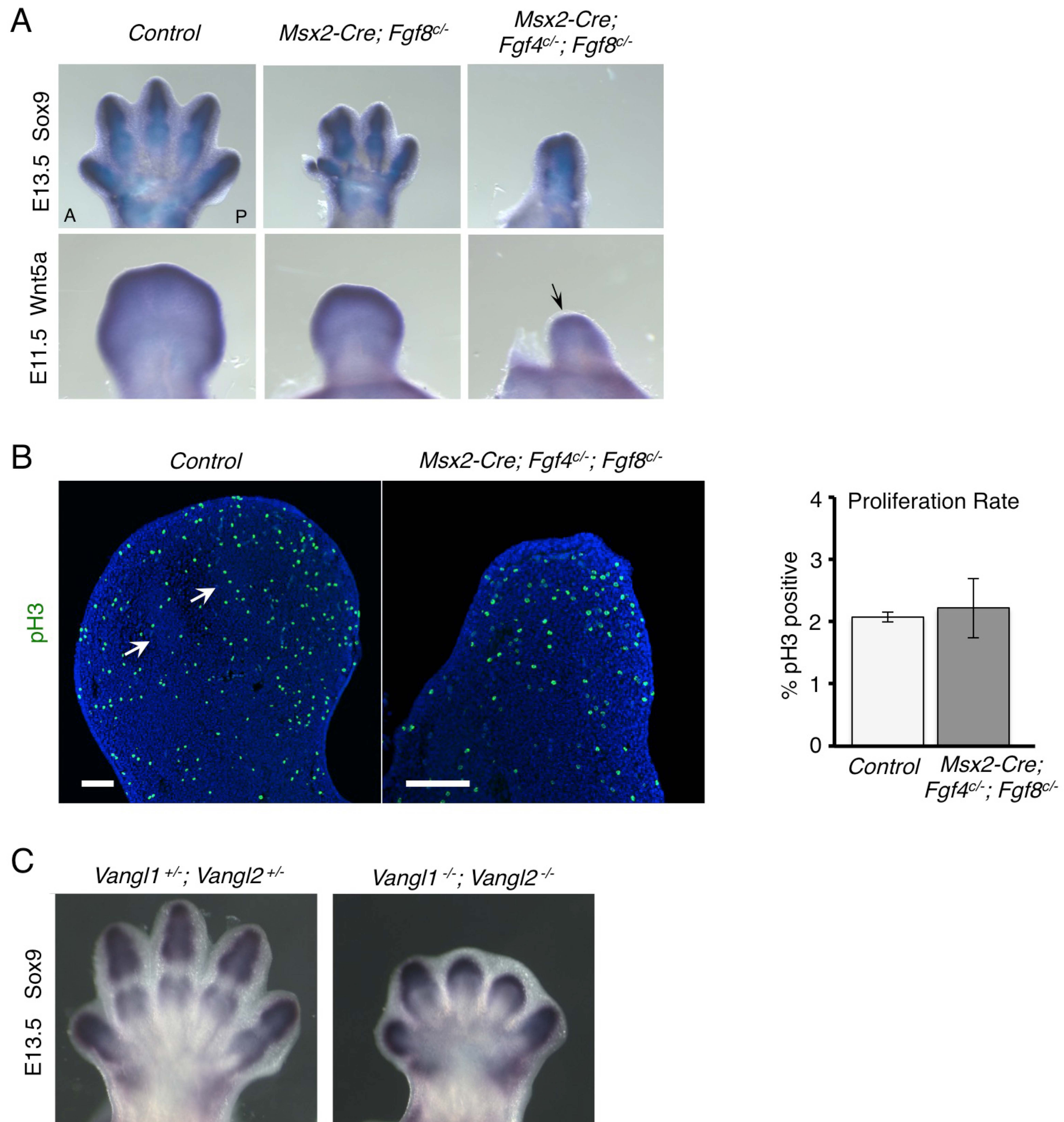
Figure S1



Analysis of *Fgf* and *Vangl* mutants. (A) Whole mount *in situ* hybridization of *Sox9* (upper panel) and *Wnt5a* (lower panel) on control and *Fgf* mutant embryonic forelimbs. Arrow points to the *Wnt5a* expression region of the *Msx2-Cre; Fgf4*$^{c/-}$*; Fgf8*$^{c/-}$ forelimb. (B) Immunodetection of phospho-histone H3 (Ser 10) in control and *Msx2-Cre; Fgf4*$^{c/-}$*; Fgf8*$^{c/-}$ distal forelimbs at E11.5 (44 som). Arrows point to forming digital rays. No statistically significant difference was found (two-tailed *t* test). (C) *Sox9* whole mount *in situ* hybridization in the mouse E13.5 forelimbs of control and *Vangl1/2* double mutant.

