Supporting Information

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SI Materials and Methods

Generation of *Ick*$^{tm1a/tm1a}$ Mice. Embryonic stem (ES) cells targeted in the *Ick* locus were generated by the trans-National Institutes of Health (NIH) Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). To produce *Ick*-targeted mice (*Ick*^{tm1a}), standard protocols for blastocyst injection and chimera production were followed (Macrogen). Briefly, ES cells were injected into blastocysts, and chimeric mice were bred with C57/BL6N wild-type mice to confirm germ-line transmission. Genotypes were confirmed using PCR with appropriate primers flanking the targeted region of *Ick*. *Ick*$^{tm1a/+}$ heterozygous mice were maintained in a C57/BL6N background. All mouse experiments were approved by the Institutional Animal Care and Use Committee of Dongguk University.

Cell Culture. NIH 3T3 cells and SHH-Light II cell lines were obtained from American Type Culture Collection. ICK-3FLAG-EGFP and SMO-EGFP stable cell lines were generated from NIH 3T3 cells. NIH 3T3 cells were seeded on six-well culture plates at 3×10^5 cells per well. The next day, cells were transfected with ICK-3FLAG-EGFP or SMO-EGFP plasmid DNA constructs. After transfected cells reached confluence, cells were subcultured in 3 μg/mL puromycin-containing media for ∼2–3 wk to isolate stably expressing clones. Stable ICK-3FLAG-EGFP and SMO-EGFP cell lines were grown following standard protocols in 10% (vol/vol) FBS containing DMEM (Cellgro).

Gene Silencing. NIH 3T3 cells were seeded on 24-well culture plates at 3×10^4 cells per well for siRNA transfection. Subcultured NIH 3T3 cells were grown in an incubator at 37 °C and 5% (vol/vol) CO_2 overnight and then transfected with the indicated ICK plasmid DNA in serum-free media using Attractene transfection reagent (Qiagen) according to the manufacturer's protocol. *Ick*-specific and scrambled siRNAs were obtained from Qiagen. The siRNA sequences used in this study were 5′-CCAAUAUCGUAAAGUUAAATT-3′ (sense) and 5′-UUUAACUUUACGAUAUAUUGGCA-3′ (antisense) for *Ick* siRNA no. 2 and 5′-CGGGAGGUUAAGUCUUUAATT-3′ (sense) and 5′-UUAAAGACUUAACCUCCCGAA-3′ (antisense) for Ick siRNA no. 4. To knockdown *Ick* expression, cells were transfected with 20 nM siRNA using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's instructions. To generate a stable shRNA cell line for Ick gene knockdown, gene-specific and control shRNA plamsids from the RNAi Consortium (TRC) library (Sigma-Aldrich) were purchased and stably integrated into the genome in NIH 3T3 cells using puromycin selection. Colonies were isolated and knockdown efficacy was determined by quantitative real-time RT-PCR with Ick-specific primers.

