Supplementary Experimental Methods

Antibodies and Reagents

Rabbit anti-Arl13b polyclonal serum was a gift from Tamara Caspary (Caspary et al., 2007) (1:500). Commercial antibodies used were against Arl13b (mAb N295B/66, Neuromab; 1:500), Gli3 (AF3690, R&D, 1:1000), α-tubulin (clone DM1A, T6199, Sigma; 1:5000), acetylated α-tubulin (mAb 6-11B-1, Sigma; 1:2000), Gli1 (L42B10, Cell Signaling; 1:1000), FoxA2 (ab40874, Abcam; 1:2000), BrdU (11170376001, Sigma-Aldrich, Fig. 5; 1:25), BrdU (BU1/75[ICR1], Abcam, Fig. S7; 1:200), Cyclin D1 (RB9041, Thermo Scientific; 1:200), and p130 (21/p130[Cas], 610271, Becton Dickinson; 1:200). Monoclonal antibodies developed by O.D. Madsen (Nkx6.1: 1:10) were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, the University of Iowa, Iowa City IA 52242, USA. Fluorescent secondary antibodies for immunofluorescence were from Jackson ImmunoResearch (1:2000 for Alexa488 labeled secondary antibodies, and 1:1000 for the rest), while IRDye 700CW and IRDye 800CW secondary antibodies for immunoblotting were from Li-cor Biosciences (used at 1:5000).

Primary cell culture, Reverse transcription and Quantitative PCR

The dissected forelimbs of E13.5 *Gpr161*$^{f/f}$ embryos were mechanically dissociated by pipetting, and by Trypsin-EDTA treatment. Dissociated cells were maintained in medium containing 10% FBS (high-glucose DMEM, 0.05 mg/ml penicillin, 0.05 mg/ml streptomycin, 2 mM glutamax, 0.1 mM MEM nonessential amino acid supplement, and freshly prepared 0.1 mM β-mercaptoethanol), and assayed within 4-5 passages in culture. For qRT-PCR, total RNA was prepared with GenElute mammalian total RNA purification kit (Sigma). RNA was used for qRT-PCR by using TaqMan one-step RT-PCR master mix reagents (Applied Biosystems). TaqMan probes for qRT-PCR were

published before (Wen et al., 2010) and an inventoried probe for *Gpr161* was from Applied Biosystems. Triplicate reactions were run and analyzed on an ABI 7500 thermocycler using murine *Rpl19* as the endogenous control.

Scanning EM

Embryos were fixed in ½ Karnovsky's fixative (2% PFA, 2.5% Glutaraldehyde in 0.1M Sodium Cacodylate buffer, pH 7.4) overnight and post fixed in 1% OsO_4 for 1 h. They were dehydrated through a series of ethanol. After three washes of hexamethyldesilazane, the samples were air dried at room temperature. The embryos were oriented and mounted on carbon tape on aluminum stubs. They were then sputter coated with 10 nm of gold/palladium mixture and viewed on an FEI XL30 SEM at 10 kV.

Supplementary References

CASPARY, T., LARKINS, C. E. & ANDERSON, K. V. 2007. The graded response to Sonic Hedgehog depends on cilia architecture. *Dev Cell,* 12**,** 767-78.

LOGAN, M., MARTIN, J. F., NAGY, A., LOBE, C., OLSON, E. N. & TABIN, C. J. 2002. Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis,* 33**,** 77-80.

RODDA, S. J. & MCMAHON, A. P. 2006. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development,* 133**,** 3231-44.

WEN, X., LAI, C. K., EVANGELISTA, M., HONGO, J. A., DE SAUVAGE, F. J. & SCALES, S. J. 2010. Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. *Mol Cell Biol,* 30**,** 1910-22.

Supplementary Figure

Fig. S1. Generation of a conditional knockout *Gpr161* allele in mice.
(A) Cartoon depicting strategy for conditional and full knockout of *Gpr161* exon 4. PCR-based genotyping for wild type, floxed (f) and knockout (-) alleles.
(B) Horizontal cryosections of *Gpr161* exon 4 heterozygote (+/-) and knockout (-/-) shows ventralization of neuroprogenitor markers FoxA2 and Nkx6.1 in lumbar neural tube at E10.25. Scale, 100 μm.
(C) Immunoblotting for Gli1 (left) and Gli3 (center) in *Gpr161* exon 4 wild type (+/+) and knockout (-/-) whole embryo lysates show increased Gli1 levels and decreased Gli3 full length (Gli3FL) and Gli3R levels when normalized to α-tubulin. qRT PCR for designated transcripts to the right. N=3 each. Data represented as mean ± SD. ***, P<0.001 **, P<0.01; *, P<0.05 by *t*-test.
(D) Primary forelimb bud mesenchymal cells cultured from E13.5 *Gpr161*$^{f/f}$ embryos were fixed after starving for 48 h. Cells were immunostained for Gpr161 (green), acetylated tub (AcTub, red), and DNA. Inset shows cilia. Arrows depict Gpr161 positive cilia, yellow arrow depicts cilia shown in inset, and arrowhead depicts cilia negative for Gpr161. Scale, 5 μm.
(E) Scanning electron micrographs of E10.25 *Gpr161* heterozygote (+/-) showing presence of both fore/hindlimb buds in lateral view (left panel), and *Gpr161* knockout (-/-) showing lack of forelimbs and presence of hindlimbs in *en face* (middle panel) and lateral view (right panel). Black arrow and white arrowheads mark forelimb and hindlimbs, respectively. White arrow marks absent forelimb. Scale, 200 μm.

