Supporting Information

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SI Materials and Methods

Zebrafish Histology, Whole-Mount in Situ Hybridization, and Immunohistochemistry. Alcian blue staining was performed as previously described (1). Briefly, 5-d-old embryos were fixed overnight in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4 °C. After washing, embryos were briefly digested with proteinase K and refixed at room temperature for 2 h. Embryos were bleached in 10% hydrogen peroxide supplemented with 0.1 M KOH for 1 h and stained overnight in 0.1% Alcian blue dissolved in acidic ethanol (70% ethanol, 5% concentrated HCl). The embryos were washed extensively in acidic ethanol, rehydrated, and stored in 80% glycerol.

In situ hybridizations were performed as described (2). To prepare digoxigenin-labeled *ip6k2* antisense RNA probe, zebrafish *ip6k2* full-length cDNA construct (catalog no. MDR1734-7601034; Open Biosystems) was linearized with PstI and used as a template for T7 RNA polymerase transcription. Previously described probes include those for *foxd3* (3), *tfap2a* (1, 4), *sox9b* (5), *dlx2* (6), *crestin* (7), *shh* (8), *ptc1* (9), and *gli1* (10). Embryos were mounted on 80% glycerol/PBS, and images were obtained using either a Retiga Exi cooled CCD camera (Qimaging) on an Olympus AX70 compound microscope and OPENLAB software (Improvision) or a Zeiss Imager microscope equipped with Axiocam digital camera and Axiovision software.

For immunohistochemistry, embryos were fixed overnight at 4 °C in 4% paraformaldehyde, 0.15 mM CaCl2, 4% sucrose in 0.1 M phosphate buffer (pH 7.3). Embryos were washed two times in PBS/0.1% Triton X-100 for 5 min, one time in H_2O for 30 min, followed by two 5-min washes in PBS/0.1% Triton ×100 for 5 min. Indirect immunofluorescence was performed using 1:100 mouse anti-engrailed (4D9) monoclonal antibody from the Developmental Studies Hybridoma Bank (DSHB) or 1:100 mouse monoclonal F59 antibody (DSHB), and 1:400 Alexa Fluor 488 goat anti-mouse conjugate (Molecular Probes). F-actin was stained using 1:25 rhodamine-phalloidin (Molecular Probes). Nuclei were visualized by counterstaining with TO-PRO3 (Molecular Probes). Embryos were mounted in ProLong Gold antifade reagent (Invitrogen), and images were obtained using a Zeiss LSM510 Meta laser scanning confocal microscope.

Time-Lapse Spinning-Disk Microscopy. *Tg(sox10(7.2):mrfp)* embryos, at 16 hpf, were dechorionated, anesthetized using 3-aminobenzoic acid ethyl ester, immersed in SeaPlaque low-melting agarose (BioWhittaker Molecular Applications; 0.8% in embryo medium), and mounted on their sides in glass-bottom culture dishes (MatTek). Time-lapse images were captured using 20× dry (NA = 0.75) objective on a motorized Zeiss Axiovert 200 microscope equipped with a PerkinElmer ERS spinning disk confocal system and heated stage and chamber maintained at 28.5 °C. Z image stacks were collected every 15 min for 6 h, and 3D data sets were compiled into composite images using Volocity software (Improvision). Sorenson 3 video-compression image sequences were played as QuickTime movie. NCC migration trajectory and distance were determined using Volocity. The center of each cell was empirically identified, and a line drawn to the corresponding cell at the next time point. The length of the combined line segments corresponds to the total distance each NCC migrated, whereas the shortest distance between start and end points represents net migration. For tracking individual cell movements, NCCs in the image sequences were marked using OpenLab (Improvision).