Quantitative Real-Time RT-PCR. Total RNA was isolated from NIH 3T3 cells using TRI reagent (Invitrogen) and 1 μg of total RNA was reverse transcribed with oligo-dT primer using a First-Strand cDNA Synthesis kit (Takara). Real-time PCR was performed using the Rotor Gene Q instrument (Qiagen) with SYBR RealHelix qPCR kit (Nanohelix). The primer sequences used in this study for quantitation of Gli1 and Ick were as follows: *Gli1* forward, 5′-GGTGCTGCCTA AGCCAGTG CCT C-3′; *Gli1* reverse, 5′-GTGCCAATCCGGTGG AGTCAGACCG-3′; and *Ick* forward, 5′-TTTCTTCCACCGGGACTTAAA-3′; *Ick* reverse, 5′-GGAGGTCTTGATCGGATTTCTC-3′. Beta-actin was used as internal control and the primer sequences used here are as follows: *β-actin* forward, 5′-GGCCCAGAGCAAGAGAGGTATCC-3′; *β-actin* reverse, 5′-ACGCACGATTTCCCTCTCAGC-3. Results were analyzed with Rotor Gene Q series software to calculate the relative mRNA levels using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting. NIH 3T3 cells or whole embryos were lysed in modified RIPA buffer [150 mM NaCl, 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40, 0.25% Na-deoxycholate] with 1 mM PMSF 1× protease inhibitor (Roche) on ice. Protein extracts were clarified for 15 min at 17,000 × g at 4 °C, and the supernatant was transferred to a fresh tube. Protein concentration was determined by the BCA Protein Assay (Pierce). Equal amounts of protein (10 μg) were loaded for electrophoresis. After electrophoresis, proteins were transferred to PVDF membranes (Millipore) using a Trans-Blot SD semidry transfer cell (Bio-Rad). Membranes were washed with Tris-buffered saline solution containing 0.2% Tween 20 (TBST) and blocked for 1 h in TBST containing 5% (vol/vol) skim milk. Membranes were then incubated overnight at 4 °C with the following primary antibodies: mouse anti-FLAG (Sigma), anti-Gli3 (kindly provided by Suzie J. Scales (Genentech), or mouse anti–beta-tubulin (Millipore). After washing with washing buffer, membranes were incubated for 2 h at room temperature with adequate secondary antibodies. Signals were detected with chemiluminescent reagent (Millipore) following the manufacturer's instructions.

Immunofluorescence Staining and Measurement of Cilia Length. Cultured NIH 3T3 cells were fixed in 4% (wt/vol) paraformaldehyde (PFA) in PBS for 10 min at room temperature followed by three washes with PBS. Fixed cells were incubated with blocking buffer (1% horse serum, 0.1% Triton X-100 in PBS) for 30 min at 4 °C. Cells were incubated in a combination of Arl13b (1), FLAG (Sigma), and acetylated-tubulin (Sigma) antibodies overnight at 4 °C. After three washes with blocking solution, cells were simultaneously incubated with FITC-conjugated donkey anti-rabbit IgG (1:1,000; Jackson Immunoresearch) and Cy3-conjugated donkey anti-mouse IgG (1:500; Jackson Immunoresearch) with DAPI (1:1,000; Sigma). Stained cells were imaged using an LSM 700 confocal laser-scanning microscope (Carl Zeiss). To analyze the localization of different antigens in double-stained tissue, different images were obtained from the same area and merged using Zen 2009 Light Edition software (Carl Zeiss). Length of cilia in Arl13b-stained cells was measured using NIS-Elements AR 3.2 software (Nikon Instruments).

Luciferase Assay. An appropriate number of cultured cells were plated. The next day, cells were transfected with *Ick* constructs, an 8 × 3′ Gli-BS luciferase construct (kindly provided by Hiroshi Sasaki, Center for Developmental Biology, RIKEN, Kobe, Japan), an an internal control Renilla luciferase construct, GL4.74 (Promega) (2). To induce ciliogenesis, we replaced media with low serum (0.5%) media 1 d after transfection. After 2 d of incubation in low serum media, SHHN-conditioned media was added to cells to activate SHH signaling. SHHN-conditioned media was made by transfecting SHHN pMT21 plasmid (kindly provided by Wendy Ingram, Queensland Children's Medical Research Institute, Brisbane, Australia) to 293FT cells in 10 mL serum media. One day after transfection, 8 mL of media was replaced with fresh 8 mL of DMEM, and cells were further incubated for 2 d at 37 °C to collect SHHN-conditioned media (3). Media was kept at −80 °C for further experiments.