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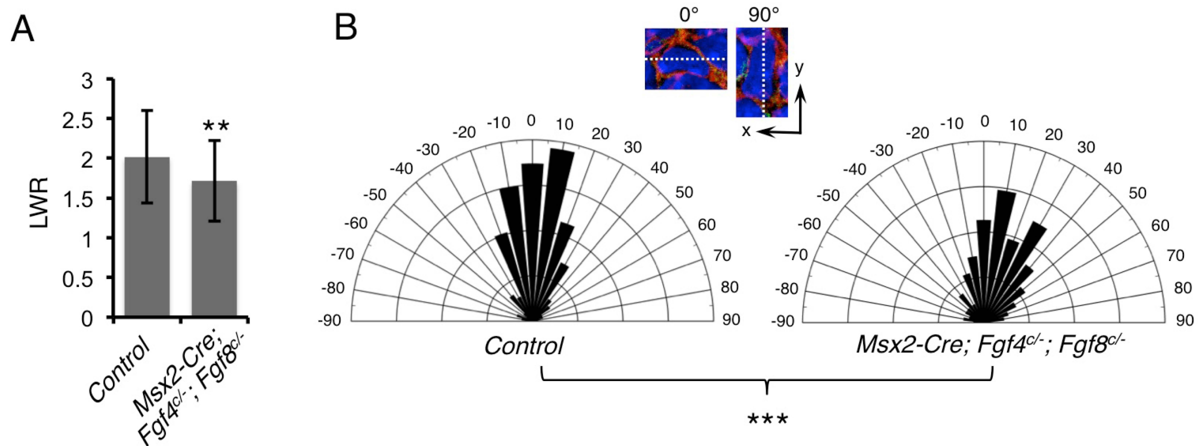
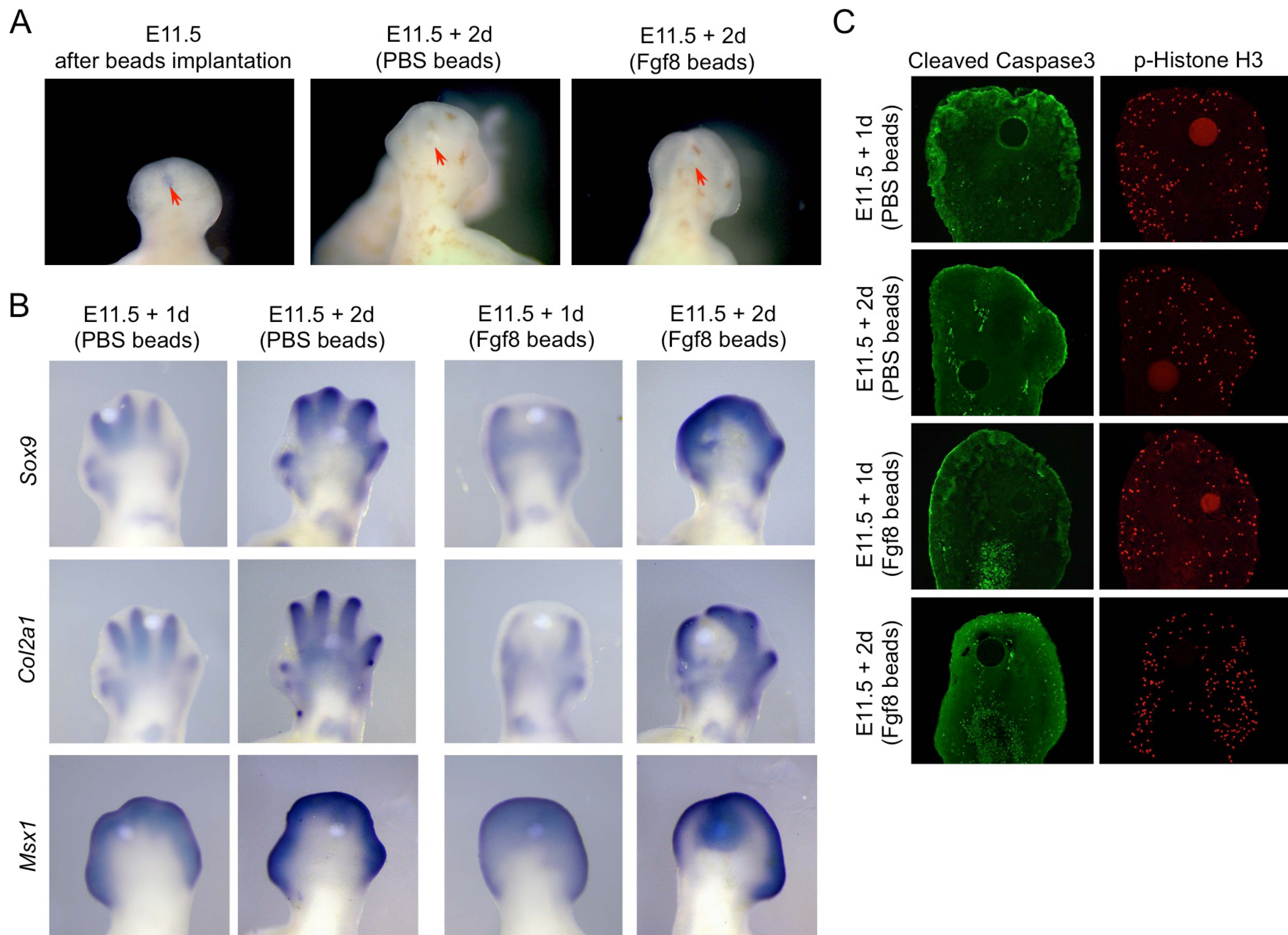
Figure S2



Analysis of Fgf4/8 mutant cell shape and orientation. (A) Statistical analysis of length-to-width ratio (LWR) of distal limb chondrocytes in E13.5 control and *Fgf4/8* double mutant shown in Figure 1B. Two-tailed *t* test, **p value=0.0012. Error bars are ±SD. Analyzed cells: control, n=46; *Msx2-Cre; Fgf4$^{c/-}$; Fgf8$^{c/-}$*, n=129. (B) Schematic diagrams summarizing the quantification of distal cells orientation in E13.5 control and *Fgf4/8* double mutant. The inset shows examples of cell orientation: 0°refers to the cell that orients horizontally, but 90°refers to the cell that orient vertically. The inset x and y axes were defined as shown in Figure 1B. Schematics x axis, angle of orientation; Schematics y axis, percentage of cells at angle x. kolmogorov smirnov test, ***p value=0.0004. Analyzed cells: control, n=167; *Msx2-Cre; Fgf4$^{c/-}$; Fgf8$^{c/-}$*, n=177.

Figure S3



Limb culture with PBS or Fgf8 beads. (A) Appearance of mouse forelimbs before and after *ex vivo* culturing. PBS or Fgf8 beads (red arrows) were implanted between mesenchymal condensations at E11.5. (B) Normal gene expression shown by whole mount *in situ* hybridization of *Sox9*, *Col2a1*, *Msx1* of limb buds cultured for 1 or 2 days. Fgf8 beads inhibited chondrogenesis, caused digit bending and induced *Msx1* expression after 2-day culturing. (C) Immunofluorescent staining of cultured limbs showing similar pattern of apoptotic (green, cleaved caspase3) and proliferating cells (red, phospho Histone H3) with PBS or Fgf8 beads. Fgf8 beads did not cause altered proliferation or cell death.

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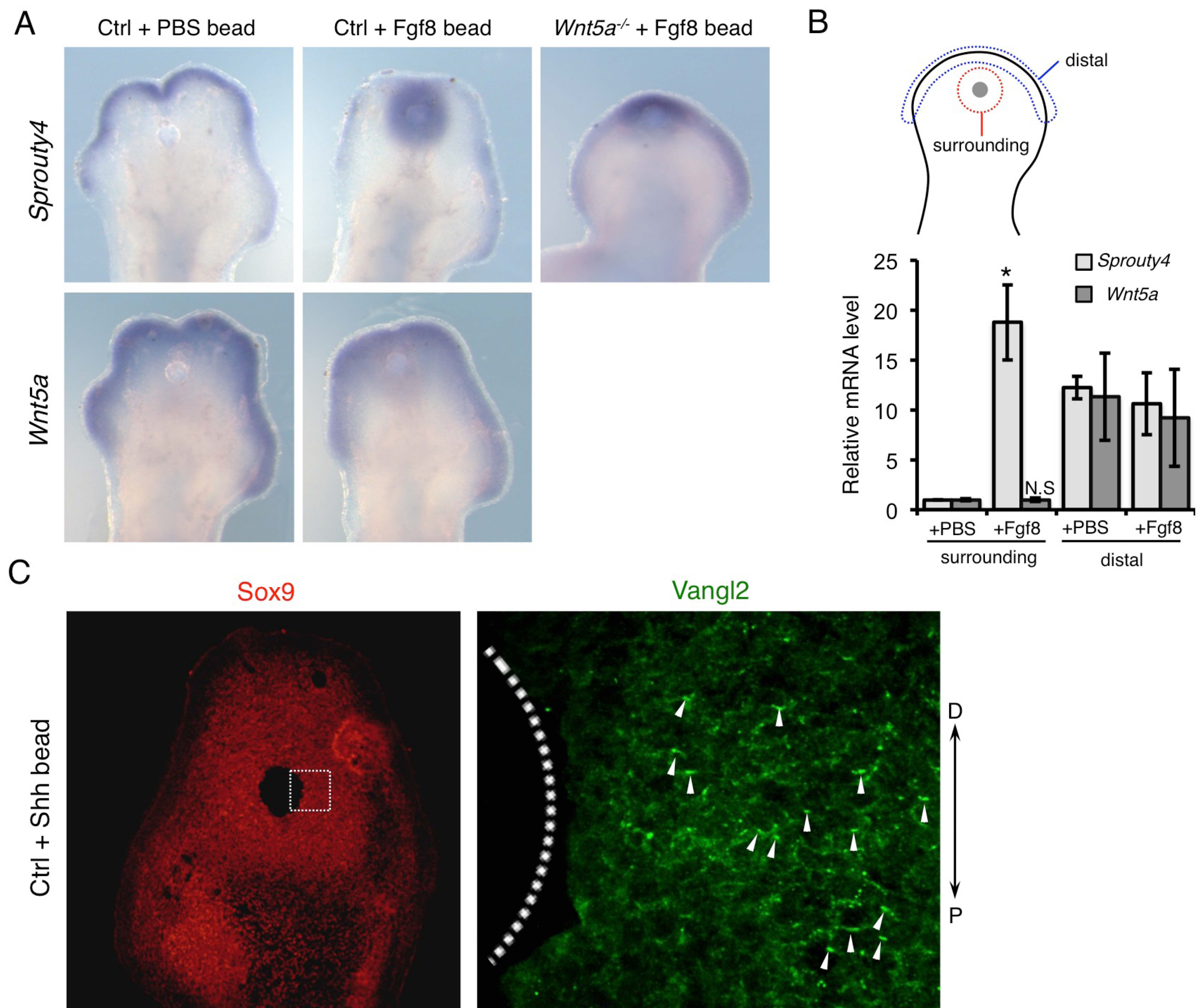
Figure S4