Cell Culture, siRNAs, Transfection, Drug Treatment, and Assays for Hh Pathway Activation. NIH 3T3 and COS-7 cells were cultured in DMEM (Invitrogen) with 10% calf serum (Invitrogen), 100 U/mL penicillin and streptomycin (Invitrogen). *ip6k2* siRNA (catalog no. sc-39072) and control siRNAs (catalog no. sc-37007) were purchased from Santa Cruz Biotechnology and transfected following manufacturer's protocol. Briefly, ∼30% confluent NIH 3T3 cells were transfected with 4 μL of 10 μM siRNA in 500 μL volume per well in a 12-well tissue culture plate. After 7 h, 500 μL of DMEM + 20% calf serum was added and cells grown for 65 h (i.e., 3 d including initial 7 h). The growth medium was replaced by Hh ligand medium [Shh-N conditioned medium (CM)], and cells were grown for an additional 24 h. To prepare Shh-N CM, COS-7 cells were transfected with Shh-N expression construct using Lipofectamine 2000 (Invitrogen) reagent and grew cells for 48 h in DMEM + 2% calf serum. The medium was harvested, passed through 0.2-μm sterile filter cartridge, snap-frozen in dry ice/EtOH bath, and stored at −80 °C. For Hh induction, the Shh-N CM was 1:4 diluted in DMEM, and 1 mL of this diluted Shh-N CM was used per well. For assays on Hh pathway activation, cells were cotransfected with 8× *gli-luciferase* (450 ng) and β-galactosidase (50 ng) reporters using siRNA transfection reagent (Santa Cruz Biotechnology). All DNA transfections without siRNA were done using Lypofectamine 2000. For assays in LIGHT-2 cells, cells were cotransfected with β-galactosidase (50 ng) reporter. Mouse full-length *ip6k2* cDNA construct in pCMV-SPORT6 expression vector (catalog no. MMM1013-7514295; Open Biosystems) was used for studying effects of *ip6k2* overexpression. Epistasis experiments were conducted using *smoM2* (250 ng), *gli1* (50 ng), or *ip6k2* (500 ng) expression plasmids. TNP, SAG, cyclopamine, or DMSO treatments were conducted in Shh-N CM simultaneously with Hh pathway induction. Luciferase and β-galactosidase assays were performed essentially as described (11). Cells were lysed in 150 μL of luciferase lysis buffer (25 mM Tris-phosphate, 2 mM EGTA, 10% glycerol, 0.5% Triton X-100, 2 mm DTT) at room temperature for 20 min. Following lysis, supernatants were used for luciferase and β-galactosidase assays. For luciferase assays, 20 μL of supernatants were diluted in assay buffer (15 mM $MgSO_4$, 15 mM K_2PO_4, 4 mM EGTA, 1 mM DTT, 1 mM ATP), and 1 mM luciferin was injected before reading in a Lumimark Plus microplate reader (BioRad). Liquid β-galactosidase assays were performed using 100 μL of supernatants and *o*-nitrophenyl β-D-galactopyranoside as the substrate according to Clontech protocols. For normalizing luciferase activities, luc:β-gal ratios were determined and expressed as relative luciferase units (RLU).

NIH 3T3 and COS-7 cells were from American Type Culture Collection. *shh-N* and *gli1* expression plasmids were kind gifts from Chin Chiang (Vanderbilt University School of Medicine, Nashville, TN), and Shh-LIGHT Z3 cells, 8× *gli-luciferase* and β-galactosidase (pCDNA-*LacZ*) reporter constructs were from Michael Cooper (Vanderbilt University Medical Center, Nashville, TN). *smoM2* expression plasmid and LIGHT-2 cells were from Philip Beachy (Stanford University School of Medicine, Stanford, CA). Sources of other reagents: TNP (Calbiochem, EMD Chemicals), cyclopamine (Toronto Research Chemicals Inc.), and SAG (12) (Vanderbilt Chemical Synthesis Core, Vanderbilt University).

Myo(1,2)[^{3}H]-Inositol–Labeled Soluble Inositol Polyphosphate Profiling. NIH 3T3 cells were seeded at 1×10^6 cells on 60-mm plates in 3 mL DMEM without inositol (MP Biomedicals) supplemented with dialyzed FBS [10% (vol/vol); Invitrogen], and

60 μCi of myo (1,2)[^{3}H]-inositol (PerkinElmer) for 4 d. Plates at 90–95% confluency were then washed with DMEM without inositol and subjected to 24 h treatments with 0.5% bovine serum (Invitrogen) + DMSO, 0.5% bovine serum + 10 μM TNP, Shh-N CM + DMSO, Shh-N CM + 10 μM TNP, or Shh-N CM + 0.5 μM cyclopamine in 10 μCi/mL myo (1,2)[^{3}H]-inositol-supplemented DMEM without inositol. DMSO concentration in treatments was equivalent to its level in 10 μM TNP. For radiolabeling siRNA-treated cells, ∼30% confluent NIH 3T3 cells on 60-mm plates were transfected with 16 μL of 10 μM siRNA in 2 mL total volume. After 7 h, 2 mL of DMEM without inositol, supplemented with 80 μCi of myo (1,2)[^{3}H]-inositol, and 20% dialyzed FBS was added. Cells were grown for 65 h. Following treatments, cells were washed with 3 mL of Dulbecco's PBS, and soluble cell extracts were prepared by adding 800 μL of 0.5 N HCl. After 5 min of HCl treatment, the samples were fractionated by centrifugation (12,000 rpm for 5 min). Supernatants were filtered through 0.45-μm nylon filter and stored at −80 °C.

