2008; Itoh *et al.*, 2000). Recent evidence suggests that distinct isoforms can interact with each other and can affect one another's subcellular localization (Bultsma *et al.*, 2010; Wang *et al.*, 2010). Given their differential expression patterns, catalytic efficiency and subcellular localization, PIP4K isoforms may serve distinct functions depending on cell type and tissue context (Clarke *et al.*, 2012). Mice homozygously deleted for the PIP4Kβ gene are mildly growth retarded and hypersensitive to insulin (Lamia *et al.*, 2004). However, knockout or knockdown phenotypes of PIP4Kα and PIP4Kγ have not been reported to date.

In the present study, we use zebrafish as a model to examine the role of PIP4Ks in vertebrate embryonic development. We identified and cloned the zebrafish orthologue of human PIP4Kα

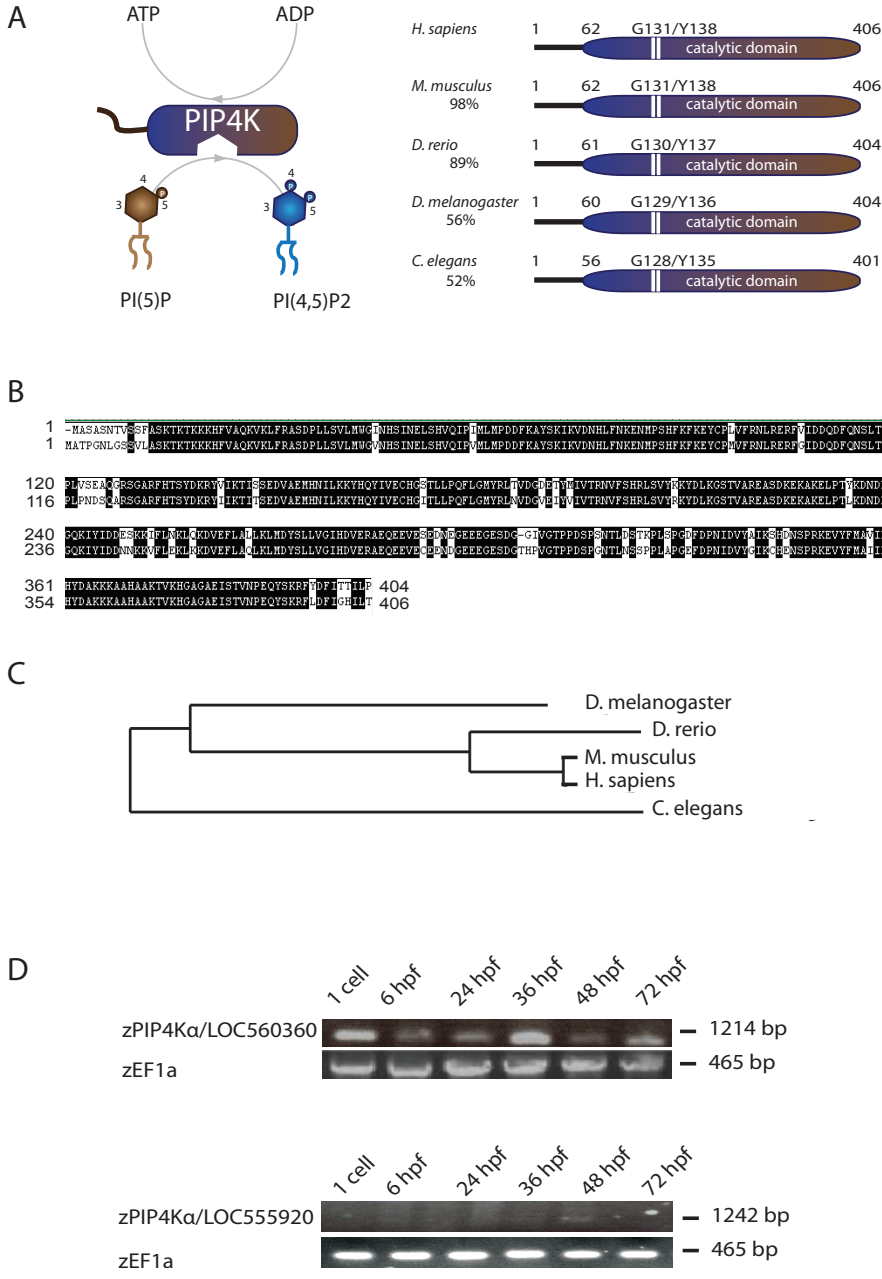

Figure 1. *Identification and developmental expression of zebrafish PIP4Kα. (A) Left panel: Scheme depicting the action of PIP4Kinases, catalyzing the phosphorylation of PI(5)P to PI(4,5)P2. Right panel: the presence of catalytic domain in zPIP4Kα and orthologs from different species. The percentage of sequence identity between human PIP4Kα and amino acids G131 and Y138, critical for catalytic activity, are also indicated. (B) Amino acid sequence comparisons between hPIP4Kα and zPIP4Kα. Sequence alignment using AlignX revealed that the predicted zPIP4Kα (ENSDARG00000003776) is* ▶

(zPIP4Kα) and describe its spatiotemporal expression during embryogenesis. We find that zPIP4Kα is maternally expressed. More importantly, phenotypic analysis of zebrafish depleted of zPIP4Kα by specific morpholino's (MOs) reveals anomalies involving primarily the development of the eye, heart and midbody axis. The importance of PIP4K activity was revealed in rescue experiments using kinase active and inactive hPIP4Kα. Thus, our study uncovers an important role for PIP4Kα in vertebrate development.

MATERIALS AND METHODS

Zebrafish maintenance

Zebrafish (*Danio rerio*) were raised and kept under standard laboratory conditions at 28°C as described previously (Westerfield, 2000). Experiments were performed in accordance with institutional guidelines and as approved by the Animal Experimentation Committee of the Royal Netherlands Academy of Arts and Sciences. Embryos were obtained from natural matings after initiation of the light cycle and staged based on hours post-fertilization (hpf) at 28°C as well as by morphological criteria (Kimmel *et al.*, 1995).