Analysis of cultured limbs (A) *Sprouty4* and *Wnt5a* whole mount *in situ* hybridization of cultured forelimbs implanted with PBS or Fgf8-soaked beads. (B) mRNA levels of *Sprouty4* and *Wnt5a* in tissues distal to (within the blue line) or surrounding (within the red circle) PBS or Fgf8 beads (grey dot) were analyzed by quantitative PCR (N=3). mRNA levels were normalized to *GAPDH* expression. Error bars are ± SD. Two-tailed *t* test, *p value =0.0146. N.S, no significance. (C) Sox9 (red) fluorescent immunostaining on cultured wild type limbs which were implanted with Shh-soaked beads and cultured for two days. The boxed region lateral to the bead was scanned by confocal microscope and representative images are shown in the right panel (Vangl2, green). The bead is outlined. Arrowheads point to some of asymmetrically localized Vangl2.

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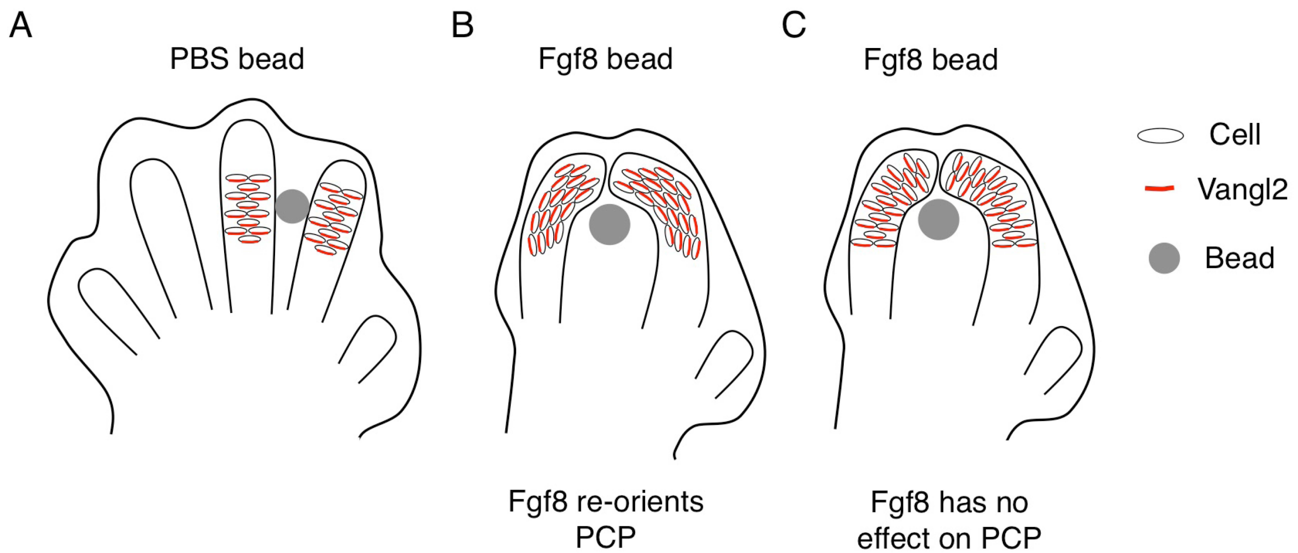
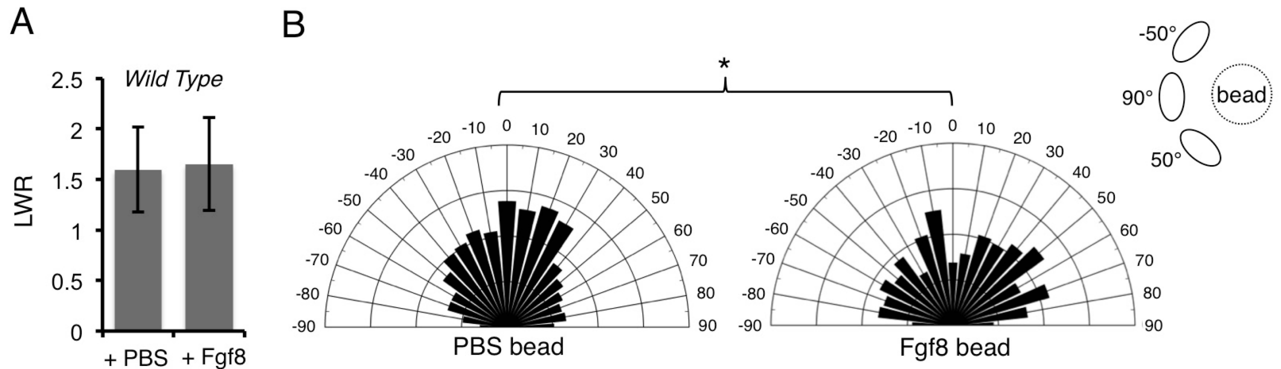
Figure S5