Hwang_Figure S1

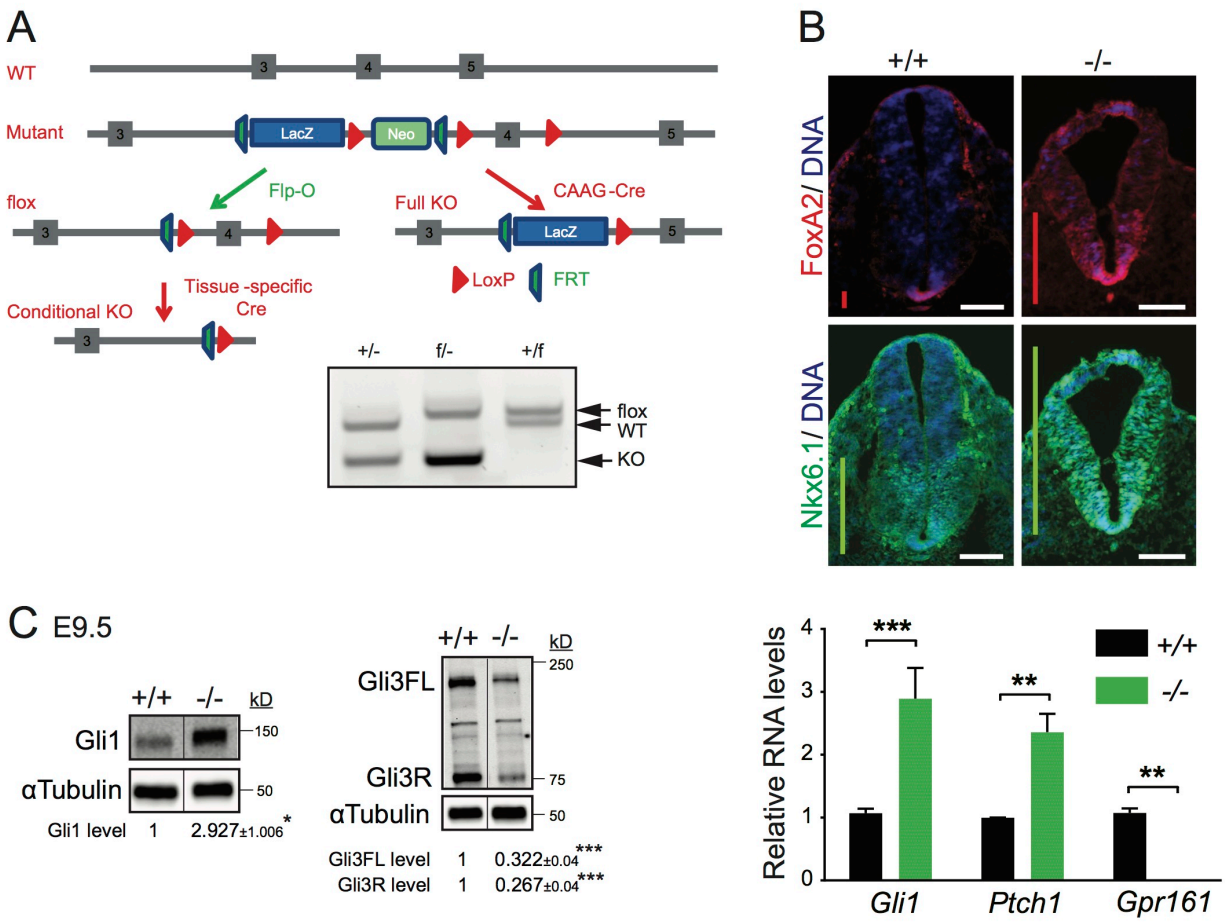


Fig. S2. *Gpr161* knockouts exhibit high Shh pathway activity.
(A) Whole-mount digoxigenin-labeled RNA in situ hybridization for *Gli3* show similar expression in anterior forelimb buds in control littermate (WT) and *Prx1-Cre; Gpr161*$^{f/f}$ (cko) embryos before *Shh* expression at E9.5. Limb buds are depicted by white arrows. N=4 (WT; *Prx1-Cre; Gpr161*$^{f/+}$) and 5 (cko) embryos each.
(B) Whole-mount digoxigenin-labeled RNA in situ hybridization for *Ptch1* and *Shh* show normal expression in E10.5 hindlimb buds of *Prx1-cre; Gpr161*$^{f/+}$ (WT) versus *Prx1-cre; Gpr161*$^{f/f}$ (cko) embryos. By E10.75, *Hoxd13* expression is anteriorly expanded in hindlimb buds in cko embryos. Abbreviations: A, anterior; P, posterior. N=4 limbs each. Scale, 500 μm (A); 200 μm (B).

