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Methods

Mice

Math1-Cre;ilk\textsuperscript{loxP/loxP} mice were generated by mating Math1-Cre\textsuperscript{+/−} [1] with ilk\textsuperscript{loxP/loxP} and backcrossing the Math1-Cre;ilk\textsuperscript{loxP/+} progeny with ilk\textsuperscript{loxP/loxP} [2] Math1-Cre;Smo\textsuperscript{loxP/loxP} mice were generated by mating Math1-Cre\textsuperscript{+/-} with Smo\textsuperscript{loxP/loxP} [3] and backcrossing the Math1-Cre;Smo\textsuperscript{loxP/+} progeny with Smo\textsuperscript{loxP/loxP}. Routine genotyping of tail DNA samples used the primer pairs for the Cre transgene and Smo LoxP transgene according to The Jackson Laboratory protocol for No. 007684 and No. 004526, respectively. For monitoring the ILK genotype, the primers used were: 5′-TTCTGTGGGAACCTGGTACA-3′ and 5′-GTGCCACCTGCAAATTACAA-3′. These primers yielded amplicons of 450 bp and 280 bp for the LoxP and wild-type alleles, respectively.

Immunohistochemistry

Whole heads from E18.5 mice were fixed in formalin for 2 days at RT. A needle-sized hole was made through the center of the head to improve permeation of the fixative. Heads were then cut down the midline and paraffin-embedded to obtain sagittal sections (4 μm) of the vermis region for IHC. Sections were dewaxed and rehydrated. Microwave antigen retrieval was carried out in 0.01 mM citrate buffer (pH 6.0) and sections were treated with 1% hydrogen peroxide for 15 mins. After blocking with normal goat serum, primary antibodies, ILK (4G9, Cell Signaling Technology; 1:200) and Gli2 (Abcam, 1:500), were added to sections and incubated at 4°C overnight, detected with avidin–biotin complex (ABC) and a color reaction product developed following addition of 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma). Sections were counterstained with H&E and mounted with DPX (Merck).

GCP isolation and FACS analysis

The cerebellum was dissected from day 7 post-partum mice, minced into small pieces and digested with Dispase (BD) for 15 min. After washing with HBSS, cells were treated with DnaseI for 10 min, triturated gently and passed through a 40 μm filter to obtain a single cell suspension, then fixed in 4% PFA. For HNK1 FACS analysis, cells were incubated with Fc Block (1:200, BD) then HNK1 (1:400, Santa Cruz) primary antibody and detected with FITC-conjugated secondary antibody (1:1000, Santa Cruz). Otherwise, cells were first permeabilized in 0.1% Triton-X before blocking (2%NDS/0.1%BSA) and incubating with Math1 (1:100, Santa Cruz) or ILK (4G9) (1:100, CST) primary antibodies, detected with AlexaFluor488- or 647-conjugated secondary antibodies (Life Technologies). All incubations were done at RT for 20-30 min. Cells incubated with secondary antibody only were included as negative controls.
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Plasmids

Split Venus plasmids (referred to as V1 and V2) were kindly provided, by Drs Stephen Michnick and Jean-François Paradis (University of Montreal). The coding region of each gene (human sequence) was cloned into one or both vectors in-frame with the fluorophore, which was N-terminal of ILK, ParvA, Pinch1 and Arrb1 and C-terminal of Smo and Arrb2.

Cell culture and transfections

IMCD3 and C3H10T1/2 cells were obtained from ATCC and maintained in DMEM medium containing 10% FCS. For siRNA experiments, cells were transfected with non- or gene-targeted siRNAs (0.1 µM, SmartPool, Dharmacon) using Lipofectamine 2000 (Invitrogen). The level of knockdown was measured using quantitative PCR and Western blot at 48 and 72 h post-transfection, respectively. For expression studies, cells were transfected on coverslips in 24-well plates with mCherry-tagged (500 ng) or Venus-tagged (150 ng of each V1 and V2) plasmids using Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. Cells were allowed to recover in culture media for one day after transfection and then serum-starved in 0.5% FCS DMEM for 24 hrs to induce primary cilia. In experiments requiring Hedgehog induction, SHh-CM (1:4 dilution), rhSHh (R&D Systems) or SAG (Calbiochem) was added to the cells for indicated times after serum starvation. Shh-CM was from HEK293 cells, engineered to express a secreted form of Shh (ShhN-pIND)[4]. Conditioned medium from untransfected 293 cells, or SHh-CM pre-incubated with 5E1 anti-Hh monoclonal antibody were used as controls.

