Supporting Information

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SI Text

Mice. *RBP-J*$^{f/f}$ mice were obtained with permission from the Riken BioResource Center, Kyoto. *Col2α1Cre* transgenic mice were interbred with *Rosa*^{Notch} and *RBP-J*$^{f/f}$ mice to obtain *Col2α1Cre;Rosa*^{Notch} and *Col2α1Cre;RBP-J*$^{f/f}$ mice, respectively. The control mice used in these studies were wild-type, *Rosa*^{Notch}, or *RBP-J*$^{f/+}$ mice without the *Col2α1Cre* transgene. Animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were used according to institutional Animal Care and Use Committee–approved protocols.

Immunohistochemistry. Antisera used included anti-Notch1 Val-1744 antibody (1:100 dilution; Cell Signaling), anti-GFP antibody (1:200 dilution; Invitrogen), MF20 antibody (1:200 dilution; Developmental Studies Hybridoma Bank), anti-Crtl1 antibody (9/30/8-A-4; Developmental Studies Hybridoma Bank), Jag1 (1:1,000 dilution; Santa Cruz Biotechnology), anti-Hes1 antibody (1:1000 dilution; generous gift of Nadean Brown), and anti–cleaved caspase-3 antibody (1:100 dilution; Cell Signaling). Sections were incubated at 4 °C overnight with primary antibodies, and then incubated with the appropriate secondary antibody at room temperature, processed further with an ultrasensitive ABC IgG Staining Kit (Pierce), and visualized with diaminobenzidine (DAB) staining or immunofluorescence. Immunofluorescence was detected using a Zeiss LSM 510 confocal microscope, and images were obtained using Zeiss LSM version 3.2 SP2 software. All images were captured in parallel using identical confocal laser settings with constant PMT filters and integration levels. PCNA labeling was performed using the Zymed PCNA Staining Kit (Invitrogen) in accordance with the manufacturer's instructions. The proliferation indices of experimental and control humerus bone sections were determined by the total number of PCNA-positive nuclei divided by the total number of nuclei within the growth plate. At least 8 comparative sections were analyzed per embryo for at least 3 embryos of each genotype. TUNEL labeling was performed using the fluorescein in situ Cell Death Detection Kit (Roche) in accordance with the manufacturer's instructions. For H&E staining, slides were incubated with hematoxylin, followed by eosin. For alcian blue–stained sections, slides were stained with 1% alcian blue 8GX (Sigma Aldrich) in 3% acetic acid and counterstained with nuclear fast red (Vector Laboratories).

In Situ Hybridization. Digoxygenin-labeled probes for *Sox9*, *Aggrecan*, *Ihh*, *Col10α1*, *Runx2*, *BSP*, *MMP9*, *Uncx4.1*, *Mesp2*, *Hes7*, *Lfng*, *Dll4*, *Mmp13*, and *Hey1* were amplified from E14.5 or E18.5 mouse limb cDNA, subcloned into pGEM T-vectors (Promega), confirmed by DNA sequencing, and generated from linearized plasmids using SP6 polymerase (Promega) or T7 polymerase (Roche). The in situ probes and PCR primers used are listed in Table S1.

RNA Isolation and Real-Time RT-PCR. Somite sections from *Col2α1Cre;Rosa*^{Notch} and control embryos were collected in TRIzol (Invitrogen). Total RNA was isolated and cDNA was generated using SuperScript II (Invitrogen) in accordance with the manufacturer's instructions. Power SyberGreen Master Mix (Applied Biosystems) was used for semiquantitative real-time RT-PCR (Opticon 2; MJ Research). At least 3 samples from each genotype were run in triplicate. *Sox9* primers 5′-AGGAAGCTGGCAGACCAGTA-3′ and 5′-CGTTCTTCAC-CGACTTCCTC-3′ were used. Gene expression levels were normalized to corresponding *L7* expression, and standard curves were generated from E10.5 somite cDNA.

Statistical Analysis. Statistical significance was determined by Student's *t* test. Data are reported as mean ± SEM.