Molecular cloning of zebrafish PIP4Ks

Total RNA was isolated from zebrafish embryos at 36 hpf using Trizol reagent (Invitrogen) according to standard protocols. Total RNA was reverse transcribed using Superscript ™ II RT (Invitrogen). We searched for zebrafish genes with homology to human PIP4Kα, beta and gamma in public databases of the zebrafish genome (the Sanger Institute http://vega.sanger.ac.uk/Danio_rerio/Info/Index) and ESTs using the program BLAST/BLAT (Ensembl). On the basis of the BLAST searches, full-length sequence of predicted zebrafish PIP4Kα http://www.ensembl.org/Danio_rerio/Info/Index/) (ENSDARG00000003776) was amplified by PCR using the following primers F 5'- GGATCCATGGCCTCGGCTAGTAACAC-3' and R 5'-GAATTCCTAGGGCAGGATGGTAGTGA-3' and cloned into pCS2+ All cloning products were confirmed by Big Dye V3 sequencing.

Morpholino antisense oligonucleotide injections

Stock morpholino oligonucleotides (MOs, Gene Tools, LLC) were dissolved in sterile water. The diluted MOs (3.5 ng) were injected into one-cell stage zebrafish embryos. The sequence of ATGMO zPIP4Kα ACAGTGTTACTAGCCGAGGCCATTG was designed against the start codon/5'UTR to block translation and the splice MO was designed against exon 2 – intron 2 (AACAAGTGAGTTCCTCTGAGACGTT) and the standard control MO (Gene Tools) was used as control. The effective concentration for each morpholino was determined through dose-

► *89% identical to hPIP4Kα (identical amino acids shaded in black). (C) Phylogenetic analysis of zPIPK4α and homologues. Zebrafish zPIP4Kα is closely related to mammalian PIP4Kα. Protein sequences used: hPIP4Kα (NP_005019.2), mPIP4Kα (NP_473392.1), zPIP4Kα (NP_001122174.1), D. melanogaster PIP4Kα (NP_001033805.1), C. elegans PIP4Kα (NP_497500.1). (D) RT-PCR analysis monitoring expression of zPIP4Kα/LOC560360 during embryonic development and the indicated stages. Control for input cDNA, EF1α. PIP4Kα expression was detected at the one-cell stage, indicative of maternal contribution. Expression of predicted zPIP4Kα/LOC555920 was not detected.*

response experiments. Specificity of the PIP4Kα morpholino-induced knockdown phenotype was verified by co-injection of 5ng p53 MO (Gene Tools).To generate mRNAs for phenotypic rescue, full-length human PIP4Kα, PIP4KαKD (G131/Y138) and GFP were subcloned into the pCS2+ vector for mRNA synthesis. Capped mRNA was synthesized using the SP6 mMessage mMAchine Kit (Ambion). The mRNAs were then co-injected into MO-treated embryos at the one-two cell stage at 100 ng /µl.

RNA isolation and RT-PCR analysis

Total RNA was extracted from zebrafish embryos at 1 cell, 6 hpf, 24 hpf, 36 hpf, 48 hpf, 72 hpf, using Trizol reagent according to a standard manufacturer's protocol (Invitrogen). For the RT-PCR detection the following forward and reverse primers were used to amplify zPIP4Kα/LOC560360 F 5'- GGATCCATGGCCTCGGCTAGTAACAC-3' and R 5'-GAATTCCTAGGGCAGGATGGTAGTGA-3' ; zPIP4Kα/LOC555920F5'-GGATCCAATGGCCTCTGCAGCCAGCAGandR5'GAATTCTTAGGACAGAA TGGTGGTGA; *zEF1α* F5'-GGCCACGTCGACTCCGGAAAGTCC-3'andR5'-CTCAAAACGAGCCTGGCT GTAAGG-3'. PCR products were analyzed by agarose gel electrophoresis.

In situ hybridization and western blotting

For *in situ* hybridization, digoxigenin-labelled antisense riboprobes were synthesized from PCR-amplified full-length zPIP4Kα cloned into the pCS2+ vector with BamH1/EcoR1 using DIG RNA labelling mix (Roche). As a control, sense riboprobes were synthesized from the opposite strand. Whole-mount in situ hybridizations of zebrafish embryos was performed as described previously (Thisse *et al.*, 1993). Embryos for ISH were fixed in 4% paraformaldehyde in Phosphate-Buffered Solution overnight at 4°C PFA/PBS and stored in 100% methanol. To detect endogenous zPIP4Kα, protein extracts were prepared from 50-dechorionated zebrafish embryos. After lysis in 50 mM Tris pH 8, 10mM EDTA, 50mM KCl and 1% NP40 supplemented with protease inhibitor cocktail (Roche) for 20 min, samples were centrifuged and pellets were resuspended in RIPA lysis buffer containing complete protease inhibitor cocktail tablet (Roche) and 1 mM DTT. After sonication for 5 min, samples were centrifuged and supernatants were separated on 4-12% bis-TRIS precast gels (NuPage). After transfer to PVDF membranes (GE heathcare/Amercham), blots were probed with primary antibodies diluted in PBS with 0.01% triton-X (PBS-T) containing 3% BSA and 2% western blot blocking reagent (Roche). Mouse anti-β-actin antibody (1:5000) was from Abcam (ab6276). Human anti-PIP4Kα polyclonal antibody against peptide CNTLNSSPPLA was raised in rabbits. Horseradish peroxidase-conjugated secondary antibodies were used for detection. Proteins were detected using enhanced chemiluminescence (Pierce Supersignal west-dura ECL) followed by exposure to high-performance autoradiography films.