For HPLC analysis, 100 μL of 50 mM $(NH_4)_2HPO_4$ (pH 4.4) was added to 400 μL of the samples. Soluble IPs were then resolved on a Partisphere SAX column (4.6 × 125 mm; Whatman) using a linear gradient from 10 mM to 1.25 M $(NH_4)_2HPO_4$ (pH 4.4) over 65 min, followed by elution with 1.25 M $(NH_4)_2HPO_4$ for 30 min. Radiolabeled IPs eluting from the column were quantified by an inline radio-HPLC detector. For siRNA-treated samples, 225 μL of 20 mM $NH_4H_2PO_4$ (pH 3.5) was added to an equal volume of sample. Soluble IPs were then separated using linear gradient from 10 mM to 1.7 M $NH_4H_2PO_4$ (pH 3.5) over 65 min, followed by elution with 1.7 M $NH_4H_2PO_4$ (pH 3.5) for 30 min.

1. Barrallo-Gimeno A, Holzschuh J, Driever W, Knapik EW (2004) Neural crest survival and differentiation in zebrafish depends on mont blanc/tfap2a gene function. *Development* 131:1463–1477.
2. Hauptmann G, Gerster T (2000) Multicolor whole-mount in situ hybridization. *Methods Mol Biol* 137:139–148.
3. Odenthal J, Nüsslein-Volhard C (1998) Fork head domain genes in zebrafish. *Dev Genes Evol* 208:245–258.
4. Knight RD, et al. (2003) lockjaw encodes a zebrafish tfap2a required for early neural crest development. *Development* 130:5755–5768.
5. Yan YL, et al. (2005) A pair of Sox: Distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development. *Development* 132: 1069–1083.
6. Akimenko MA, Ekker M, Wegner J, Lin W, Westerfield M (1994) Combinatorial expression of three zebrafish genes related to distal-less: Part of a homeobox gene code for the head. *J Neurosci* 14:3475–3486.
7. Luo R, An M, Arduini BL, Henion PD (2001) Specific pan-neural crest expression of zebrafish Crestin throughout embryonic development. *Dev Dyn* 220:169–174.
8. Krauss S, Concordet JP, Ingham PW (1993) A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75:1431–1444.
9. Concordet JP, et al. (1996) Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* 122:2835–2846.
10. Karlstrom RO, et al. (2003) Genetic analysis of zebrafish gli1 and gli2 reveals divergent requirements for gli genes in vertebrate development. *Development* 130:1549–1564.
11. Bolger TA, Folkmann AW, Tran EJ, Wente SR (2008) The mRNA export factor Gle1 and inositol hexakisphosphate regulate distinct stages of translation. *Cell* 134:624–633.
12. Ehtesham M, et al. (2007) Ligand-dependent activation of the hedgehog pathway in glioma progenitor cells. *Oncogene* 26:5752–5761.
13. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680.
14. Kucenas S, et al. (2008) CNS-derived glia ensheath peripheral nerves and mediate motor root development. *Nat Neurosci* 11:143–151.