Primary Mouse Embryonic Fibroblast Lines

Primary MEFs were isolated from E13.5 wild-type and ILKloxP/loxP mice. At confluence, cells were split 1:5 into 6-well culture dishes containing polylysine-coated coverslips. After reaching 70% confluence, cells were infected with 1 µl AdCre-GFP virus (Gene Transfer Vector Core, University of Iowa, 6x10^9 pfu/ml) for 4 h. The following day, cells were serum-starved for 4 days before fixing in formalin for immunofluorescence.

Quantitative RT-PCR

Messenger RNA was extracted using innuPREP RNA mini kit (Analytik Jena AG, Germany) and cDNA synthesized using either Cells-to-Ct reverse transcription (Applied Biosystems) or Transcriptor High Fidelity cDNA Synthesis (Roche) kits. Transcript levels measured using SYBR Green (Invitrogen) probes were normalized to 18S ribosomal RNA. PCR using SYBR Green (Invitrogen) was run on the 7900HT Fast RT-PCR System, and gene expression data acquisition
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and analyses were performed using the Sequence Detection System Version 2.3 software (Applied Biosystems). Primer sequences for murine gene products were:

18S forward 5’-GTAACCCGTTGAACCCCATT-3’,
18S reverse 5’-CCATCCAATCGGTAGTAGCG-3’;
ilk forward 5’–AAGGTGCTGAAGGTTCGAGA- 3’
ilk reverse 5’–AGTGTGTGACCTACGGCATA- 3’
Gli1 forward 5’-AATTCGTGTGCCATTTGGGAG-3’
Gli1 reverse 5’-AGTCAGTCTGTCTCTCTTCCCT-3’
Gli2 forward 5’-CAAGCAGAAACAGCGAGTCAG-3’
Gli2 reverse 5’-CCTCAGCCCTCAGTCTTGACC-3’

In some cases, transcript levels were measured using Taqman probes (Applied Biosystems; ILK: Mm00439671, Gli1:Mm00494645, Gli2, Mm01293116) and normalized to GAPDH (Mm003302249) levels, giving similar results to the SYBR Green analyses.

All samples were assayed in triplicate, in two independent SYBR Green experiments.

**Western blots**

Cells were lysed in RIPA buffer with protease inhibitors 3 days after siRNA transfections and 4 days after AdCre infection and Western blots conducted using standard protocols for the Odyssey system (Licor). Primary antibodies, ILK (4G9 clone, CST), Gli2 (Abnova), PanActin (Ab-5, NeoMarkers) and GFP (detecting V2 or V1 fluorophore, Roche) were detected with either AlexaFluor 680 (Invitrogen) or IRDye800 (Rockland) secondary antibodies and membranes were scanned on Odyssey Scanner (Licor). Protein levels were quantitated relative to actin levels.

**Immunofluorescence**

Cells were fixed in formalin for 10 min at RT, permeabilized with 0.1% TritonX/PBS followed by 1%SDS/PBS for 5 min at RT, washed briefly in PBS and then re-fixed in formalin. Following six washes in PBS (to ensure all SDS was removed), cells were blocked in blocking buffer (PBS with 2% normal donkey serum and 0.1% BSA) and incubated with the relevant primary antibodies for 1 h at RT. Primary antibodies included rabbit ILK (4G9 clone, CST), mouse ILK (65.1 clone, Santa Cruz), Smo (LifeSpan), acetylated-α-tubulin (Sigma), γ-tubulin (Sigma), paxillin (BD Bioscience), and GFP (detecting V2 fluorophore, Roche). Acetylated α-tubulin was directly conjugated with the fluorophore using Mix-n-Stain™ CF™ Dye Antibody Labeling Kit (Biotum). Other antibodies were detected with Alexafluor (Invitrogen) secondary antibodies before mounting with Prolong Gold Antifade (Invitrogen). Immunofluorescence data for Smo translocation was collected using a Nikon C1 laser scanning confocal microscope, and that for Venus experiments was collected using
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A Deltavision confocal microscope. A 60x 1.4NA objective was used and images taken at 1024x1024 12 bit resolution and 0.3µm z-step size. Scans were performed in a sequential manner to minimize fluorophore crosstalk. Duplicate coverslips were analyzed for each treatment and a minimum of 100 cells counted per coverslip. Each experiment was conducted three times.