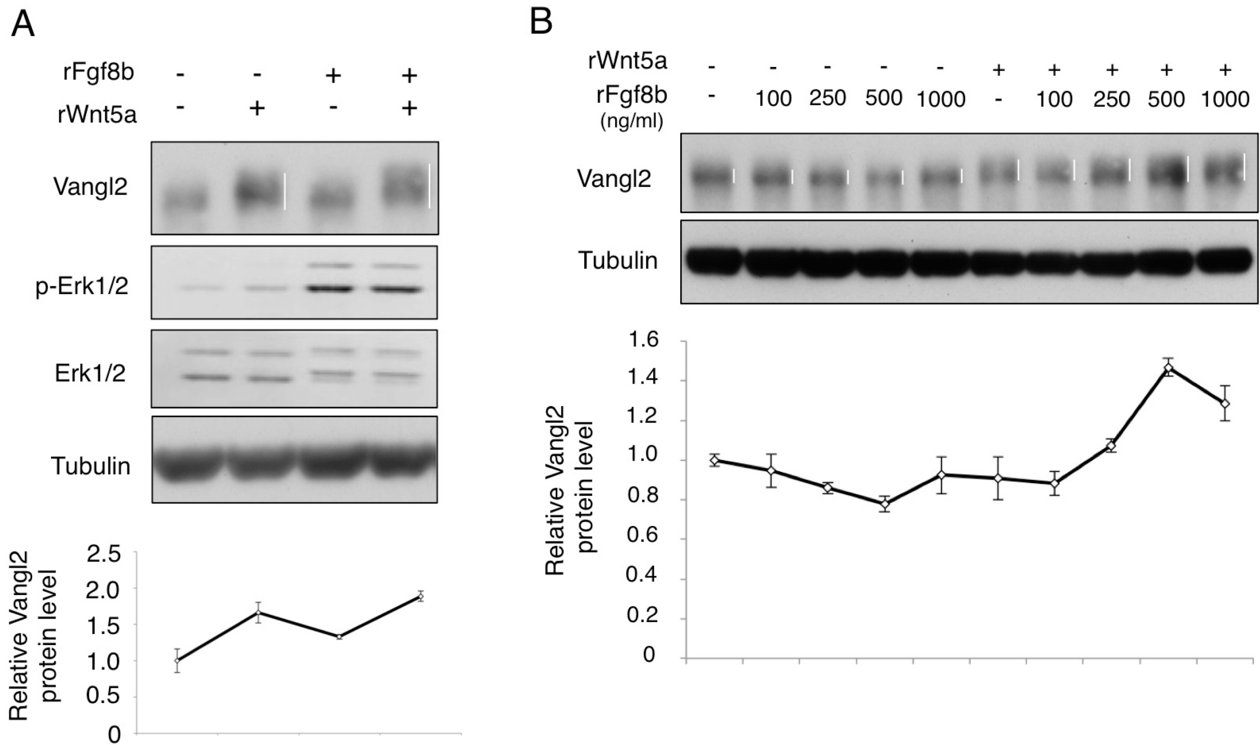
Schematics of beads experiment. (A) PBS bead does not change digit morphogenesis or PCP. Asymmetric localization of Vangl2 is shown along the axis of the digit outgrowth (proximal-distal axis). (B) and (C) Fgf8 bead implantation causes digit bending. Because our observed reorientation of asymmetric Vangl2 (B) is different from the expected Vangl2 localization pattern if Fgf8 has no effect on PCP (C), the digit bending is likely due to the combined effects of Fgf8 on both chondrogenesis and PCP.

Figure S6



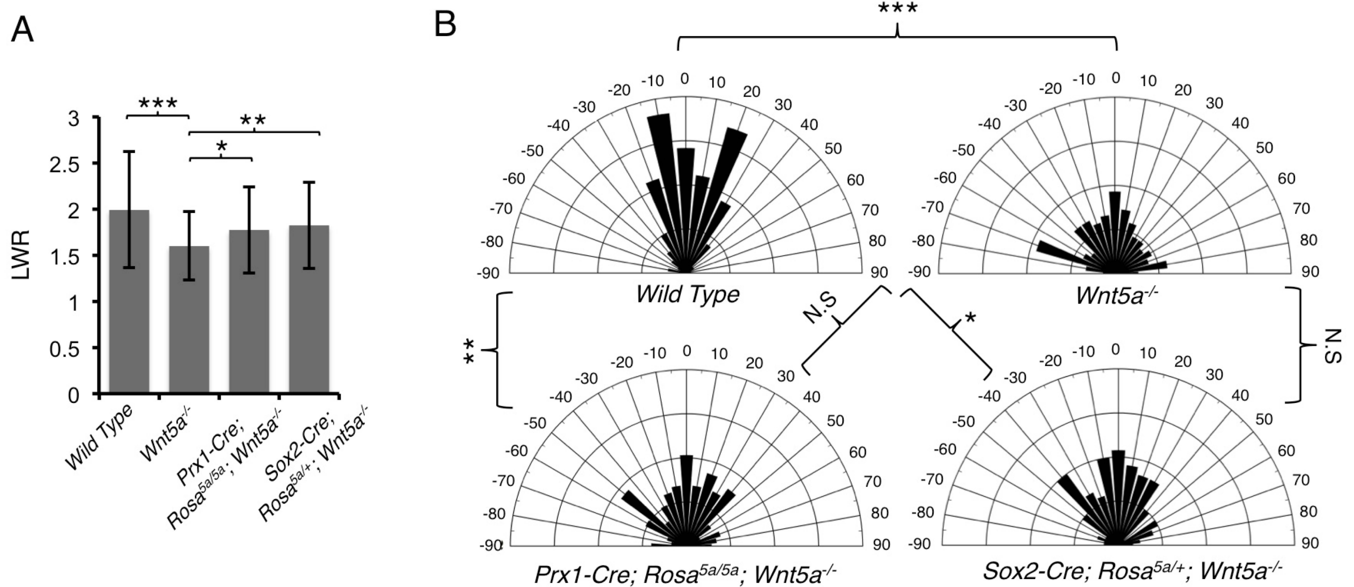
Analysis of Fgf8-regulated cell shape and orientation. (A) Statistical analysis of length-to-width ratio (LWR) of limb chondrocytes implanted with PBS or Fgf8 beads shown in Figure 2A . No significance between PBS and Fgf8 soaked beads (two-tailed *t* test). Error bars are ±SD. Analyzed cells: PBS beads, n=316; Fgf8 beads, n=274. (B) Schematic diagrams summarizing the quantification of cells orientation with implanted PBS or Fgf8 beads. x axis, angle of orientation; y axis, percentage of cells at angle x. kolmogorov smirnov test, *p value=0.0183. The inset show examples of cells surrounding the beads with different angles. Analyzed cells: PBS beads, n=615; Fgf8 beads, n=485.