Luciferase assays were performed according to manufacturers' protocols. Briefly, cells were lysed in 100 μL passive lysis buffer

(Promega) for 15 min on a shaker. Sample extracts were transferred to a fresh tube. Extracted samples were transferred into 96-well plates, and 50 μL firefly substrate (GoldBio Technology) was added to the samples. After measuring firefly luciferase activity, 1 μL Renilla substrate (200 μM; GoldBio Technology) was diluted with 49 μL Stop & Glo buffer (1.1 M NaCl, 2.2 mM Na2 EDTA, 0.22 M $KxPO_4$ (pH 5.1), 0.44 mg/mL BSA, 1.3 mM NaN_3) and added to cell lysates. Dual-luciferase activity was measured using a Glomax luminometer following the manufacturer's instructions (Promega). Statistical analysis was performed using Student's *t*-tests (two tailed, equal variance).

Histology and Skeleton Preparation. Embryos were dissected on the indicated days and fixed in 4% (vol/vol) PFA overnight. Embryos were then embedded in Tissue-Tek OCT compound, frozen, and sectioned at 12 μm. Sections were stained with hematoxylin and eosin (H&E) using a standard protocol. For limb skeleton preparation, alcian blue and alizarin red staining was performed using standard procedures. Briefly, we eviscerated the embryos, including peritoneal content, followed by scalding with hot tap water (65–70 °C). Embryos were fixed in 95% (vol/vol) ethanol overnight and then transferred to acetone overnight to remove fat. Embryos were then transferred into 0.03% alcian blue (Sigma) solution for cartilage staining. After a few washes with 70% (vol/vol) ethanol for 6–8 h, samples were placed in 1% KOH until they became transparent. Next, embryos were transferred into 0.05% alizarin red (Sigma) overnight for bone staining. Embryos were cleared by placing them in 1% KOH/20% (vol/vol) glycerol for 2 d and stored in a 1:1 mixture of glycerol and ethanol until image processing.

Whole Mount in Situ Hybridization. Whole mount in situ hybridization was performed as previously described (4). Briefly, specimens were fixed overnight in 4% (vol/vol) PFA in PBS, dehydrated in graded methanol, and permeabilized by proteinase K. Specimens were then hybridized with digoxigenin (Dig)-labeled antisense RNA probes followed by incubation in anti-Dig antibody conjugated with alkaline phosphatase. Colorimetric reaction was carried out using NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3′-indolyphosphate p-toluidine salt) as the substrate. Riboprobes for *Gli1* (5) and *Ptch1* (6) were prepared as previously described. A riboprobe for mouse *Hoxd13* was generated from 407-base pair (bp) mouse *Hoxd13* cDNA containing the +925 to +1,020 coding region and 311 bp 3′ untranslated region (NM_008275.3).

Electron Micrographs. For scanning electron micrographs, limb buds of embryonic day (E) 11.5 $Ick^{+/+}$ *and* $Ick^{tm1a/tm1a}$ embryos were fixed with 2.5% (vol/vol) glutaraldehyde and 2% (vol/vol) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4 °C overnight, and rinsed with 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% (vol/vol) glutaraldehyde and 2 mM calcium chloride, three times at 4 °C for 20 min. The specimens were postfixed with 1% osmium tetroxide at 4 °C for 1 h and immersed in saturated thiocarbohydrazide at room temperature for 20 min. The specimens were then dehydrated using a graded ethanol, dried using a critical point dryer (HCP-2; Hitachi), affixed on an adhesive tape, and coated with gold by ion sputter coater (IB-3; Eiko). Specimens were examined and photographed using scanning electron microscopy (S-4700; Hitachi).

For transmission electron micrographs, limb buds of E11.5 $Ick^{+/+}$ and $Ick^{tm1a/tm1a}$ embryos were fixed with the same procedure as the scanning electron micrograph. The specimens were postfixed with 1% osmium tetroxide at 4 °C for 2 h, dehydrated using graded ethanol, and infiltrated with propylene oxide. Specimens were embedded using Poly/Bed 812 kit (Polysciences). After fresh resin embedding and polymerization in an electron microscope oven (TD-700; Dosaka) at 60 °C for 24 h, the specimens were initially sectioned in 350-nm thickness using Leica EM UC-7 Ultramicrotome (Leica Microsystems) and stained with toluidine blue for light microscopy. The specimens were then sectioned in 80-nm thickness and double stained with 7% (vol/vol) (20 min) uranyl acetate and lead citrate for contrast staining. All of the thin sections were observed by transmission electron microscope (JEM-1011, 80 Kv; JEOL) at the acceleration voltage of 80 kV.