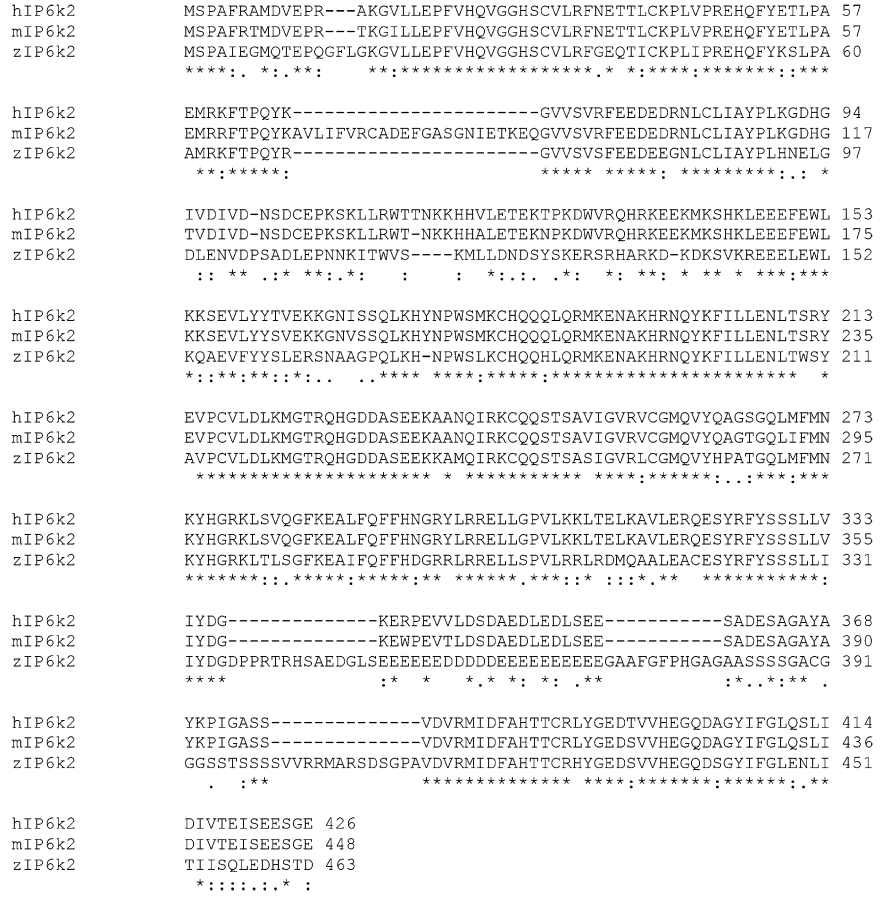


Fig. S1. IP6K2 enzymes from fish to mammals exhibit high sequence conservation in their amino acid sequences. Sequence alignment of human (hIP6K2), mouse (mIP6K2), and zebrafish (zIP6K2) using the ClustalW program (13). Identical residues (*), conserved (:), and semiconserved (.) substitutions are marked. Predicted amino acid sequences of the IP kinases exhibit 60.47% identity (identical residues) and 79.91% similarity (identical, conserved, and semiconserved residues).

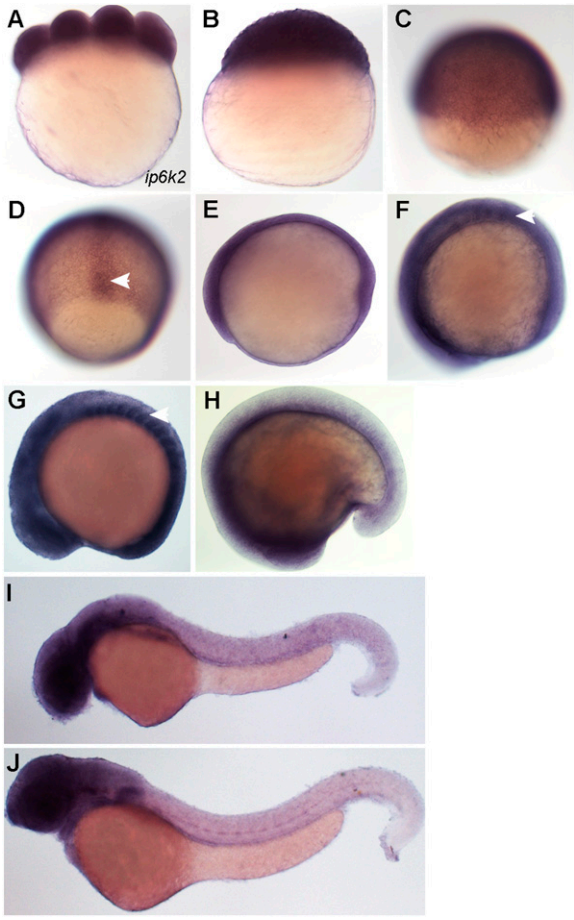


Fig. S2. *ip6k2* is expressed during zebrafish embryogenesis. Gene expression was analyzed during early embryogenesis. (*A*) Eight-cell-stage embryo showing maternally expressed *ip6k2* mRNA. (*B* and *C*) Lateral views of blastula stage (4 hpf) embryo (*B*) and embryo at early gastrulation stage (6 hpf) (*C*) showing ubiquitous *ip6k2* expression. (*D*) Dorsal view showing *ip6k2* expression in axial cells (arrowhead) at midgastrulation (8 hpf). (*E*) Lateral view showing ubiquitous *ip6k2* expression at 10 hpf. (*F–H*) Lateral views showing *ip6k2* expression during segmentation stages. At 11 hpf, *ip6k2* expression is detected in the neural plate and somites (arrowhead) (*F*). *ip6k2* expression is enriched in somites (arrowhead), both forebrain and hindbrain regions, and developing eyes at 15 hpf (*G*); expression is reduced at 18 hpf (*H*). (*I* and *J*) Lateral views showing *ip6k2* expression in retina, otic vesicle, and pronephric duct at 40 hpf (*I*) and in central nervous system at 60 hpf (*J*).