Hwang_Figure S2

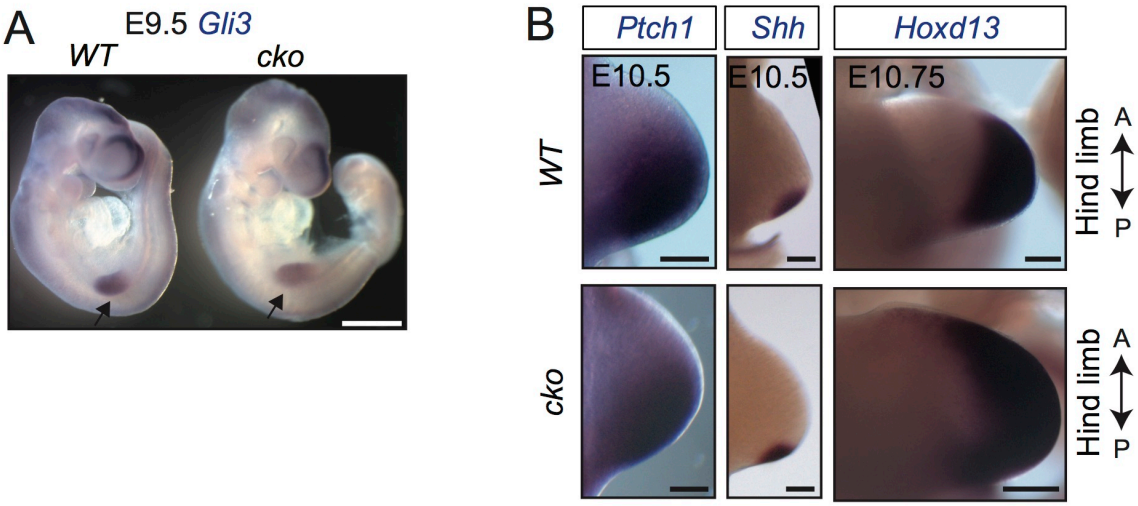


Fig. S3. Gpr161 determines endochondral and intramembranous bone formation.
(A) *Prx1-cre; Rosa26-loxP-STOP-loxP-tdTomato* (*Rosa-LSL-tdT*) embryos depict tdTomato positive fore/hindlimb buds and cranial mesenchyme (arrowhead) (Logan et al., 2002). All lateral views, except right panel (*en face*). Scale, 1 mm.
(B) Alcian blue and alizarin red staining of E16.5 forelimbs (bottom view, left; side view, right) in *Prx1-cre; Gpr161*$^{f/f}$ (*Gpr161* cko) (N=19) shows complete lack of mineralization in humerus (h), radius (r) and ulna (u). Scale, 2 mm.
(C) Horizontal sections from E18.5 *Prx1-cre; Gpr161*$^{f/+}$ (WT) and *Prx1-cre; Gpr161*$^{f/f}$ (*Gpr161* cko) at thoracic level (left two panels) and at abdominal level (right two panels) show ribcage lacking ventral rib fusion, anterior abdominal wall defects, and protruding internal organs in *Gpr161* cko. Scale, 2 mm.
(D) Alcian blue and alizarin red staining of base of the skull from E18.5 of (i) *Prx1-Cre; Gpr161*$^{f/+}$ (WT) (N=20) and (ii) *Prx1-Cre; Gpr161*$^{f/f}$ (*Gpr161* cko) (N=19), show intact endochondral bones in the base of the skull. Abbreviations: bo, basiocciput; bs, basisphenoid; ps, presphenoid.