Immunofluorescence

Wildtype 7 dpf larvae were fixed in 40% ethanol, 5% acetic acid, and 10% formalin (EAF) for 2 h at room temperature, followed by three washes in PBS with Triton-X (PBT) before being embedded in 1.5% low melting agarose. Agarose pellets were dehydrated in ethanol, cleared in xylene, and processed into paraffin. For immunofluorescence analysis, sections were deparaffinized and hydrated, followed by microwave antigen retrieval for 15 min in preheated 1.9 mM citric acid, 8.2 mM sodium citrate, pH 6.0, or in preheated 10 mM Tris, 1 mM EDTA, pH 9.0. Sections

were blocked in PBS containing 5% normal goat serum for 30 min at room temperature and incubated in primary antibody overnight at 4°C. Anti-PIP4Kα polyclonal antibody was used as primary antibody (1:200) and anti-rabbit Alexa Fluor 568 was used as secondary antibody (1:200; Molecular Probes). Nuclei were stained with DAPI and were imaged using a Leica TCS SP2 laser scanning microscope.

PIP4K activity assay

Fifty morphant and control MO-injected zebrafish embryos (48 hpf) were dechorionated and pelleted. For enzyme purification, the extracts applied to a heparin agarose column. After washing, the column was eluted with 25 µl of 1M NaCl. PIP4K activity was measured using liposomes with 1 nmol of PI(5)P and 10 nmol of Pser (phosphatidylserine) as a substrate, 20 µM ATP, 10 µCi [^{32}P]ATP and the heparin sepharose eluate in 100 µl of PIP kinase buffer [50 mM Tris/HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM EGTA and 70 mM KCl] for 10 min as described previously (Jones *et al.*, 2006). [^{32}P]PI(4,5)P_2, the product of PIP4K, was separated by thin layer chromatography (TLC) and quantified using a phosphoimager (Bio-Rad).

RESULTS

Cloning and characterization of zPIP4Kα

To identify the zebrafish orthologs of the different isoforms of human PIP4Ks, we performed database searches (*Danio Rerio* Genebank genomic database) using the sequence of human PIP4K isoform α, β and γ. Analysis of the PIP4Kα isoform identified genomic locus LOC560360 on zebrafish chromosome 24. The predicted gene encodes a transcript that shows very high similarity to human PIP4Kα (77% at the RNA transcript level and 88% at the protein level Figure 1B). PIP4Kα has a highly conserved PIP kinase catalytic domain and has orthologs in many other organisms (Figure 1A; right panel). Phylogenetic analysis of amino acid sequences performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw/index.html) showed a close relationship of zebrafish PIP4Kα to mammalian PIP4Kα (Figure 1C). The LOC560360 gene is distributed among 10 exons, which is similar to the human equivalent, while the intron-exon boundaries are conserved as well. Due to partial genome duplication, the zebrafish genome often contains different paralogs of genes that are present in a single copy in mammals. In this case, a related gene for PIP4Kα, named "novel protein similar to vertebrate phosphatidylinositol-4-phosphate 5-kinase, type II" or LOC555920 was predicted and mapped to chromosome 2. To validate the predicted transcripts, the coding sequence was amplified and sequenced from RNA purified from zebrafish tissue. ClustalW sequence alignment of LOC560360 revealed 5 silent mutations that are probably due to genetic variation between different zebrafish strains. However, we could not retrieve any transcripts corresponding to LOC555920 from either adult fish or pools of embryos (Figure 1D). This suggested that LOC555920 might be a pseudogene; therefore, we set out to study the developmental expression of LOC560360 (hereafter referred to as zPIP4Kα). To investigate the temporal expression of zPIP4Kα during zebrafish development, we performed RT-PCR analysis on cDNA obtained from different developmental stages. A predicted PCR product of 1214 bp was detected by gel electrophoresis and its identity was confirmed by sequencing. zPIP4Kα was readily detectable at the one-cell stage, indicating maternal

deposition of zPIP4Kα, whereas no mRNA splice variants were detected in the expression analysis. zPIP4Kα expression was detected also at post-gastrulation stages; it peaked at 36 h post-fertilization (hpf) and remained expressed until 72 hpf, the latest developmental time point examined (Figure 1D). Ef-1α gene expression was amplified as a control for cDNA input.

In addition, through database analysis, we identified zebrafish orthologs of PIP4K isoforms β and γ and identified for both a predicted protein. Since mammalian PIP4Kβ shows approx. 2000-fold less catalytic activity than PIP4Kα and PIP4Kγ likely lacks catalytic activity (Bultsma *et al.*, 2010c; Wang *et al.*, 2010c), we focused our study on the zPIP4Kα isoform.

Spatiotemporal expression of zPIP4Kα during zebrafish development

We examined zPIP4Kα mRNA expression by whole-mount in situ hybridization (WISH) in embryos from the 4-cell stage to 48 hpf. A corresponding sense probe was used as negative control (Suppl. Figure S1). Similar to the RT-PCR results, WISH confirmed zPIP4Kα expression during early zebrafish development. zPIP4Kα was ubiquitously expressed from the 1-cell stage to gastrulation (Figure 2A). At 24 hpf, zPIP4Kα was abundant in the brain, CNS, retina and part of the somites. At 48 hpf, robust expression was found in the eye, CNS, brain and pectoral fin buds.

zPIP4Kα knockdown

To analyze the role of zPIP4Kα in zebrafish development, we inhibited the function of zPIP4Kα by injecting antisense morpholinos (MO) at the one-cell stage. We designed two different MOs targeting two different sites of the zPIP4Kα mRNA, so that either splicing or translation events would be disrupted. The splice morpholino (MO1) was aimed at impairing the exon 2- intron 2 splicing in nascent zPIP4Kα mRNA. The ATG MO (MO2) was designed to affect the translation process of both maternal and newly synthesized zPIP4Kα mRNAs by targeting the 5'UTR (Suppl. Figure S2). To test the efficacy of the knockdown approach, we assayed by western blotting the protein levels of zPIP4Kα in MO-injected embryos at 48 hpf. Because zPIP4Kα differed by only four amino acids in the epitope used to generate an antibody to hPIP4Kα we attempted to use this antibody to detect zPIP4Kα by Western blotting. Figure 2B demonstrates that, at 48 hpf, MO1 injection reduced zPIP4Kα expression, whereas zPIPK4α was undetectable in MO2-injected embryos. The region of the genomic DNA corresponding to the MO1 target site was cloned and sequenced to confirm the specificity of the splice MO. On the basis of the sequencing data, we can exclude polymorphisms that might hinder MO1 to bind specifically and efficiently to its target region (data not shown). Therefore, we conclude that the residual zPIP4Kα protein expression in MO1-injected embryos likely arises from translation of residual maternal mRNA.