Figure S7



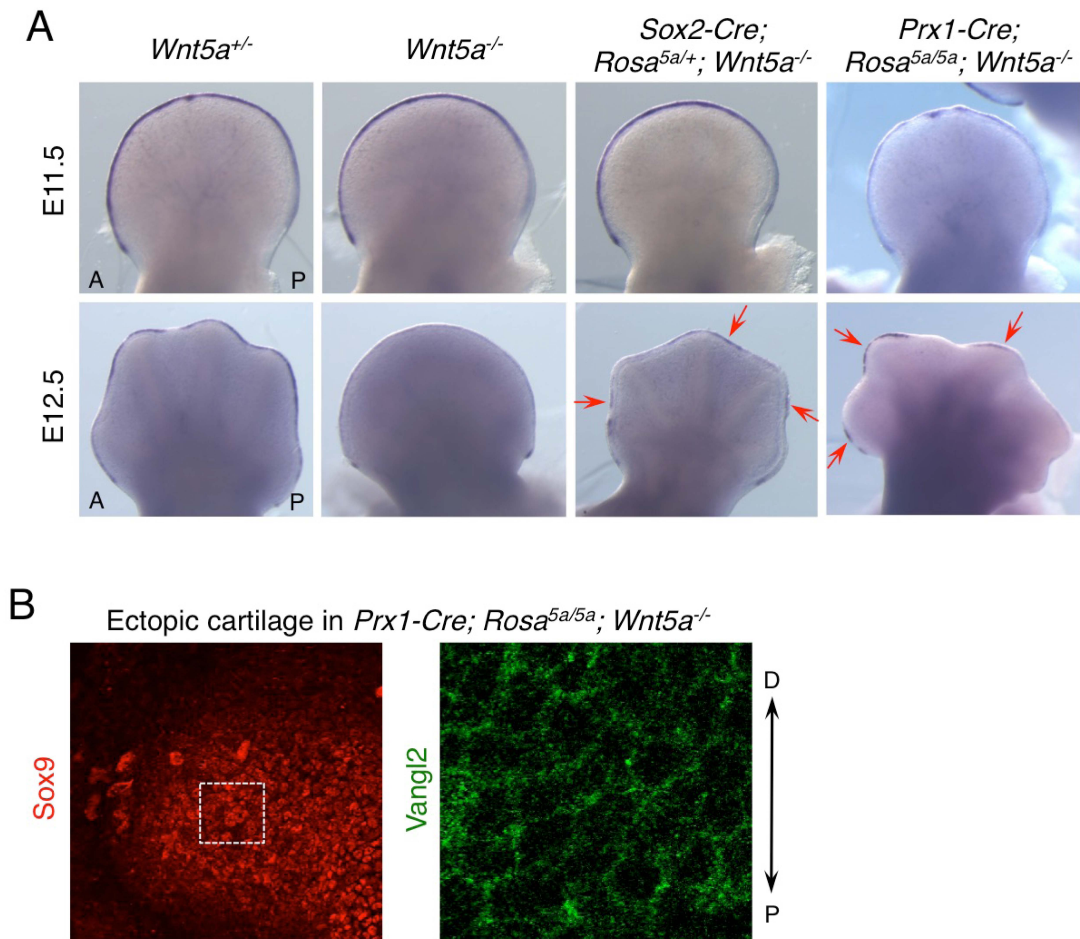
Fgf regulation of Vangl2. Vangl2 phosphorylation and protein level were analyzed in the cultured mesenchymal cells isolated from the E10.5~E11.5 mouse limb buds. Fgf8b recombinant proteins (250ng/ml) appeared to enhance Vangl2 phosphorylation induced by Wnt5a recombinant proteins (500ng/ml) (A) and in a dose-dependent manner (100ng/ml recombinant Wnt5a) (B). The white bars demarcate phosphorylation shift. The relative total Vangl2 protein level was quantified and shown in the lower panel. Fgf8b also enhanced the Vangl2 protein level in the presence of Wnt5a.

Figure S8



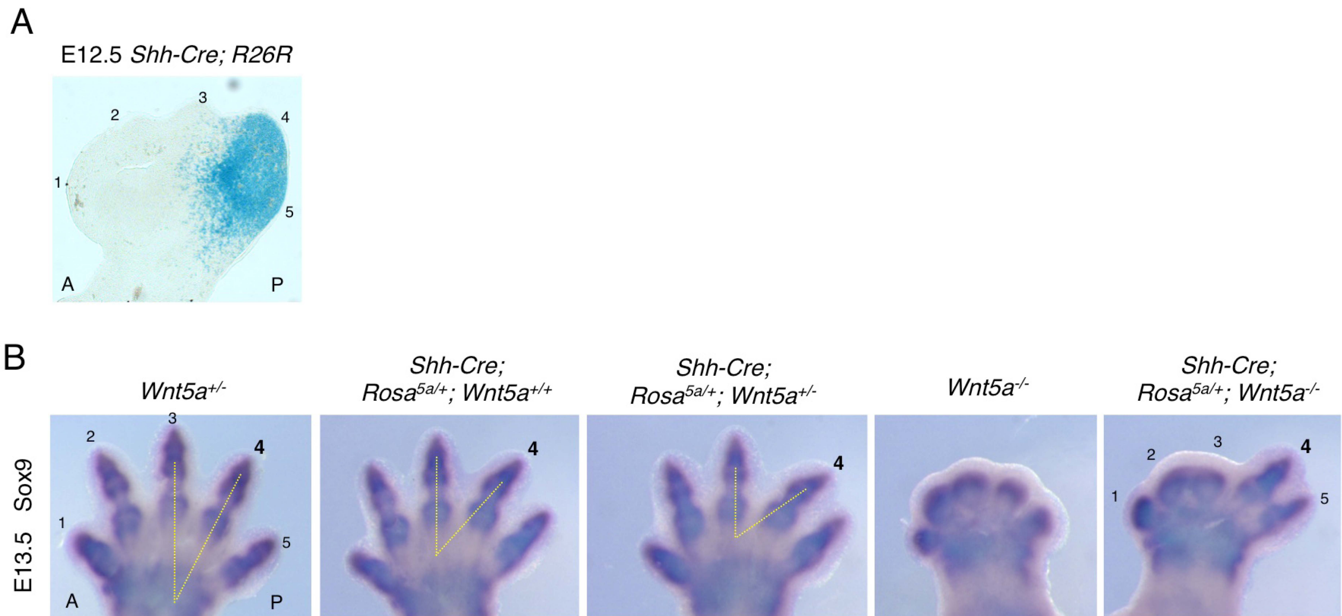
Analysis of cell shape and orientation with non-graded Wnt5a. (A) Statistical analysis of length-to-width ratio (LWR) of distal limb chondrocytes in E13.5 embryos in indicated genotypes. Significances between different genotypes are indicated (two-tailed *t* test, ***p<0.0001; **p=0.00066; *p=0.00576). Error bars are ±SD. Analyzed cells: wild type, n=165; *Wnt5a*$^{-/-}$, n=122; *Prx1-Cre; Rosa*$^{5a/5a}$*; Wnt5a*$^{-/-}$, n=84; *Sox2-Cre; Rosa*$^{5a/+}$*; Wnt5a*$^{-/-}$, n=75. (B) Schematic diagrams summarizing the quantification of cells orientation in each genotype. x axis, angle of orientation; y axis, percentage of cells at angle x. kolmogorov smirnov test, ***p value=0.0025; **p value=0.0144, *p value=0.0422. N.S, no significance (p value>0.05). Analyzed cells: wild type, n=99; *Wnt5a*$^{-/-}$, n=151; *Prx1-Cre; Rosa*$^{5a/5a}$*; Wnt5a*$^{-/-}$, n=175; *Sox2-Cre; Rosa*$^{5a/+}$*; Wnt5a*$^{-/-}$, n=121.

Figure S9



Analysis of mouse models with non-graded *Wnt5a* expression. (A) *Fgf8* expression in *Sox2-Cre;Rosa*$^{5a/+}$*;Wnt5a*$^{-/-}$ and *Prx1-Cre;Rosa*$^{5a/5a}$*;Wnt5a*$^{-/-}$ forelimbs (red arrows). A: anterior; P: posterior. (B) Representative images of fluorescent Immunostaining of Vangl2 (green) and Sox9 (red) in the Wnt5a-induced ectopic cartilage of *Prx1-Cre; Rosa*$^{5a/5a}$*; Wnt5a*$^{-/-}$ limbs. No Vangl2 asymmetric localization was observed. P: proximal; D: distal..