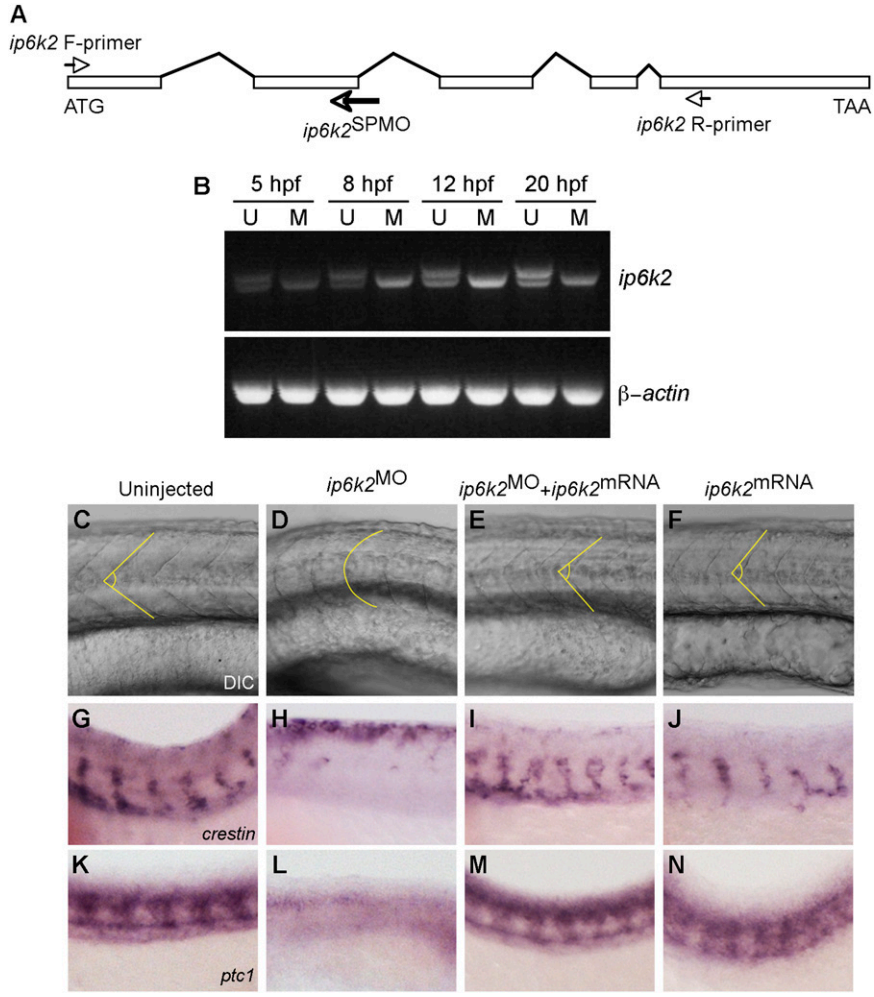


Fig. S3. ip6k2 mRNA rescues the Hh-related phenotypes in ip6k2^{SPMO}-injected embryos. (*A*) Schematic of *ip6k2* genomic organization with exons (rectangular boxes) and introns (lines). Arrows indicate relative positions of *ip6k2*^{SPMO} and oligonucleotide primers used to amplify *ip6k2* cDNA. (*B*) *ip6k2*^{SPMO} blocks splicing at exon2-intron2 junction. Total RNA prepared from uninjected (U) and *ip6k2*^{SPMO} injected (M) embryos at different stage (5–20 hpf) were analyzed by two-step RT-PCR using *ip6k2*-F and -R primers (shown above). Reduced sized, aberrantly spliced *ip6k2* product was detectable at 5 hpf and later time points in *ip6k2*^{SPMO}-injected embryos (*Upper*). PCRs with β-actin-specific primers served as controls (*Lower*). (*C–N*) *ip6k2* mRNA injection reverses inhibitory effects of *ip6k2*^{SPMO} on somite development (*C–F*), NCC migration (*G–J*), and Hh target gene expression (*K–N*). Anterior is toward the left and dorsal toward the top. (*C*) Lateral view of chevron-shaped somites of a live uninjected embryo at 24 hpf. (*D* and *E*) Rescue of U-shaped somites in embryos injected with *ip6k2*^{SPMO} (*D*) by coinjecting *ip6k2* mRNA (*E*). (*F*) *ip6k2* mRNA injection alone did not have any effect. Dotted lines indicate somite boundaries. (*G–J*) Whole- mount in situ hybridizations showing *crestin* expression in trunk NCC in uninjected (*G*), *ip6k2*^{SPMO}-injected (*H*), *ip6k2*^{SPMO} + *ip6k2* mRNA-coinjected (I), and *ip6k2* mRNA-injected (*J*) embryos at 24 hpf. Expression of *crestin* in *ip6k2*^{SPMO}-injected embryos was restored by *ip6k2* mRNA coinjection (*H* and *I*). *ip6k2* mRNA injection alone did not alter *crestin* expression (*J*). (*K–N*) Whole-mount in situ hybridizations showing *ptc1* expression in uninjected (*K*), *ip6k2*^{SPMO}-injected (*L*), *ip6k2*^{SPMO} + *ip6k2* mRNA-coinjected (*M*), and *ip6k2* mRNA-injected (*N*) embryos at 24 hpf. It is apparent that *ip6k2* mRNA coinjection rescued *ptc1* expression in embryos injected with *ip6k2*^{SPMO} (*L* and *M*), whereas *ip6k2* mRNA injection alone had no effect (*N*).

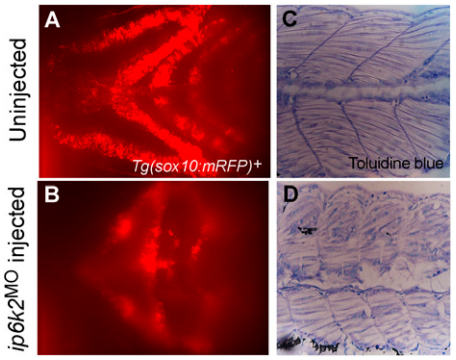


Fig. S4. *ip6k2* knockdown perturbs craniofacial development and somite structures. (*A* and *B*) Ventral view of the head region showing cartilage fluorescence of 3-d-old *Tg(sox10(7.2):mrfp)* embryo (*A*) and embryo injected with *ip6k2*^{ATGMO} (*B*). Reduced mRFP expression in the *ip6k2*^{ATGMO} embryos reflects a lack of pharyngeal arches. (*C* and *D*) Toluidine blue staining of the trunk region of 5-d-old uninjected (*C*) and *ip6k2*^{ATGMO}-injected (*D*) embryo. The embryos were fixed and embedded in plastic resin, sectioned, and stained with toluidine blue. Sagittal sections through the midtrunk region were shown. The somites in the *ip6k2*^{ATGMO} embryo were defective in shape and muscle fiber organization.