Developmental effects of zPIP4Kα knockdown

Next, we investigated whether zPIP4Kα expression is critical for normal zebrafish development. We analyzed the effects of zPIP4Kα deficiency by comparing the gross phenotypic alterations produced by the MO1 and MO2 injections into one-cell stage fertilized eggs. MO1-injected embryos were practically indistinguishable from controls, while MO2 injection led to several phenotypic alterations (Figure 2C e-f and i-j). Control injections with an unrelated MO

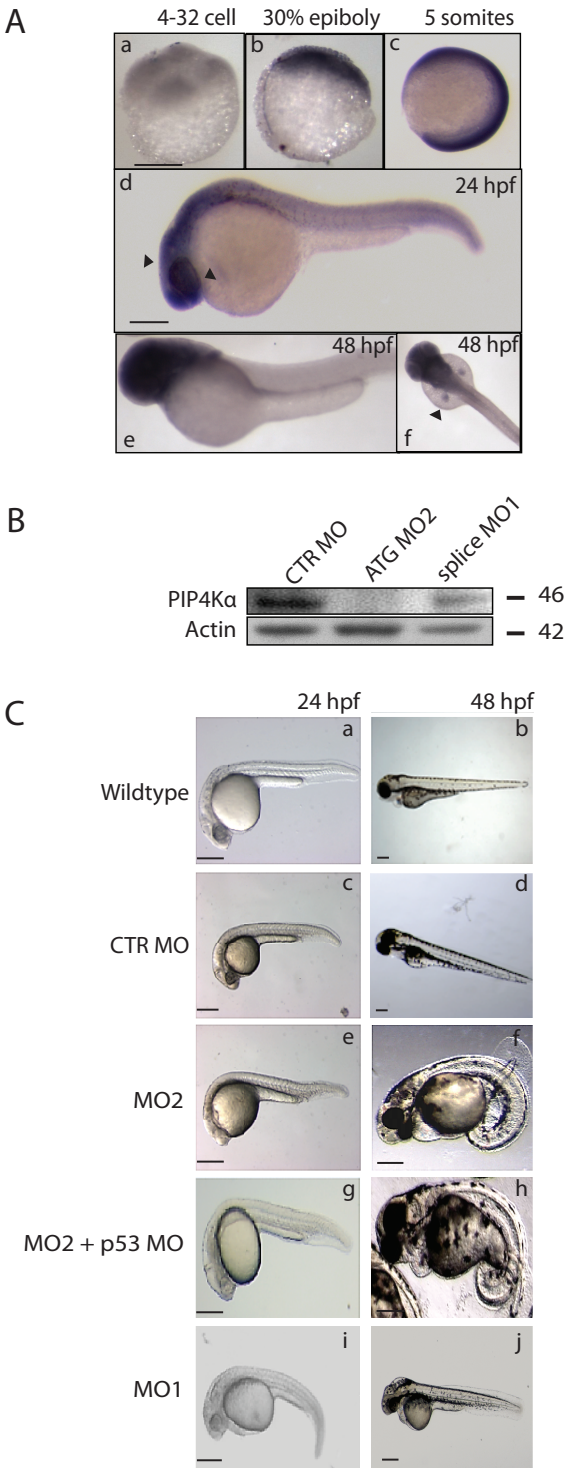

Figure 2. *Knockdown of PIP4Kα in zebrafish. (A) Whole-mount in situ hybridization at the indicated stages. (a) 4-32 cell stage; (b) 30% epiboly; (c) 5 somites; (d) 24 hpf; (e-f) 48 hpf. PIP4Kα mRNA expression was detected in the head region, tail, eyes and pectoral fins (arrowheads) in wild-type embryos. Scale bar, 250 µm. (B) Western blot analysis of two PIP4Kα morpholino-injected embryos. Protein expression PIP4Kα in control MO-injected embryos. Protein levels are strongly reduced in the PIP4Kα ATG MO2 morphants. Splice MO1 injected morphants only showed partial decrease in PIP4Kα expression. MW markers in kDa. (C) Morphological defects in PIP4Kα MO-mediated knockdown at 24 and 48 hpf. (a-b) wild-type non-injected embryos; (c-d) control MO-injected embryos show normal morphology; (e-f) PIP4Kα MO2 morphants display impaired midbody development and severe cardiac oedema; (g-h) co-injection of p53 MO did not affect the PIP4Kα phenotypes; (i-j) PIP4Kα MO1-injected embryos, showing normal morphology. Scale bar, 250 µm.*

(standard control MO, Gene Tools) led to embryos that developed normally and were identical to wild-type at 24 and 48 hpf (Figure 2C a-d). It has previously been shown that some ATG-directed MOs can activate the p53 pathway resulting in apoptosis (Robu *et al.*, 2007), a phenotype that is unrelated to the function of the gene targeted by the MO (off-target effects). To further confirm the specificity of the effects observed by PIP4Kα knockdown, we co-injected the zPIP4Kα MO2 with a p53 MO into zebrafish embryos and found that the embryonic defects of co-injected embryos did not differ from embryos injected with the zPIP4Kα MO2 alone (Figure 2C e-h). At 24 hpf, a variety of developmental defects were observed in the MO2 injected zPIP4Kα morphants. These included heart oedema, eye malformations and strikingly severe midbody axis curvatures (Figure 3). We classified the strong phenotype (severe) as having severe developmental deficiencies with midbody curvatures ranging from over 90 degrees to over 360 degrees and severe cardiac edema. The weak phenotype (mild) was determined as having features with degrees of midbody curvatures ranging till 90 degrees and enlarged pericardium with defects in cardiac looping (Figure 3). Microscopic examination of zPIP4Kα morphants (n=309) revealed that at a dose of 3.5 ng MO2, approximately 75% of injected embryos displayed a severe phenotype: about 20% showed a mild phenotype and <5% had no gross morphological defects (Table 1). However, these phenotypes were not evident in MO1-injected embryos (up to 5 dpf) and therefore were not investigated further (Figure 2C i-j).