Hwang_Figure S3

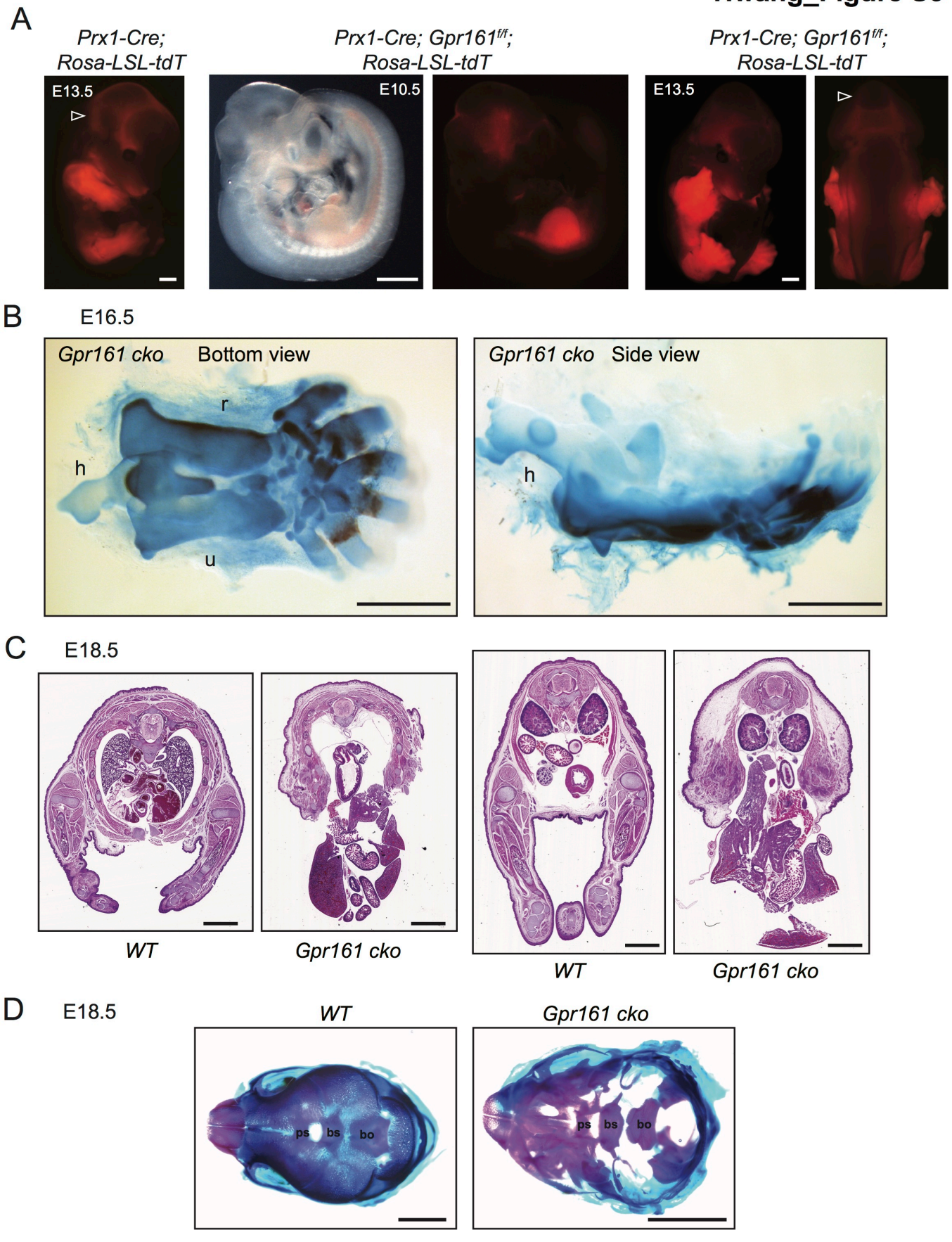


Fig. S4. Lack of maturation and sustained proliferation of periarticular/round chondrocytes in *Gpr161* cko.
(A) Magnified regions of designated region " c "or " c' " from the *Prx1-cre; Gpr161$^{f/f}$* (cko) ulna stained by Safranin O, and von Kossa. Only a few columnar and hypertrophic chondrocytes are visible. Serial sections of this region show faint Ihh expression (Fig. 6A).
(B) E14.5 *Prx1-cre; Gpr161$^{f/+}$* (WT) and *Prx1-cre; Gpr161$^{f/f}$* (cko) forearm cryosections were immunostained for Cyclin D1 (green) and p130 (red). Proximal radius for WT and the whole extent of long bone in cko zeugopod is shown. Note that proliferating chondrocytes in cko are Cyclin D1 positive and lack p130. N=3 each. Scale, 100 μm.
(C) E17.5 *Prx1-cre; Gpr161$^{f/+}$* (WT) (N=1) and (ii) *Prx1-cre; Gpr161$^{f/f}$* (cko) (N=2) hindlimbs sectioned horizontally were stained by Safranin O (left), and von Kossa (right). Note lack of ossification in tibia in *Gpr161* cko (bracket/asterisk). Scale, 1 mm.