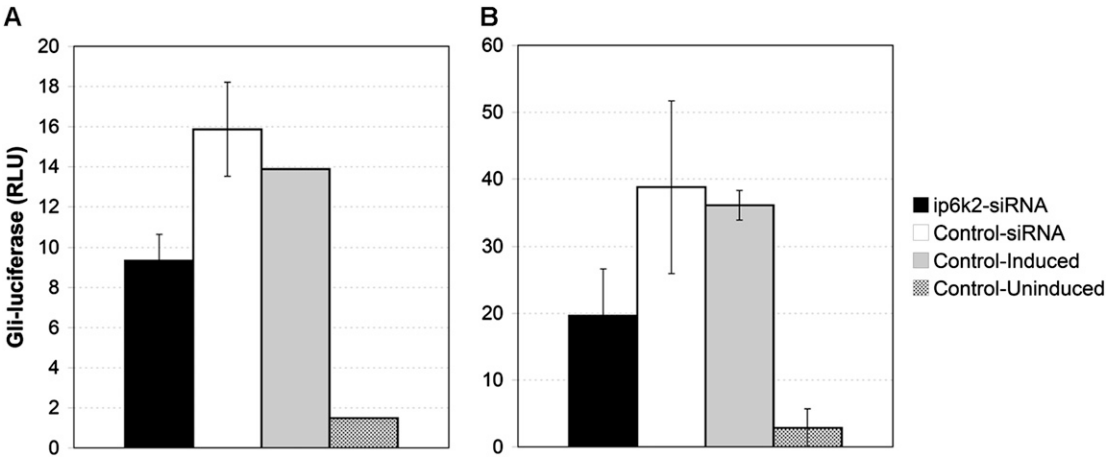


Fig. S5. Depletion of *ip6k2* mRNA levels inhibits Shh- induced Hh pathway response. Bar graph showing Shh-dependent Gli-luciferase activity in either Shh-LIGHT2 cells with 8× *gli-luciferase* reporter (*A*) or Shh-LIGHT Z3 cells with Gli-dependent firefly *luciferase* and stably maintained constitutive β-galactosidase reporters (*B*). The cells were subsequently treated with *ip6k2* siRNA or a nontargeting control siRNA for 72 h and induced with Hh ligand (Shh-N CM) for 24 h. Gli-luciferase activity