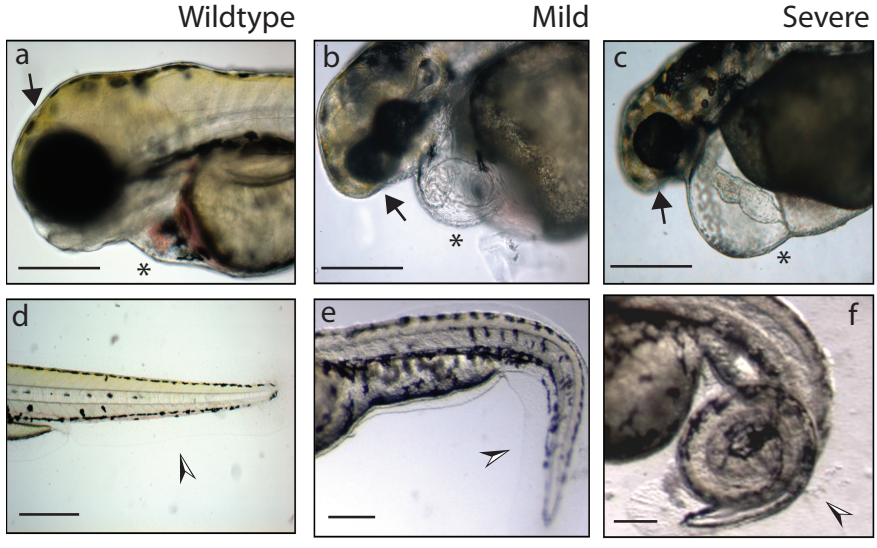

Figure 3. *PIP4Kα knockdown phenotypes. Morphological defects in PIP4Kα morpholino-mediated knockdown at 3 dpf. Left to right displays the range of phenotypic severity observed with PIP4Kα MO. PIP4Kα morphants show overall delay in development and a phenotype affecting several structures. PIP4Kα morphants show curved midbodies with alterations in somatic structure (arrows heads), smaller head with eye malformations (arrows), and pericardial oedema (asterisks). Table 1 summarizes quantification of the indicated developmental defects in PIP4Kα morphants. Scale bar, 300 μm.*

Table 1. *Quantitative analysis of developmental defects in PIP4Kα morphants.*

Phenotype	Control MO (*n* = 126)	MO2 (*n* = 309)		
		Severe	Mild	Wild type
Heart oedema	2	216	65	28
Midbody curvature	0	244	58	7
Eye defects	1	185	92	32

The lack of a phenotype in MO1-injected embryos might be attributed to the presence of maternally supplied mRNA, which may generate enough zPIP4Kα protein to enable normal early embryo development as assessed by western blot (Figure 2B).

The zPIP4Kα morphants eventually died at around 8 dpf, probably due to the severe midbody defects and trunk curvature, which compromises movement of the embryo. Normally, 72 hpf embryos respond to touch stimuli with a rapid and vigorous escape contraction of their trunk and tail, followed by swimming, which propels the embryo forward and results in swimming out of the microscope viewing field. Upon touching, the zPIP4Kα morphants showed rapidly alternating contraction of the trunk and tail, but they failed to propel forward in a straight line and instead swam in circles (data not shown). The altered swimming pattern and delayed hatching from the chorion were most likely due to the midbody curvature. Whether the zPIP4Kα morphants have patterning or structural defects in skeletal somite muscles remains to be determined, but immunohistochemical staining of PIP4Kα in 7 dpf zebrafish larvae (using anti-hPIP4Kα antibody) revealed PIP4Kα localization staining in inter somite structures (Figure Suppl. S3).

Rescue of the zPIP4Kα knockdown phenotype

To examine whether zPIP4Kα and mammalian PIP4Kα are interchangeable, we sought to rescue the knockdown phenotype by exogenous wild-type human PIP4Kα mRNA. Human PIP4K (hPIP4Kα) mRNA was co-injected together with the MOs into one-to two cell stage embryos and the phenotype was scored at 48 hpf (Figure 4A). Exogenously provided hPIP4Kα mRNA partially rescued the zPIP4Kα MO2 phenotype, as the midbody curvatures and reduction in eye size were less severe than in the morphants. About 80% of the MO2-injected embryos exhibited the "severe" zPIP4Kα knockdown phenotype, which was reduced to 38% upon co-injection of hPIP4Ka mRNA with MO2 (n=60 from two independent experiments; Figure 4B; left panel). The defects in cardiac development were less efficiently rescued by hPIP4Kα mRNA (Figure 4A). Injection of hPIP4Kα mRNA into wild-type or control MO-injected embryos did not result in any embryonic abnormalities. Proper expression of hPIP4Kα was confirmed by western blot (Figure 5A).