Hwang_Figure S4

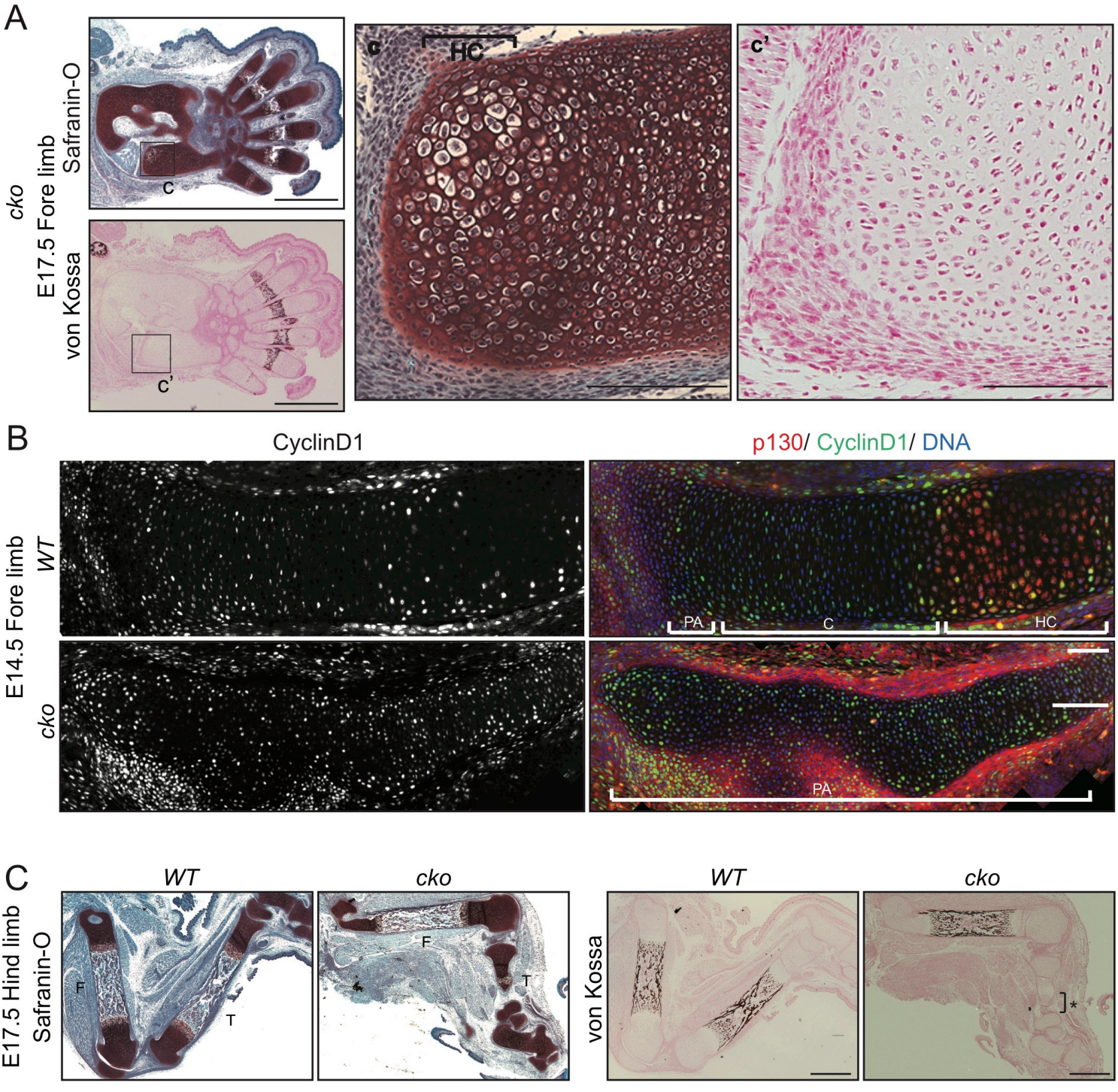


Fig. S5. Decreased Ihh signaling in *Gpr161* cko.
(A) Steps in chondrogenesis and osteogenesis, and role of Ihh signaling during endochondral bone morphogenesis (Rodda and McMahon, 2006).
(B) Full forearm including autopods as shown in Fig. 6A. Note that adjacent autopod metacarpals exhibit Ihh targets *Ptch1* and *Gli1* (perichondrium and proliferating chondrocytes), *Ihh* (prehypertrophic and hypertrophic chondrocytes), *Col X* (hypertrophic chondrocytes), *Runx2* (osteoblast progenitors) and *Osx* (immature osteoblasts). Skin hair follicles also exhibit Shh pathway activation (*Ptch1*, *Gli1* expression). Abbreviations: R, radius; U, ulna. Scale, 1 mm.
(C) Embryos sectioned horizontally at forelimb levels from (i) *Prx1-Cre; Gpr161*$^{f/+}$ (WT) (N=2 sides) and (ii) *Prx1-Cre; Gpr161*$^{f/f}$ (cko) (N=8 sides), with designated insets as shown in Fig. 6B.
(D) E17.5 (i) *Prx1-cre; Gpr161*$^{f/+}$ (WT) (N=1) and (ii) *Prx1-cre; Gpr161*$^{f/f}$ (cko) (N=2) hindlimbs sectioned horizontally were probed for expression of designated transcript levels as in Fig. 6. Note that hindlimbs of *Gpr161* cko were less or not affected as in forelimbs (Fig. 6). Abbreviations: F, femur; T, tibia; Fi, fibula. Scale; 1mm.