was measured and normalized by either total protein content (*A*) or β-galactosidase activity (*B*). Control-induced represents non–siRNA-treated cells, whereas control uninduced represents non–siRNA-treated uninduced cells. *ip6k2* siRNA treatment resulted in significant reduction of Shh-induced Gli-luciferase activity in comparison with control siRNA cells. Data are represented as mean ± SD of three independent assays.

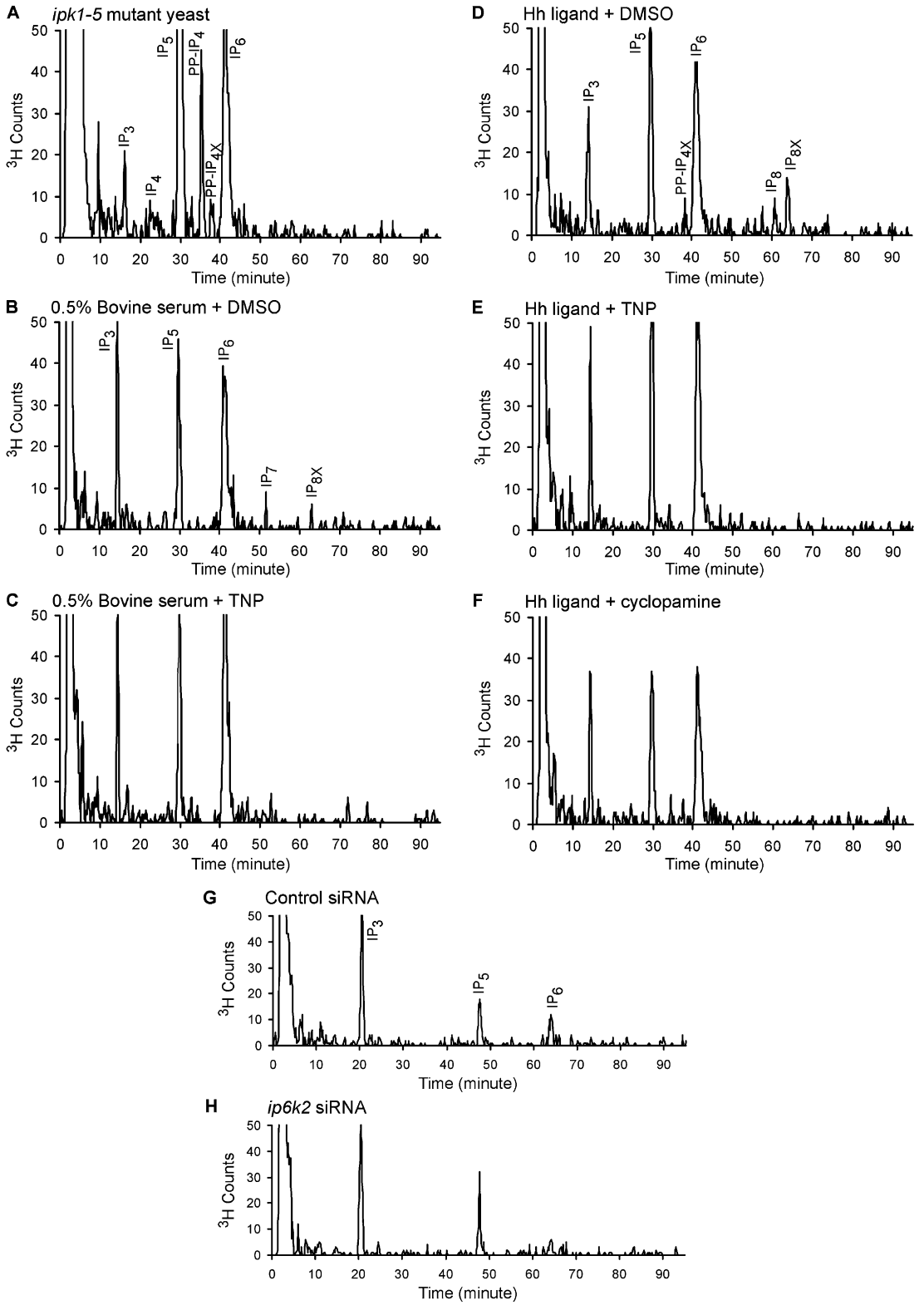
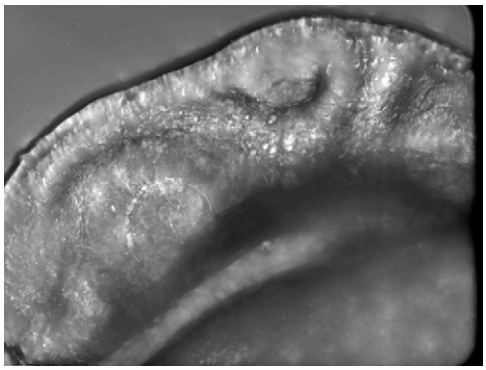
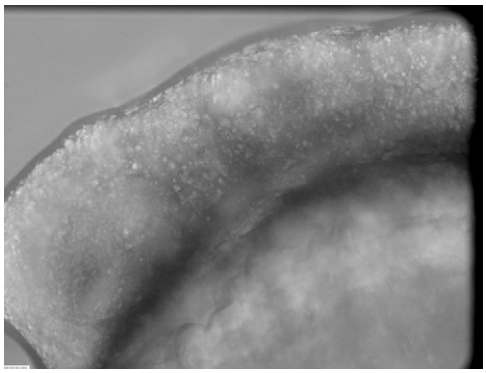