Kinase activity of zPIP4Kα is indispensable for normal zebrafish development

PIP4Kα is the isoform with the highest catalytic activity (Bultsma *et al.*, 2010; Wang *et al.*, 2010). To confirm that zPIP4Kα can phosphorylate PI(5)P, we measured zPIP4Kα catalytic activity after its partial purification from lysates of 48 hpf zebrafish embryos by heparin sepharose

chromatography. As shown in Figure 5B, morpholino-mediated knockdown of zPIP4Kα led to a significant decrease in the synthesis of PI(4,5)P_2 compared to control MO-injected embryos, indicating that zPIP4Kα is able to phosphorylate PI(5)P. To further confirm the requirement of

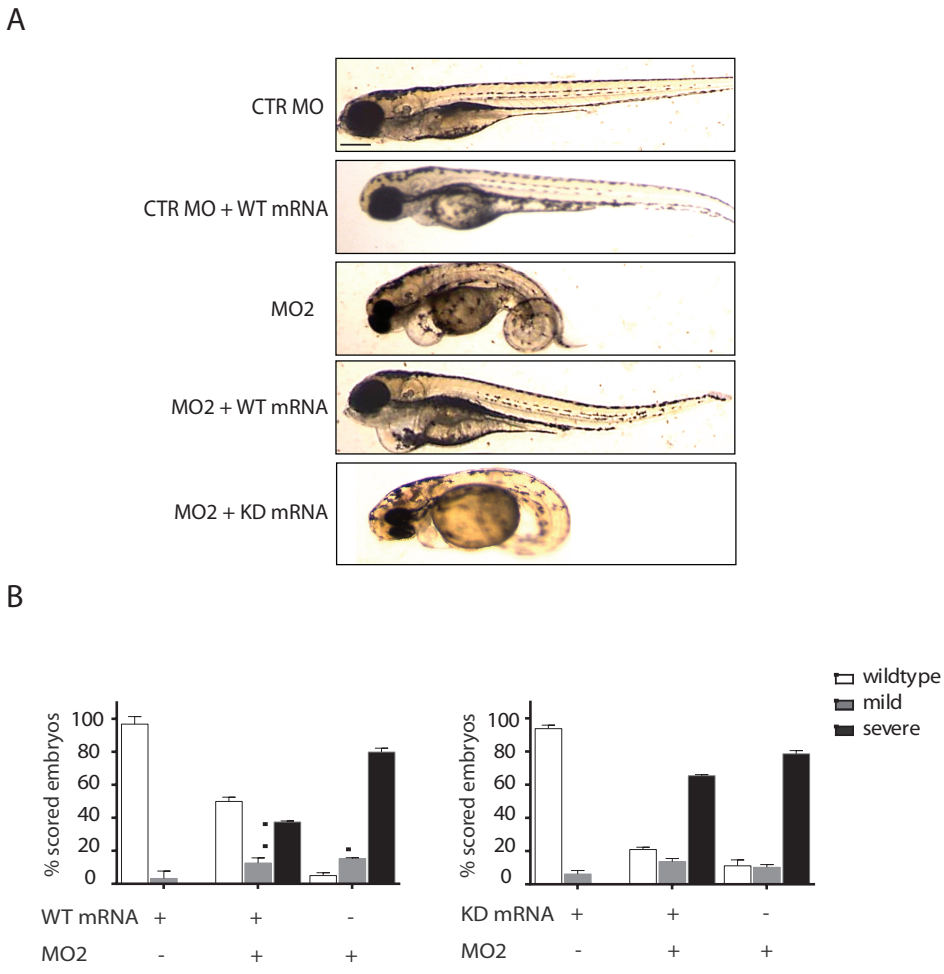

Figure 4. *Rescue of phenotype of zPIP4Kα-deficient embryos by human PIP4Kα RNA variants. (A) Lateral view of embryos at 48 hpf. Expression of hPIP4Kα partial rescues the PIP4Kα morphants from developmental defects. The rescue effect of catalytically dead PIP4Kα mRNA was less than observed with wt PIP4Kα mRNA. Scale bar, 300 µm. (B) Left panel; Quantification of the phenotypes observed upon expression of wt PIP4Kα mRNA in PIP4Kα morphants. Black indicates proportion of PIP4Kα with the classified "severe" morphological defects. Grey indicates the scored embryos displaying a "mild" phenotype and white display a wild type phenotype. Right panel: Quantification of the morphological phenotype obtained upon expression of catalytically dead PIP4Kα mRNA in PIP4Kα morphants with the same classification of phenotypes. Error bars represent standard error (n = 4; total number of embryos analyzed: 415).*