**Venus quantification and Primary Cilia analyses**

Venus interactions and primary cilia identification and quantitation were performed using Imaris analysis software (Bitplane AG, v7.0). Briefly, the fluorescence signal of acetylated α-tubulin was used to trace out enclosed volumes. To eliminate background, any defined volumes with a longitudinal length of <0.7 µm as well as containing below background levels of γ-tubulin signal were excluded from the analysis. Each remaining volume was then considered an individual primary cilium and analyzed.

Split Venus interactions were assessed with interaction maps generated by the following formula calculated in a per-pixel 32-bit fashion, then re-scaled into 16-bit for display:

\[
\frac{I_{\text{Venus}} \cdot Bg_{\text{Venus}}}{I_{\text{GFP}} \cdot Bg_{\text{GFP}}} = \frac{I_{\text{Venus}} - Bg_{\text{Venus}}}{I_{\text{GFP}} - Bg_{\text{GFP}}}, \text{ where } I = \text{pixel intensity and } Bg = \text{background intensity.}
\]

Background values were determined by quantifying the fluorescence of samples transfected with non-interacting split Venus components and secondary antibody alone anti-GFP immunofluorescence, for Venus and anti-GFP backgrounds respectively. The mean fluorescence + 2 standard deviations were then used as the background intensity for subtraction in the formula. Thus, the interaction map displays the relative level of split Venus interaction relative to the amount of split Venus components present. Cilia length analysis was performed by Imaris v7.6.1 (Bitplane AG). Briefly, diffraction limited confocal images of acetylated tubulin were modelled in 3D, whereby primary cilium was defined as acetylated tubulin fiber with >1450 intensity units (IU) and >0.5µm in length and adjacent to high intensity gamma tubulin signal (>4095 IU in 12-bit space).

**Alkaline phosphatase induction by Shh and SAG in C3H10T1/2 cells**

AP assay was conducted as previously described [5]. Briefly, C3H10T1/2 cells were plated in 96-well plates at 5000 cells/well and serum-starved in 0.5% FCS DMEM media the next day. After 24 hours, SHh (SHh-CM or rhSHh) or SAG was added and the cells incubated for a further 5 days. Inhibitors were added 1 h prior to the addition of SHh and remained in the media for the duration of the experiment. For siRNA experiments, C3H10T1/2 cells were seeded at 10000 cells/well and reverse transfected with siRNA as described above. At the end of the 5 days of treatment, cells were lysed and assayed for AP activity using the chromogenic substrate p-nitrophenylphosphate.
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(Fischer Biotech) and read at 405 nm. Values were normalized to cell numbers measured on
duplicate plates using an ATP assay (Promega). Each assay was performed a minimum of three
times, with treatments assayed in triplicate.

Statistical Analysis
Data were analyzed using Student’s t-test, as appropriate (n > 2), for comparing means between
treatment groups or time points, as indicated in Figure Legends (GraphPad Prism 4.0).

1. Lumpkin, E.A., et al., Math1-driven GFP expression in the developing nervous system of
2. Terpstra, L., et al., Reduced chondrocyte proliferation and chondrodysplasia in mice
3. Long, F., et al., Genetic manipulation of hedgehog signaling in the endochondral skeleton
4. Chen, J.K., et al., Inhibition of Hedgehog signaling by direct binding of cyclopamine to
5. Williams, K.P., et al., Functional antagonists of sonic hedgehog reveal the importance of
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Supplementary Figure Legends

Figure S1: Math1-Cre-mediated deletion of ILK GCPs comprising the EGL. Representative sagittal sections of cerebellae from E18.5 ilkloxP/+ (control) and Math1-Cre;ilkloxP/loxP mice labeled with rabbit monoclonal antibody to ILK. Arrows indicate GCPs bounded by dashed line indicating the EGL boundary. Scale bar = 50 µm.

Figure S2. ILK is not required for ciliogenesis. (A) Confocal images of Scr control siRNA-, Kif3a siRNA- and ILK siRNA-transfected IMCD3 and 10T1/2 cells labeled with anti-α-tubacet antibody (cilia, green), γ-tubulin (centrosome, red) and DAPI (nucleus, blue). scale bar = 15 µm. (B, C) Quantitation of the number of cells with cilia in respective siRNA-transfected (B) IMCD3 and (C) C3H10T1/2 cells. The level of mRNA knockdown relative to scrambled siRNA levels for each cell line is indicated. Data are presented as mean ± SEM. **p<0.001, n=3-4 (Student’s t-test). (D) Single-plane confocal images of ilkloxP/+ and ilkloxP/loxP primary MEFs transfected with GFP-tagged adeno-Cre (Ad-Cre) and labeled with anti-α-tubacet antibody (cilia, red). Arrows point to cilia. Scale bar = 15 µm. (E) The number of Ad-Cre positive cells with cilia were counted in ilkloxP/+ and ilkloxP/loxP primary MEFs, confirming that deletion of ILK does not effect ciliogenesis. 100% = 111 and 114 cells, respectively. (F) Western blot showing depletion of ILK protein in ilkloxP/loxP but not ilkloxP/+ cells after AdCre infection. (G) Cilia length was counted in serum-starved IMCD3 cells transfected with ILK or scrambled control siRNA, and stimulated with SHh-CM for 6 hr as indicated. Cilia were identified by proximity of α-tubacet and γ-tub markers, and IMARIS software was used to measure cilia length. Data points from two independent experiments are presented, with red lines indicating means of these experiments, with 315-627 cilia counted in each group.