Figure S10



Reoriented *Wnt5a* expression gradient altered digit morphogenesis. (A) The cells derived from *Shh*-expressing cells are shown by X-gal staining of the E12.5 forelimb of the *Shh-Cre; R26R* embryo. Note that digit 3 is partially contributed by X-gal positive cells. (B) *Sox9* whole mount *in situ* hybridization in the mouse E13.5 forelimbs. The angles between longitudinal axes of digit 3 and bending distal part of the digit 4 (yellow dotted lines) were used to indicate the growth direction of digit 4. The increased angles suggest posteriorly biased growth deviation of digit 4 in *Shh Cre; Rosa*$^{5a/+}$*; Wnt5a*$^{+/+}$ and *Shh-Cre; Rosa*$^{5a/+}$*; Wnt5a*$^{+/-}$ compared to that in *Wnt5a*$^{+/-}$ control. Each digit is numbered. A: anterior; P: posterior.

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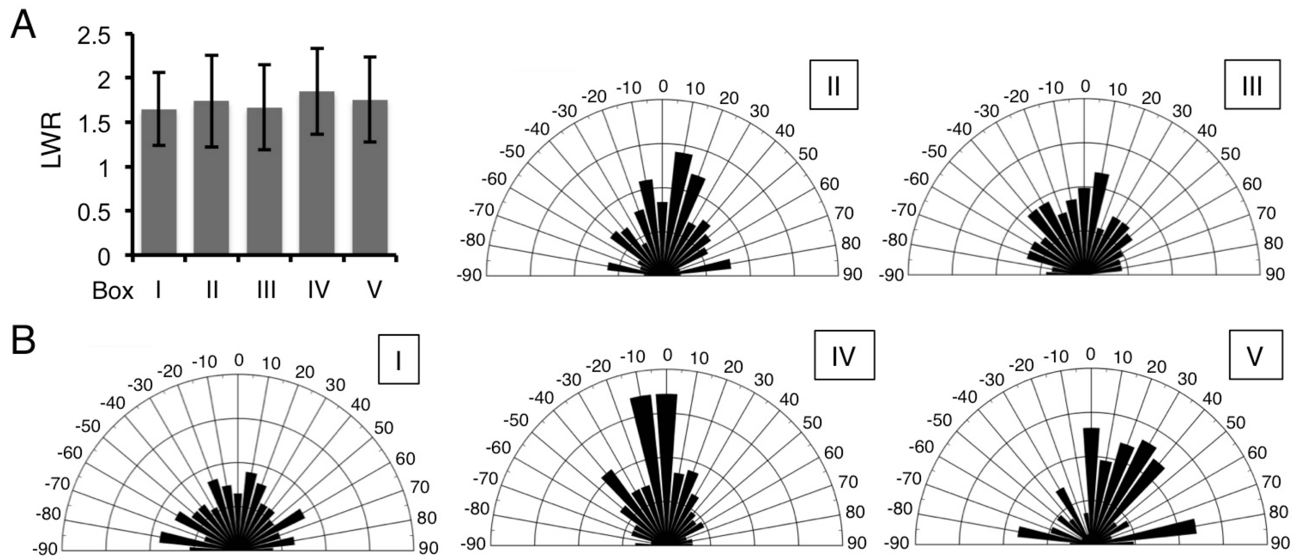
Figure S11



Analysis of cell shape and orientation with reoriented Wnt5a. (A) Length-to-width ratio (LWR) of chondrocytes of E12.5 *Shh-Cre;Rosa$^{5a/+}$;Wnt5a$^{-/-}$* embryos shown in Figure 6A (boxed areas I-V). Error bars are ±SD. Analyzed cells: n=120 for area I; n=191 for area II; n=101 for area III; n=126 for area IV; n=50 for area V. (B) Schematic diagrams summarizing the quantification of cells orientation in each boxed area. x axis, angle of orientation; y axis, percentage of cells at angle x. Analyzed cells: n=251 for area I; n=239 for area II; n=203 for area III; n=211 for area IV; n=104 for area V.