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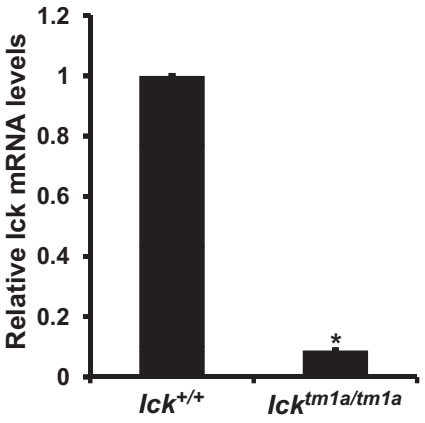

Fig. S1. Quantitative RT-PCR analysis for *Ick* gene expression in embryos. To measure relative amount of Ick gene expression in E11.5 wild-type or Ick mutant embryos, quantitative RT-PCR analysis was performed and the expression level of *Ick* in mutant embryo significantly reduced.

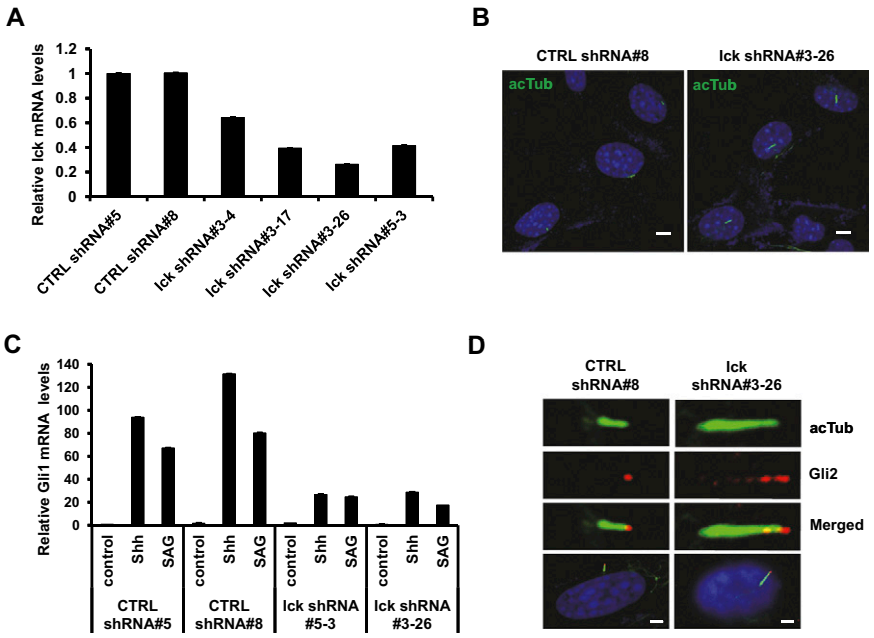

Fig. S2. *Ick* gene knockdowns in tissue culture cells using gene-specific shRNAs show elongated cilia and reduced Shh responsiveness. (*A*) Using two different *Ick* gene-specific shRNAs (ICK shRNA 3 and 5) from the RNAi Consortium (TRC), we generated stable cell lines that constitutively knockdown *Ick* in NIH 3T3 cells. The relative knockdown efficacies of *Ick* shRNAs compared with control shRNA stable cell lines were measured by quantitiative RT-PCR. ICK shRNA 3–26 clone most effectively reduces the endogenous ICK gene expression. (*B*) *Ick* shRNA stable cell line shows elongated cilia. (Scale bars, 5 μm.) (*C*) Shh responsiveness of *Ick* shRNA stable cell lines by SHHN conditioned medium or Smo agonist (SAG) treatment was reduced compared with control shRNA stable cell lines. Gli1 mRNA levels were measured to determine the Shh responsiveness. (*D*) Immunofluorescent staining of endogenous GLI2 proteins in *Ick* shRNA stable cell lines show that GLI2 proteins display punctate accumulation in the axoneme of elongated cilia. In contrast GLI2 proteins in control shRNA cells were localized at the tips of primary cilia, which were stained with acetylated tubulin (acTub) antibody. (Scale bars, 2.5 μm.)