Figure S3: Specific ILK interactions detected in BiFC assays localize correctly in fibroblasts. Focal adhesion binding partners of ILK were used to verify the specificity of BiFC assays in C3H10T1/2 cells. (A, B) Stacked confocal images of V1-ILKwt/V2-ParvA and V1-ILKwt/V2-PINCH transfected cells demonstrate reconstituted Venus signal (green) in focal adhesions marked using an anti-paxillin antibody (red). Nuclei are labeled with DAPI. Scale bar = 10 µm. (C, D) Venus signal is not reconstituted in cells transfected with V1-ILKΔANK/V2-PINCH. Transfected cells were identified by immunofluorescence with a GFP antibody specifically directed to the V2 tag (red) and a second GFP antibody that recognizes the V1 tag (magenta). Scale bar = 15µm.
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Figure S4: Specific ILK interactions detected in BiFC assays localize correctly in epithelial cells. Focal adhesion binding partners of ILK were used to verify the specificity of BiFC assays in IMCD3 cells. (A-D) as in Figure S3. (E) Western blots showing expression of Venus constructs transfected in IMCD3 cells. The membrane was probed with GFP antibody specific to V1-tag, then stripped and re-probed with GFP antibody specific to V2-tag. (F) IMCD3 cells were transfected with V1-arrB1 and V2-Smo (green). GFP antibody confirmed expression of both fusions (red), and γ-tub (blue) and α-tub acet (magenta) marked basal bodies and cilia, respectively. Inset is shifted overlay of ciliary signals. (G) V1-ILK and V2-arrB1 were co-transfected into IMCD3 cells, with basal bodies and cilia marked as in (F). ILK-arrB interaction (green) localized between cilia and basal body, in transition zone with high signal intensity calculated by interaction density mapping. Scale bar = 15µM.

Figure S5: Interactions of ILK with focal binding partners ParvA and PINCH do not localize ILK to cilia. (A) Serum-starved IMCD3 cells were transfected with mCherry-ILK<sup>M402A/K403A</sup>, which does not bind ParvA. Co-localization of mCherry signal with α-tub acet (green) and γ-tub (magenta) in the cilium juxtaposed to basal body. IMCD3 cells were co-transfected with (B) V1-ILKΔANK and V2-Smo, and (C) with V1-ILKΔANK and V2-arrB2. Cilia and basal bodies were marked as in (A). ILKΔANK lacks the N-terminal ankyrin repeat domain of ILK, which mediates binding to PINCH. Scale bar = 5µM.
Supplementary Figure S3

A  V1-ILK + V2-ParvA  anti-Paxillin  merge

B  V1-ILK + V2-Pinch  anti-Paxillin  merge

C  V1-ILK<sup>ΔANK</sup> + V2-ParvA  anti-GFP-V2  anti-GFP-V1

D  V1-ILK<sup>ΔANK</sup> + V2-Pinch  anti-GFP-V2  anti-GFP-V1
Supplementary Figure S4

F: V1-arrb1 + V2-SMO
   anti-GFP
   γ-tub/α-tub acet
   merge

G: V1-ILK + V2-arrb1
   anti-GFP
   γ-tub/α-tub acet
   merge

ILK-arrb1/γ-tub interaction density map

bar scale
A mCh-ILKM402A/K403A/α-tubacet/γ-tub
mCh-ILKM402A/K403A/α-tubacet/merge
mCh-ILKM402A/K403A/γ-tub

B V1-ILKΔANK + Smo-V2
anti-GFP α-tub acet/γ-tub merge shifted overlay
cilia positive for venus signal = 42/65

C V1-ILKΔANK + V2-Arrb2
anti-GFP α-tub acet/γ-tub merge merge