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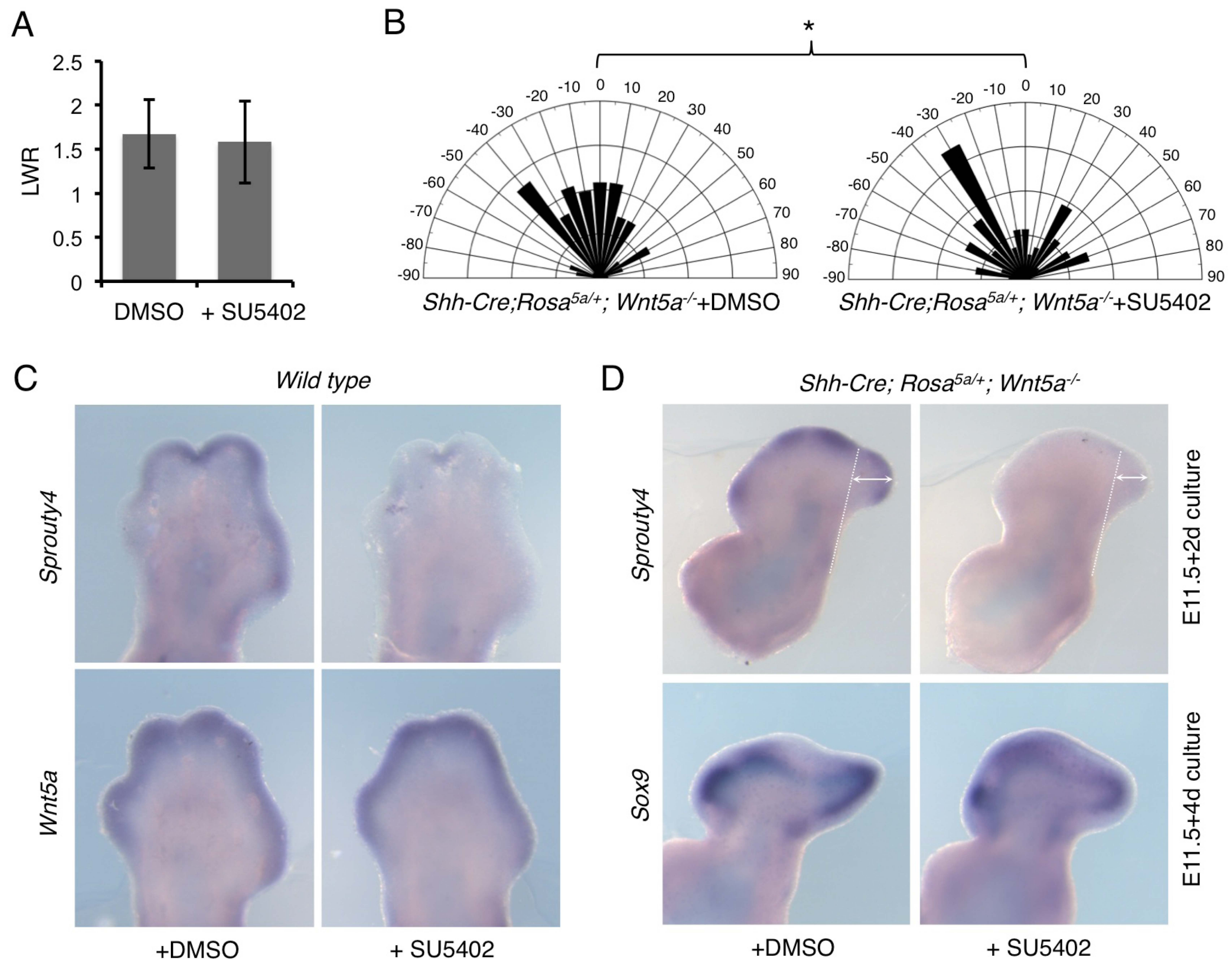
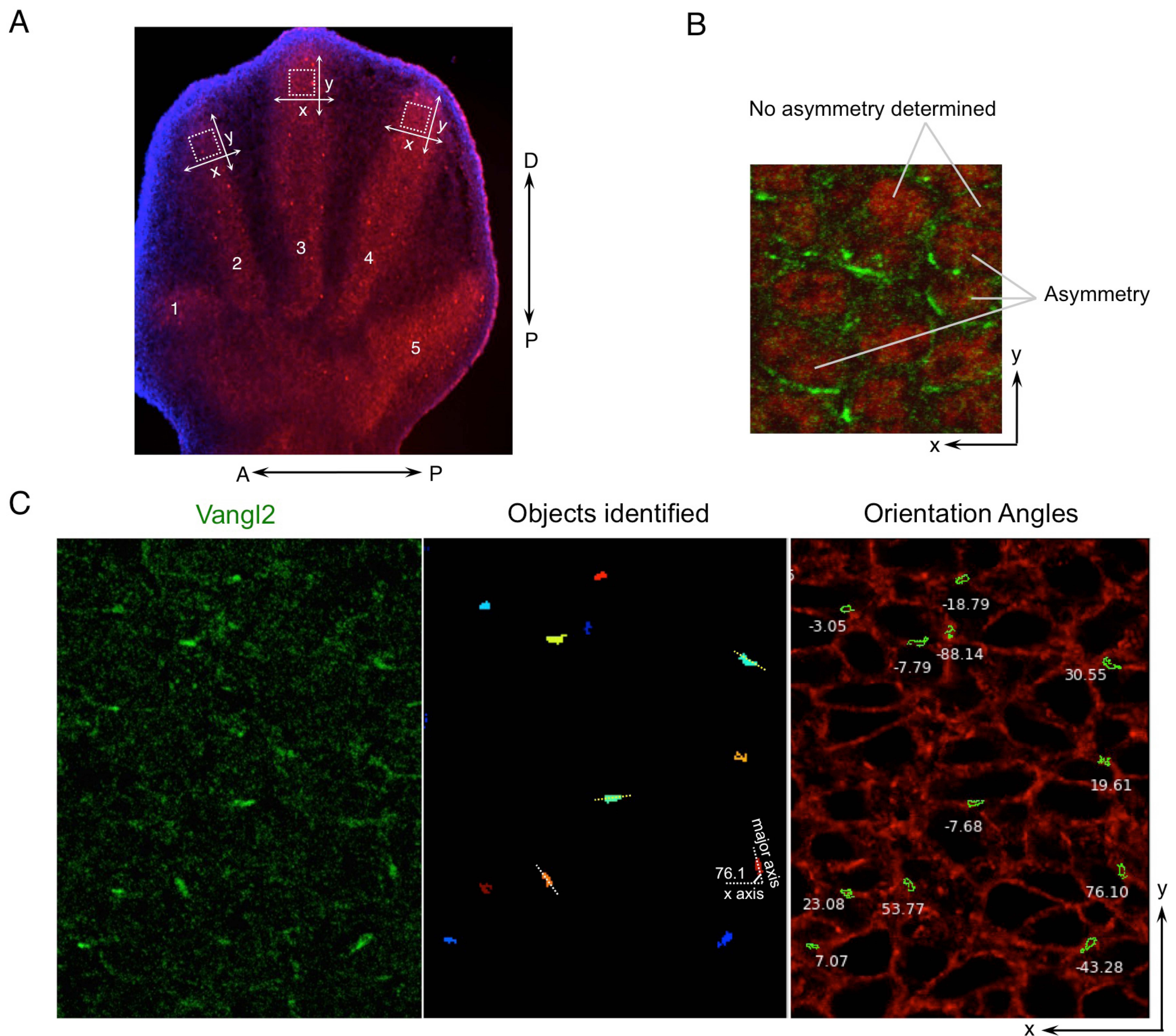
Figure S12