Hwang_Figure S5

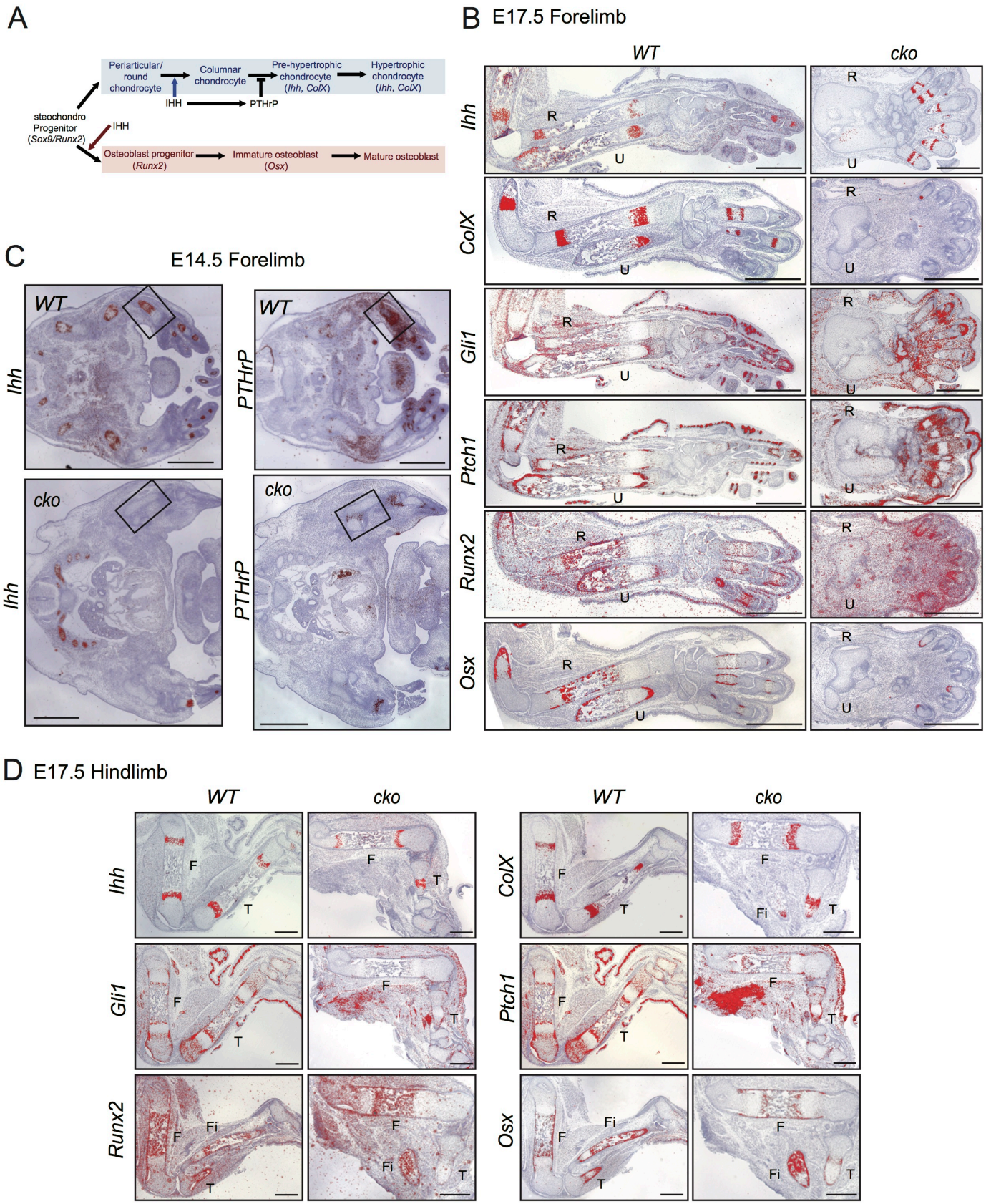


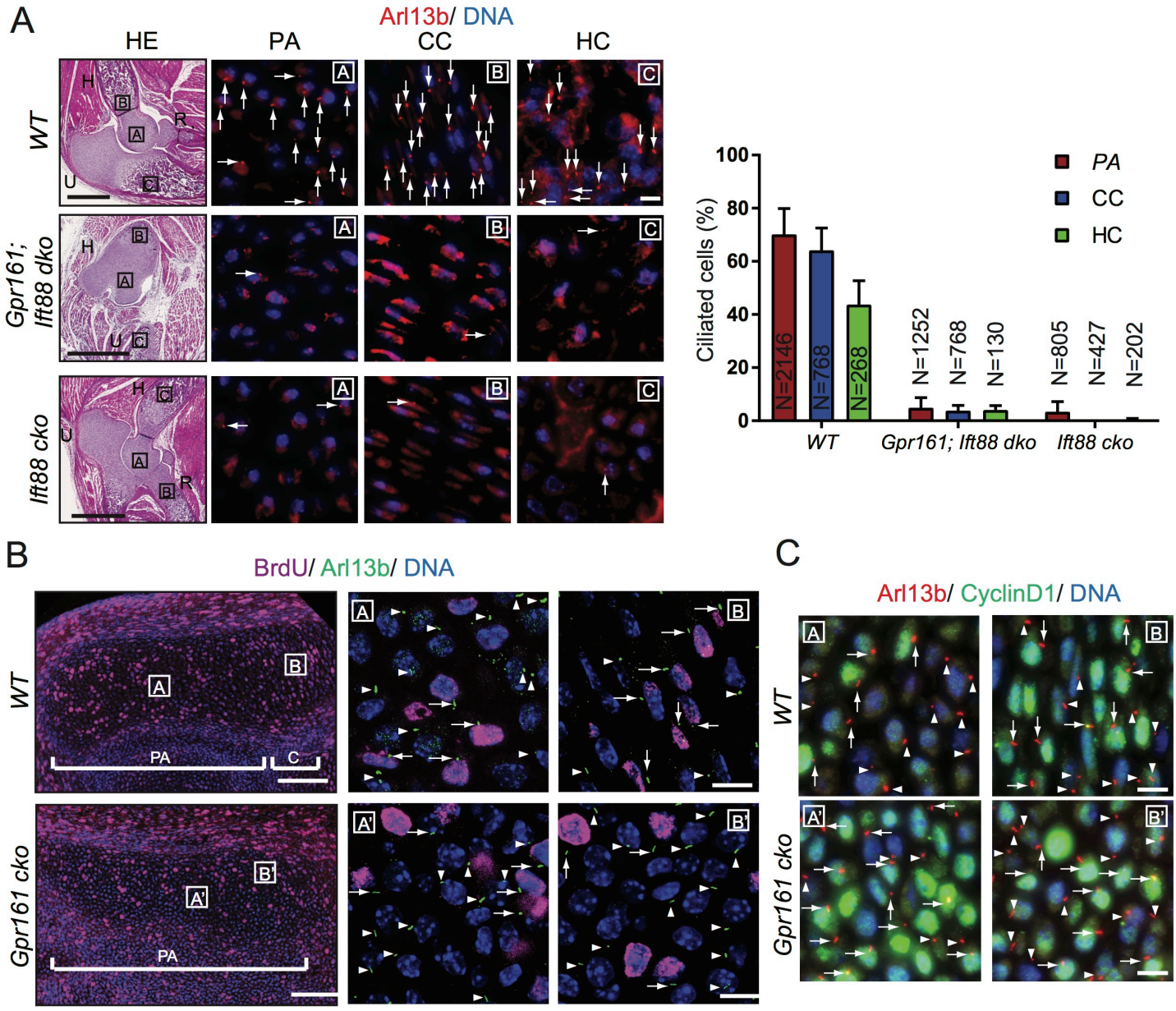
Fig. S6. Gpr161 determines limb patterning and skeletogenesis in a cilia-dependent manner.

(A) Designated regions in sections consecutive to HE stained sections (left) from P0 *Prx1-Cre; Gpr161*$^{f/+}$ (WT), *Gpr161 Ift88* dko, and *Ift88* cko forelimbs were quantified for Arl13b+ cilia after immunostaining for Arl13b (red) and DNA (blue). Arrows refer to cilia. Quantification to the right. N=3 sections each. Data represented as mean ± SD. Scale, 1 mm (left HE panel), 10 μm (right panels). Abbreviations: PA, periarticular/round chondrocytes; CC, columnar chondrocytes; HC, hypertrophic chondrocytes.

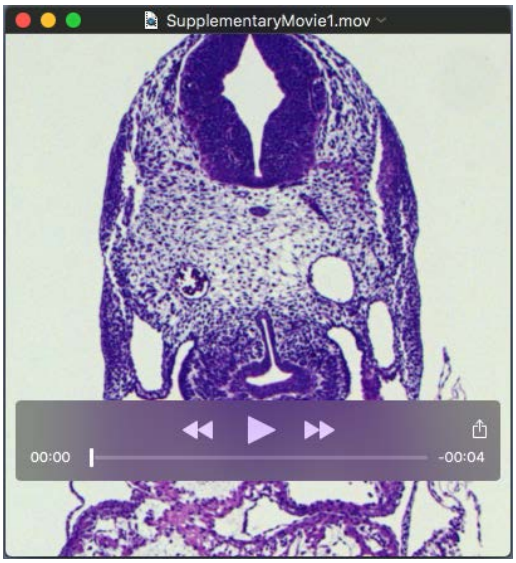
(B) Designated regions from E17.5 *Prx1-cre; Gpr161*$^{f/+}$ (WT) and *Prx1-cre; Gpr161*$^{f/f}$ (cko) forearm cryosections were immunostained for BrdU (3 h pre-labeled before harvesting embryos) (magenta), and Arl13b (green). Note that proliferating chondrocytes in cko are ciliated, irrespective of BrdU labeling. Arrows and arrowheads refer to BrdU +ve ciliated cells and BrdU -ve ciliated cells, respectively. N=3 each. Scale, 100 μm (left), 10 μm (right).