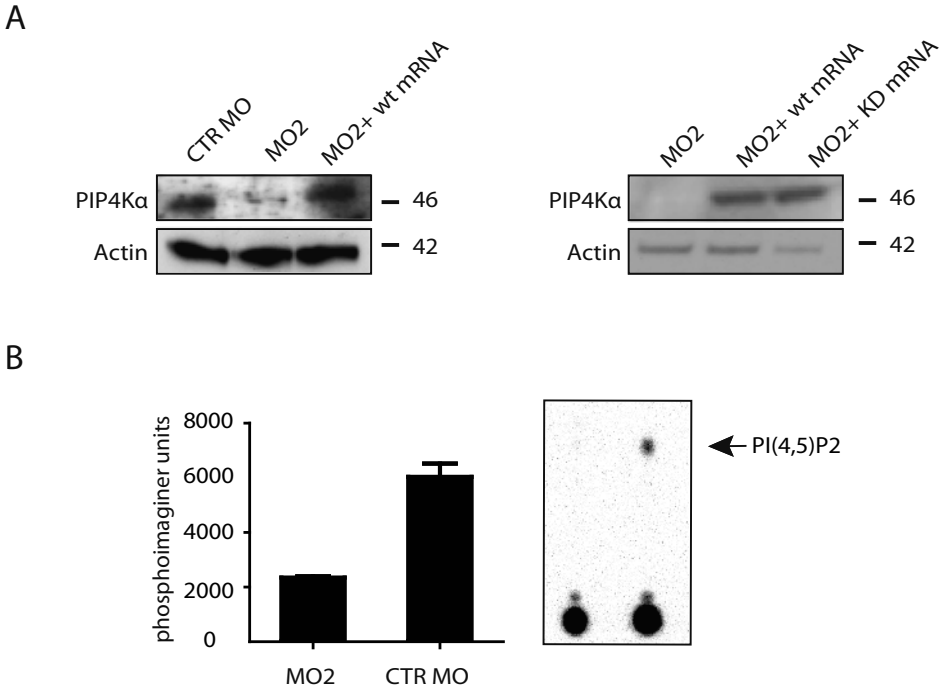

Figure 5. *PIP4K activity in PIP4Kα morphants. (A) Western blot analysis of PIP4Kα expression in "rescued" embryos, as detected by using anti-PIP4Kα polyclonal antibody. Left panel; control MO-injected embryos show PIP4α protein expression, PIP4Kα levels were strongly reduced in the ATG MO2 PIP4Kα morphant embryos. Co-injection of hPIP4Kα mRNA resulted in PIP4Kα expression at 48 hpf. Right panel; reduced PIP4Kα levels in ATG MO2 PIP4Kα morphant embryos at 48 hpf. Co-injection of hPIP4Kα and PIP4Kα-KD mRNA resulted in PIP4Kα expression. MW markers in kDa. (B) PIP4K activity measurements of control and PIP4Kα MO-injected embryos (50 embryos/group). Total lysates were incubated with PtdSer- and PI(5)P-containing liposomes and [^{32}P]ATP. Left panel; quantification of kinase activity of wild-type and PIP4Kα knockdown embryos at 2 dpf. Right panel; autoradiograph showing amount of [^{32}P]PI(4,5)P2, a measure of PIP4K activity, as measured by thin-layer chromatography. Error bars represent standard deviation (n=2). For details see 2.*

zPIP4Kα activity in early development, we examined whether the kinase activity of zPIP4Kα is a prerequisite for normal development. We generated a kinase-dead mutant (G131L/Y138F) (Bultsma *et al.*, 2010) of hPIP4Kα (hPIP4KαKD), and examined whether co-injection of its mRNA with the zPIP4Kα MO2 could rescue the knockdown phenotype. Co-injection of wild-type hPIP4Kα mRNA with zPIP4Kα MO2 significantly reduced the number of embryos that showed gross morphological defects. The hPIP4KαKD mutant also rescued the phenotype, when compared to zPIP4Kα MO injection alone however to a much lesser extent than the kinase active hPIP4K (Figure 4A and B; right panel). Expression of hPIP4KαKD was confirmed by western blot (Figure 5A; right panel). Taken together, these results show that zPIP4Kα catalytic activity is important for normal embryogenesis in zebrafish.

DISCUSSION

Here we report the molecular cloning of a zebrafish orthologue of human PIP4Kα. The high degree of amino acid conservation in the kinase domain, including amino acids G131 and Y138 that are essential for catalysis, strongly suggested that zPIP4Kα has conserved catalytic activity. We found ubiquitous expression of zPIP4Kα during gastrulation and at later stages expression in the eye, brain, somites and pectoral fins. Knockdown of zPIP4Kα caused developmental defects when zPIP4Kα was targeted with a translation-interfering morpholino. Using a morpholino (MO1) that targets the splicing of newly synthesized zPIP4Kα mRNA, zPIP4Kα expression was partly reduced but that did not result in embryonic abnormalities. Using other splice MO's targeting different exon-intron junctions in zPIP4Kα, we also did not detect gross morphological defects (data not shown). The severe phenotypes of the injection of MO2 could be partially rescued by the expression of hPIP4K suggesting that they are unlikely due to off-target mRNA suppression. From these results, we conclude that the translation of maternal zPIP4Kα mRNA provides sufficient levels of zPIP4Kα to ensure normal embryonic development. This may explain why only a morpholino targeting the ATG start codon can induce a knockdown phenotype.

At 48 hpf, zPIP4Kα morphants show developmental defects, namely heart failure, malformation of the eyes and severe midbody winding. These phenotypes can be attributed to zPIP4Kα deficiency, since co-injection of MO2 along with MO2-resistant hPIP4Kα mRNA could partially rescue the phenotype. That hPIP4Kα can complement reduced zPIP4Kα function supports the notion that human and zebrafish PIP4Kα share similar functions. Importantly, catalytically-dead PIP4Kα mRNA rescued the zPIP4Kα knockdown phenotype to a much lesser extent, indicating the importance of PIP4Kα catalytic activity. The partial rescue by the kinase inactive hPIP4K might suggest important scaffolding functions for PIP4K or may be a consequence of residual PIP4K activity in the mutant protein. Taken together, these results establish the specificity of the phenotype, its dependence on catalytic activity, and conservation of PIP4Kα function among vertebrates.

The zPIP4Kα morphants, after 48 hpf, showed an altered swimming pattern and delayed hatching from the chorion. These features were most likely due to the defects in midbody curvature in the zPIP4Kα morphants. It is known that the midbody shape is largely dependent on structurally and functionally intact muscle, especially in somites. Interestingly, several reports have implicated phosphoinositide-metabolizing enzymes in skeletal muscle disorders, especially phosphoinositide phosphatases (McCrea *et al.*, 2009; Nicot *et al.*, 2008). Myotubularins (MTM) are phosphatases that can specifically convert PI(3)P to PI and PI(3,5)P2 to PI(5)P (Robinson *et al.*, 2006) and patients with MTM mutations suffer from severe skeletal myopathy (Jungbluth *et al.*, 2008; Laporte *et al.*, 2000; Tosch *et al.*, 2006). The role of MTMs has been examined in zebrafish muscle development, and knockdown of MTM1 and MTM14 resulted in severe skeletal muscle defects (Dowling *et al.*, 2009; Dowling *et al.*, 2010). The delayed chorion hatching and morphologic changes of the zPIP4Kα morphants are indicative of impaired muscle function. As both PIP4K and MTM's are involved in the regulation of PI(5)P levels, it is tempting to hypothesize a possible link between PI(5)P metabolism and skeletal muscle development. However, whether the zPIP4Kα morphants have patterning or structural defects in skeletal somite muscles remains to be determined. Figure 6 shows a schematic diagram of PIP4K and other 3- or 5- phosphatases in the regulation of PI(5)P and its possible physiological effects relevant to development.