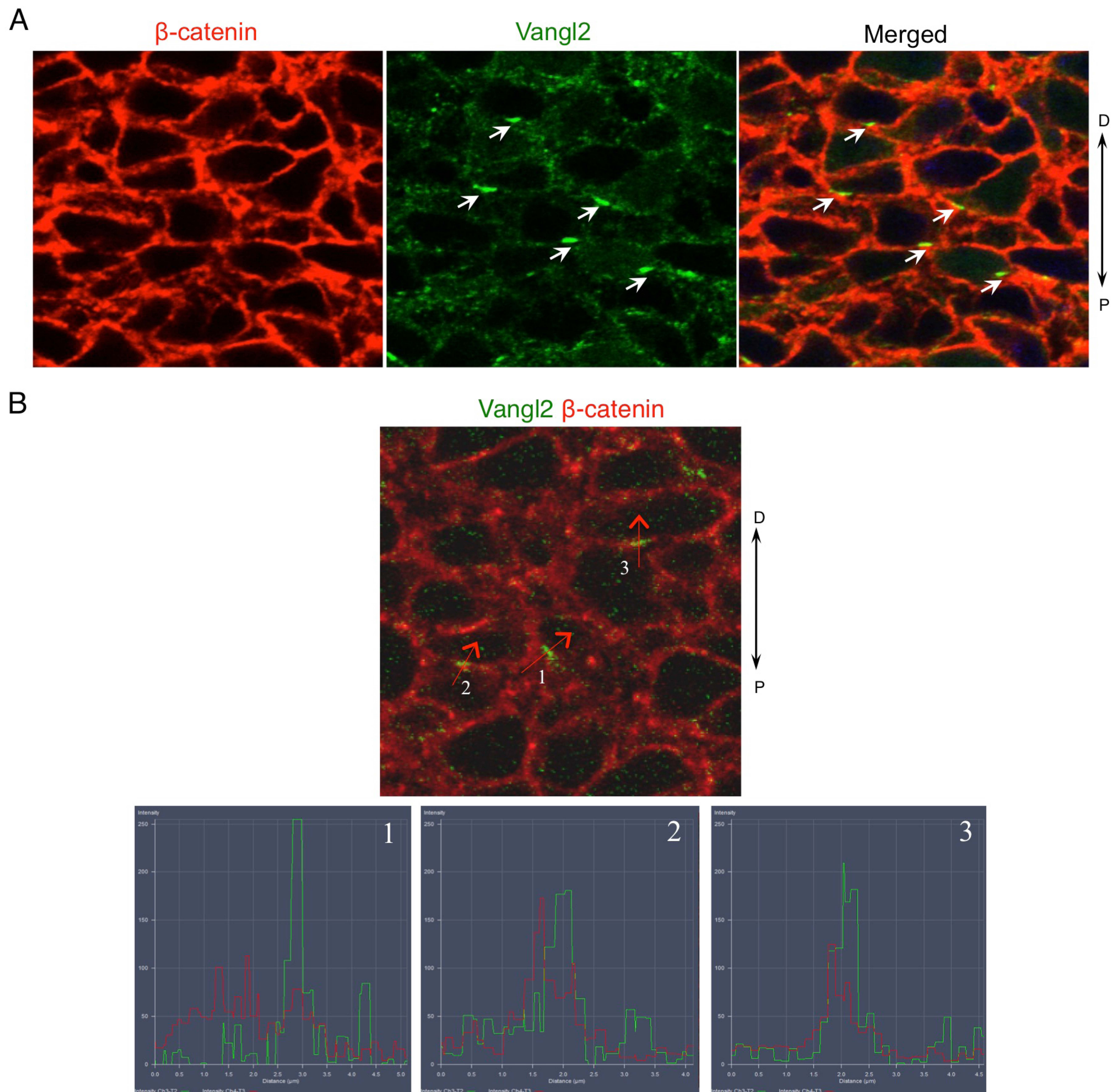


Inhibition of Fgf signaling in cultured limbs. (A) Statistical analysis of length-to-width ratio (LWR) of distal cells in the *Shh-Cre;Rosa$^{5a/+}$;Wnt5a$^{-/-}$* cultured forelimbs with DMSO or SU5402 treatment. Error bars are ±SD. Analyzed cells: DMSO, n=134; SU5402, n=120. (B) Schematic diagrams summarizing the quantification of distal cells orientation in each group. x axis, angle of orientation; y axis, percentage of cells at angle x. kolmogorov smirnov test, *p value=0.0354. Analyzed cells: DMSO, n=111; SU5402, n=106. (C, D) *Sprouty 4, Wnt5a or Sox9* whole mount *in situ* hybridization in cultured limbs. SU5402 inhibited Fgf signaling, but not *Wnt5a* expression. (C) Wild type limb; (D) *Shh-Cre;Rosa$^{5a/+}$;Wnt5a$^{-/-}$* limb. Double-headed arrows indicate the length of digit outgrowth, which was compromised in the *Shh-Cre;Rosa$^{5a/+}$;Wnt5a$^{-/-}$* cultured forelimbs with SU5402 treatment. Dotted lines indicate the baseline of measurement. The difference is more obvious shown by *Sox9* whole mount *in situ* hybridization when the limbs were cultured for 4 days.

Figure S13

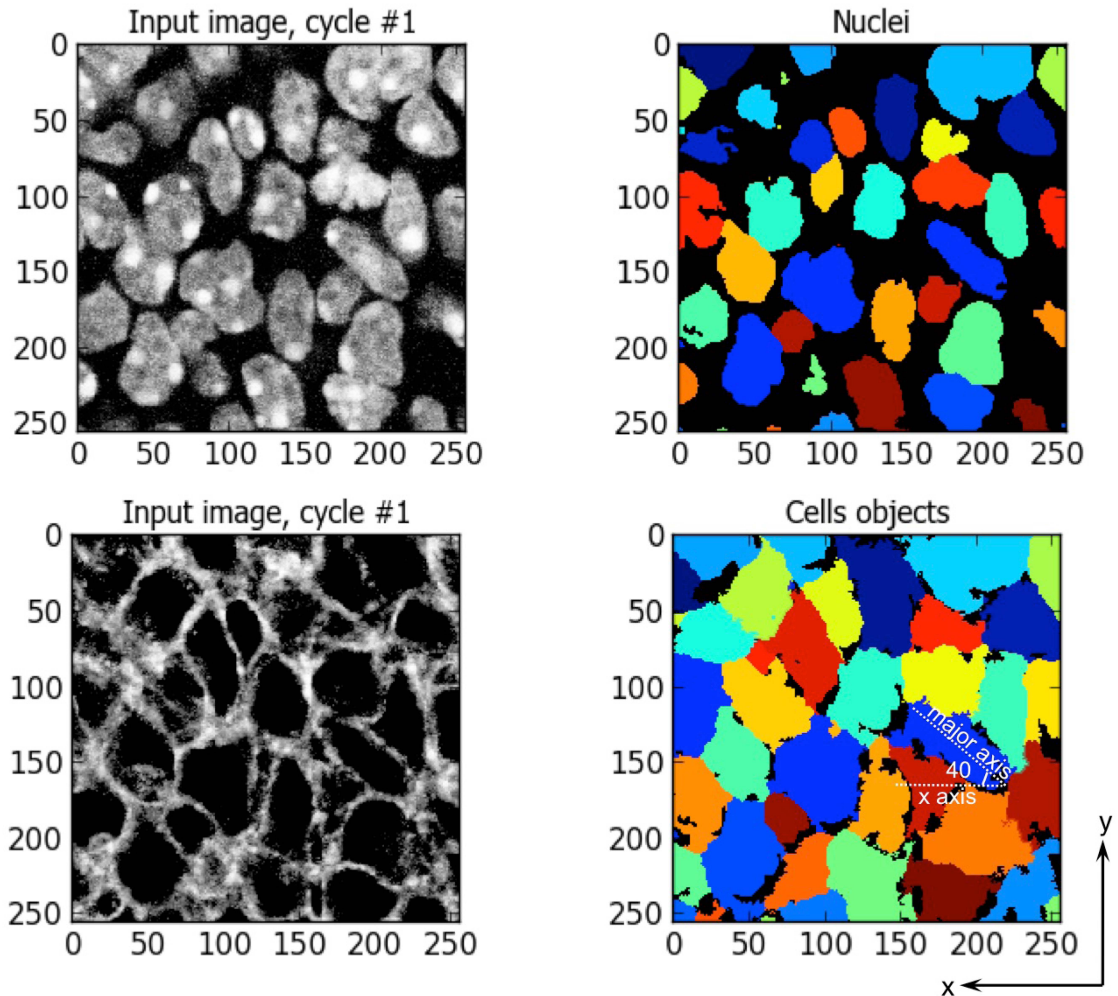


Quantification of Vangl2 orientation. (A) Schematics of vector placement to measure the Vangl2 and cell orientation. The longitudinal axes of the target digits were defined as the y axis. A-P and P-D are anterior-posterior and proximal-distal axis of the whole limb, respectively. The boxed areas were scanned. Digits 1-5 are labeled. (B) Examples of cells with "Asymmetry" or "No asymmetry determined". "Asymmetry" means a cell has Vangl2 polarized to any direction. "No asymmetry determined" means a cell has no polarized Vangl2. (C) A confocal scanning picture of Vangl2 staining was analyzed by CellProfiler to identify the objects. The orientation was determined by the angle between the x axis and the major axis of the identified object (indicated by dotted lines in the middle picture). The angle was automatically measured by CellProfiler. The angle of each identified object was labeled, and the red signal was co-stained β-catenin to indicate the cell membrane.



Localization of Vangl2. (A) In the distal limb, the localization of Vangl2 proteins on the membrane (arrows, green) of the cell of interest or its neighbor can be distinguished by its co-localization with β–catenin (red), which marked the cell membrane. (B) Line scan in the indicated proximal to distal direction (red arrows, #1-3) using ZEN 2012. The Vangl2 signal (green) is located to the distal side of the cell boundary shown by β–catenin signals (red), indicating that Vangl2 belongs to the distal cells and located to the proximal side of that distal cell. D-P, distal-proximal axis.

Figure S15



Measurement of cell shape and orientation. Illustration of cell morphology recognized by CellProfiler software. The nucleus of the cell (DAPI or Sox9 staining) was first recognized and imposed to the cell membrane (β-catenin staining) for generation of cell object, which will be subjected to cell shape and orientation analysis by CellProfiler. The orientation is determined by the angle between the x axis and the major axis (indicated by dotted line) of the identified object.