Fig. S6. Hh pathway activation accompanies changes in cellular IP flux. (*A–F*) NIH 3T3 cells (*B–F*) were grown for 4 d in DMEM without inositol supplemented with 10% dialyzed FBS and 20 μCi/mL myo(1,2)[^{3}H] inositol. Cells were washed with DMEM without inositol and subsequently treated for an additional 24 h with myo(1,2)[^{3}H] inositol supplemented 0.5% bovine serum and DMSO (*B*), 0.5% bovine serum and 10 μM TNP (*C*), Hh ligand and DMSO (*D*), Hh ligand and 10 μM TNP (*E*), or Hh ligand and 0.5 μM cyclopamine (*F*). Soluble cell extracts were prepared and IPs separated by PartiSphere strong-anion exchange HPLC using linear gradient from 10 mM to 1.25 M $(NH_4)_2HPO_4$ (pH 4.4) over 65 min, followed by elution with 1.25 M $(NH_4)_2HPO_4$ for 30 min. IP elution profiles are presented as ^{3}H-counts (*y* axis) vs. time (min) (*x* axis) graphs. Labels indicate IP elution positions. HPLC profile of an *ipk1-5* mutant yeast strain (*A*) confirms IP elution positions. (*G* and *H*) NIH 3T3 cells were treated with *ip6k2* siRNA (*H*) or a nontargeting control siRNA (*G*) for 72 h in DMEM without inositol, supplemented with 10% dialyzed FBS, and 20 μCi/mL myo(1,2)[^{3}H] inositol. Soluble IPs were extracted and resolved by HPLC using a linear gradient from 10 mM to 1.7 M $NH_4H_2PO_4$ (pH 3.5) over 65 min, followed by elution with 1.7 M $NH_4H_2PO_4$ for 30 min. IP_X, IP isomer.



Movie S1. Migration of cranial neural crest cells in real time in a zebrafish embryo. CNCCs from mid- and hindbrain regions in a *Tg(sox10(7.2):mrfp)* (14) embryo migrate anteriorly and/or ventrally toward optic vesicle (Movie S1). The red fluorescence image sequence is from a lateral view, focused on the head region with dorsal up and anterior to the left. Images were collected every 15 min beginning at 16 hpf and ending at 22 hpf. The movie runs at two frames per second.

Movie S1



Movie S2. Migration of cranial neural crest cells in real time in a IP6K2 depleted embryo. Directional migration of CNCCs is affected in *ip6k2*^{ATGMO}-injected *Tg(sox10(7.2):mrfp)* (14) embryos (Movie S2; see text for details). The red fluorescence image sequence is from a lateral view, focused on the head region with dorsal up and anterior to the left. Images were collected every 15 min beginning at 16 hpf and ending at 22 hpf. The movie runs at two frames per second.

Movie S2