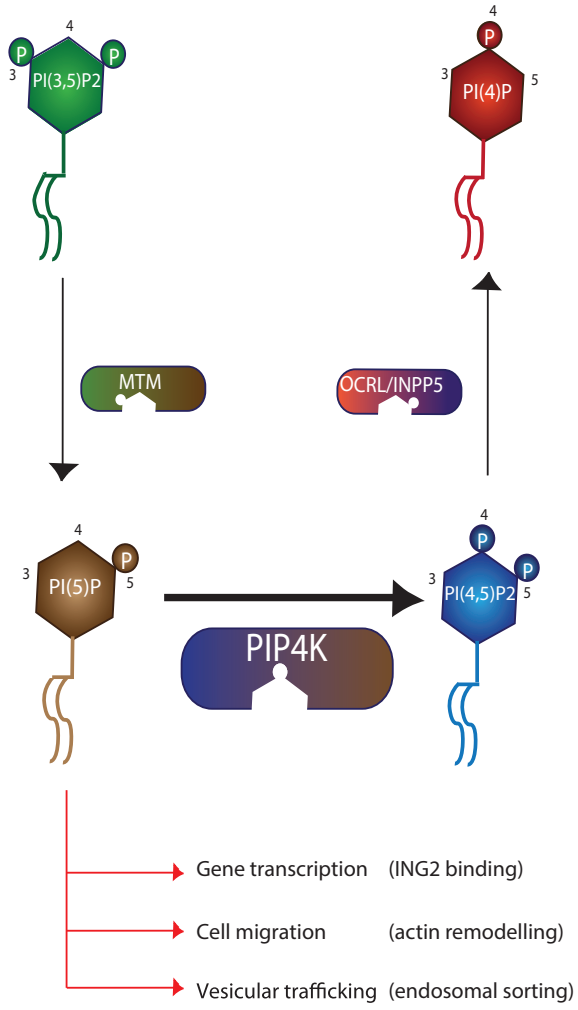

Figure 6. *Scheme of PIP4K and phosphoinositide phosphatases in the regulation of PI(5)P and its possible physiological effects. PI(5)P has been implicated in the regulation of nuclear signalling, gene transcription, membrane dynamics and actin remodelling. To what extent alterations in the pool(s) of PI(5)P and/or PI(4,5)P_2 may underlie the observed phenotypic defects in zPIP4Kα-deficient embryos remains to be established. See text for futher details and references.*

Other phosphoinositide-metabolizing enzymes, notably the inositol polyphosphate 5-phosphatases that can hydrolyze PI(4,5)P_2 (and its water-soluble metabolite Ins(1,4,5)P_3), have also been associated with genetic disorders (Conduit *et al.*, 2012). In particular, *INPP5E* and *OCRL* are associated with Joubert and Lowe's syndrome, respectively, and have been linked to primary cilia defects. Interestingly, OCRL1 knockdown in zebrafish caused developmental defects consistent with disruption of ciliary function, including body axis curvature and pericardial

oedema, similar to what was observed in the zPIP4Kα morphants (Coon *et al.*, 2012; Luo *et al.*, 2012; Ramirez *et al.*, 2012). Collectively, these findings suggest a general role for PI(5)P/PI(4,5)P2-metabolizing enzymes in the formation of cilia, which are implicated in translating signalling cues into the coordinated development of diverse organs.

In conclusion, our study provides the first description of how lack of PIP4Kα expression and activity affects vertabrate development. Our data show that PIP4Kα plays an important role in tissue homeostasis and organ developments. To what extent changes in the levels of PI(5)P and/or those of $PI(4,5)P_2$ in specific tissues may account for the PIP4Kα-deficient phenotype remains to be examined.

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SUPPLEMENTARY INFORMATION

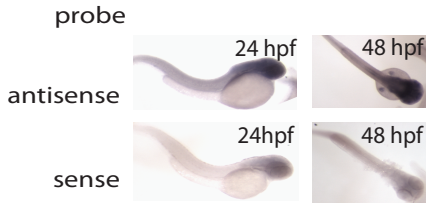

Figure S1. *Whole-mount in situ hybridization; Expression of PIP4Kα at 48 hpf with antisense and sense probe.*

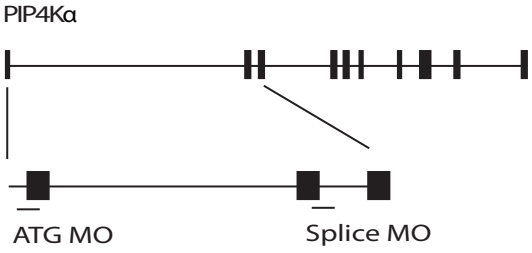

Figure S2. *Schematic representation of splice MO and ATG MO target regions in the PIP4Kα transcript. The sequences of both MOs are shown.*

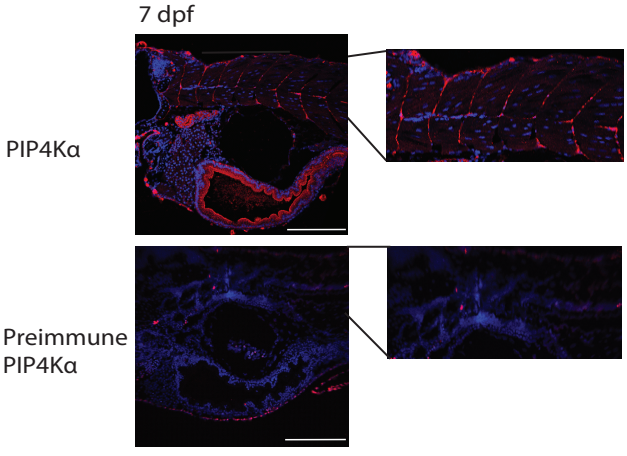

Figure S3. *Analysis of transverse sections of 5 dpf wild-type zebrafish larvae stained by anti-PIP4Kα antibody or preimmune serum (red); nuclei were stained by DAPI (blue). Immunofluorescence reveals expression of PIP4Kα in the lumen, kidneys and intersomite boundaries. Scale bar, 150 μm.*