(C) Designated regions similar to **(B)** from E14.5 *Prx1-cre; Gpr161*$^{f/+}$ (WT) and *Prx1-cre; Gpr161*$^{f/f}$ (cko) forearm cryosections were immunostained for Cyclin D1 (green) and Arl13b (red). Note that proliferating chondrocytes in cko are ciliated, irrespective of Cyclin D1 labeling. Arrows and arrowheads refer to cyclin D1 +ve ciliated cells and cyclin D1 -ve ciliated cells, respectively. N=2 each. Scale, 10 μm.

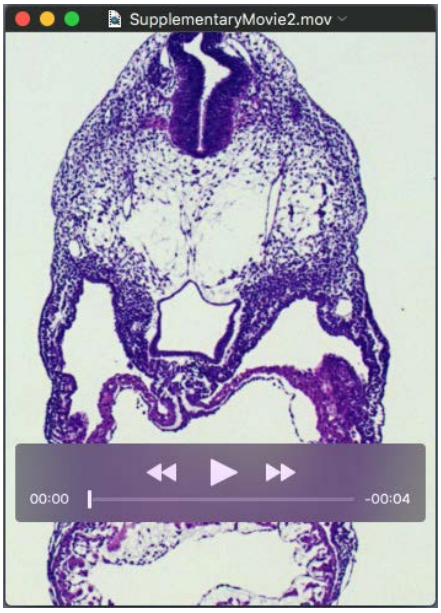
Hwang_Figure S6



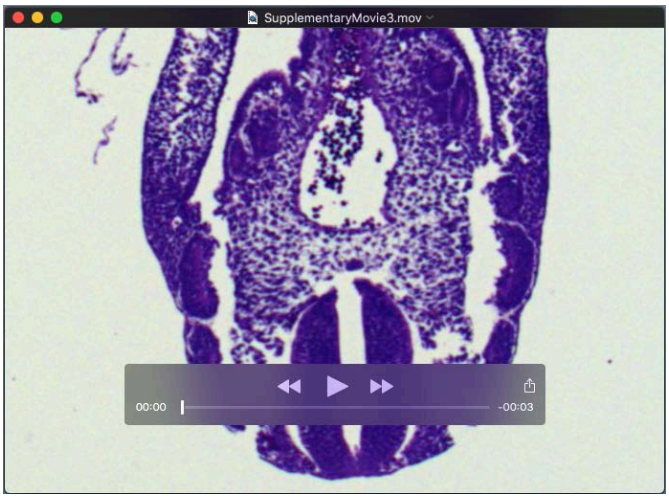
Supplementary Movies



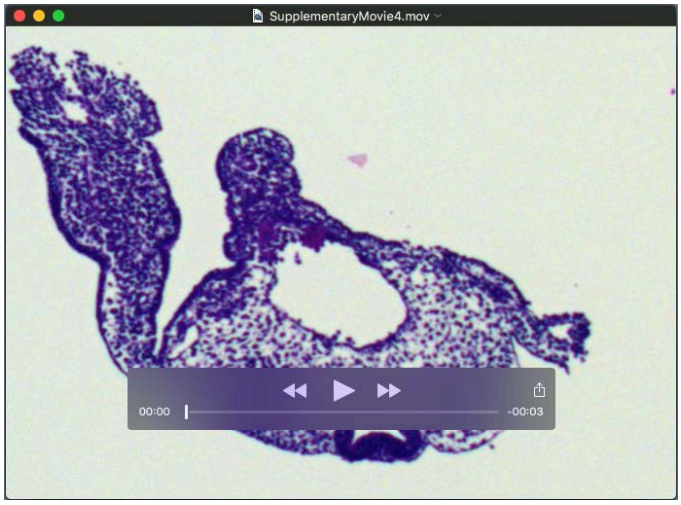
Movie 1. Forelimb buds in wild type embryo at E10.25. Paraffin sections were made at 5 μm thickness starting from the cardiac level and collected every 30 μm for hematoxylin and eosin staining, along with 5 more serial sections for mounting to unstained slides. Serial sections show forelimb mesenchyme. Orientation of embryo section as in Figure 1B.



Movie 2. Lack of forelimbs in *Gpr161*$^{-/-}$ embryo at E10.25. Serial sections collected and processed as in Movie 1 starting from the cardiac level show lack of forelimb mesenchyme. Orientation of embryo section as in Figure 1B'.



Movie 3. Hindlimb buds in wild type embryo at E10.25. Serial sections collected and processed as in Movie 1 starting from the lumbar level show hindlimb bud. Later caudal sections are missing in the video. Orientation of embryo section as in Figure 1B.



Movie 4. Hindlimb buds in *Gpr161*$^{-/-}$ embryo at E10.25. Serial sections collected and processed as in Movie 1 starting from the lumbar level show hindlimb bud. Later caudal sections with open neural tube are missing in the video. Orientation of embryo section as in Figure 1B'.