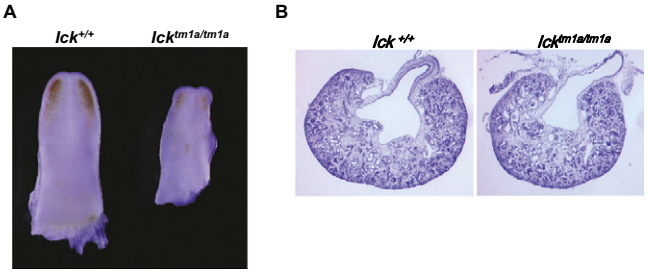

Fig. S3. ICK mutant mice phenotype. (*A*) Gross morphology of E15.5 wild-type or ICK mutant embryo tongues. Tongues were surgically removed and fixed in 4% (vol/vol) PFA before photography. Mutant embryo tongues were smaller than those of wild type and malformed. (*B*) Histological examination of $Ick^{tm1a/tm1a}$ embryo kidneys, which showed cyst-like tubule expansion. Sections of 4% PFA-fixed E16.5 embryonic kidneys were counterstained with hematoxylin.

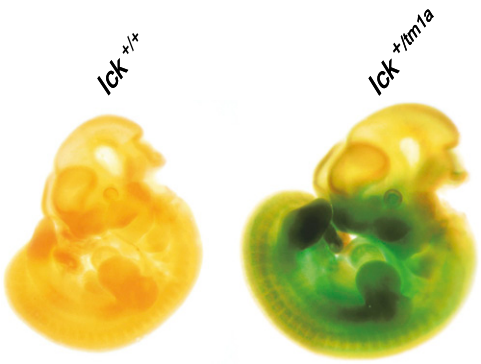

Fig. S4. Tissue-specific *Ick* gene expression in embryo. Whole mount X-gal staining for E10.5 wild-type or heterozygote $Ick^{+/tm1a}$ embryo was performed to visualize the tissue-specific distribution of *Ick* gene expression in embryo.

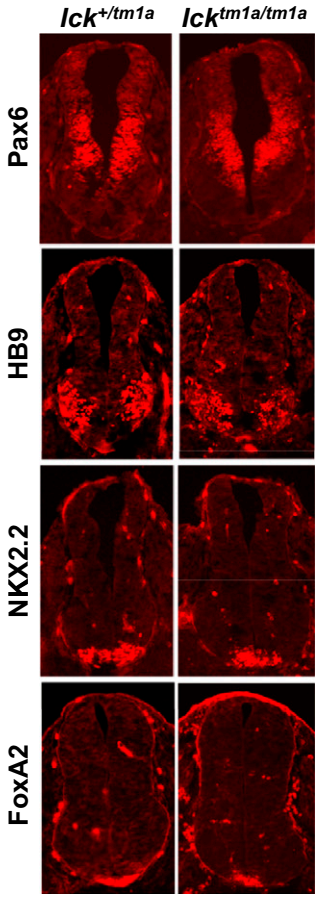

Fig. S5. Spinal neural tube patterning in wild-type and *Ick* mutant mouse embryos ($Ick^{tm1a/tm1a}$). Ventral neural tube patterning in E10.5 *Ick* mutant embryos was examined using the SHH-regulated neuronal cell identity markers FoxA2, Nkx2.2, HB9, and Pax6. The expression domains of these markers in neural tubes of mutant embryos were identical to those of wild-type embryos.