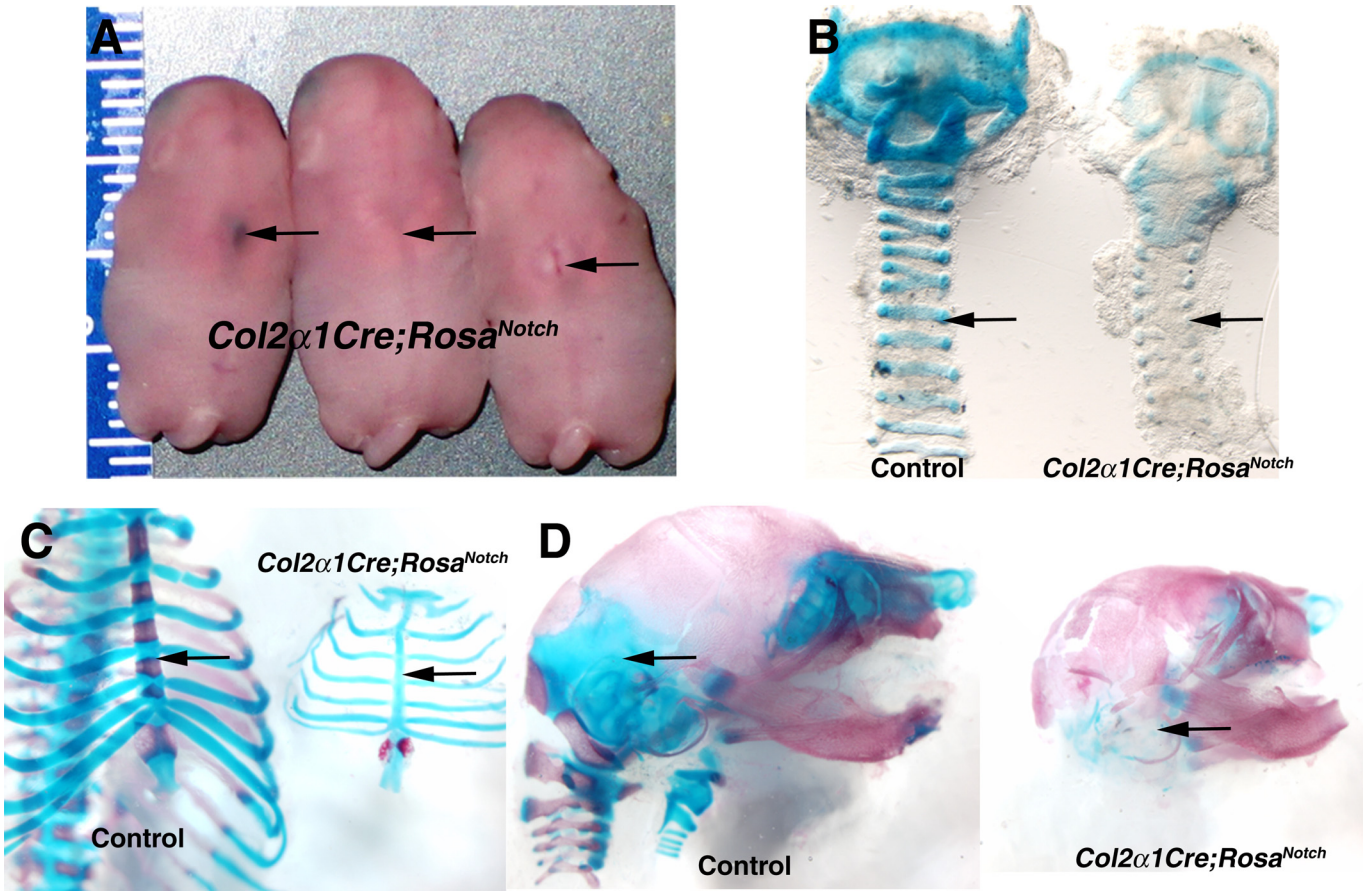

Fig. S1. Cartilage malformations associated with gain of Notch1 function in the chondrocyte lineage. (*A*) *Col2α1Cre;Rosa*^{Notch} mice exhibit vertebral defects. (*B–D*) *Col2α1Cre;Rosa*^{Notch} mice exhibit overall decreased chondrogenesis, as indicated by alcian blue staining in the trachea (*B*), rib cage (*C*), and skull (*D*) (arrows).

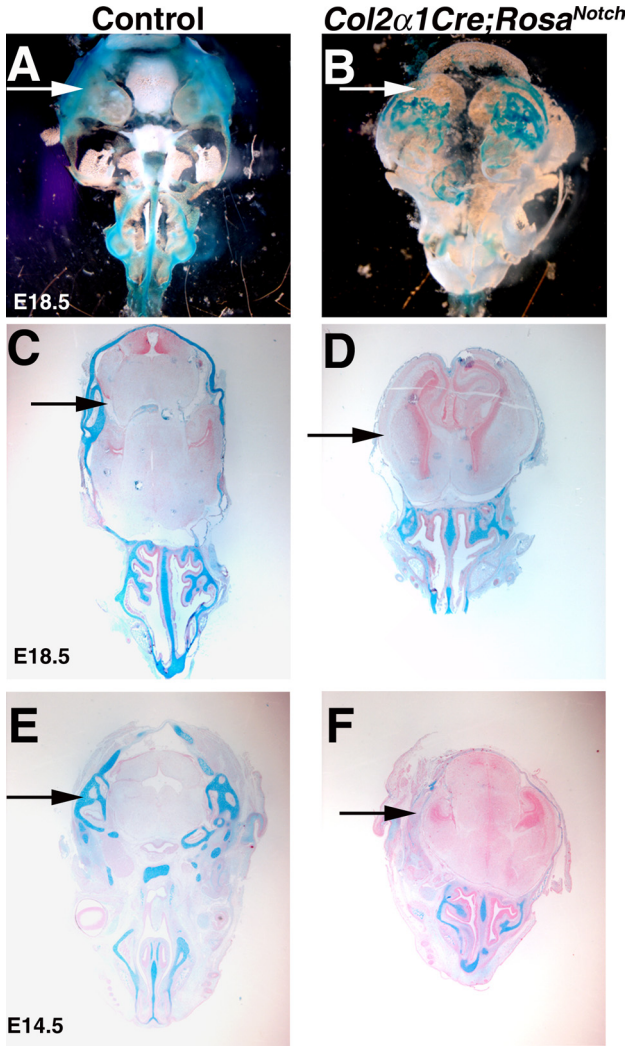

Fig. S2. Skull defects associated with decreased cartilage in *Col2α1Cre;Rosa*^{Notch} mice. (*A* and *B*) Skull preparations of *Col2α1Cre;Rosa*^{Notch} mice exhibit decreased alcian blue–positive staining, indicating a loss of cartilage (arrows). (*C–F*) Sections of E18.5 and E14.5 skulls show a decrease in alcian blue–positive staining of the cranium (arrows). Membranous bones of the skull appear to be unaffected in *Col2α1Cre;Rosa*^{Notch} mice. Red represents nuclear fast red.

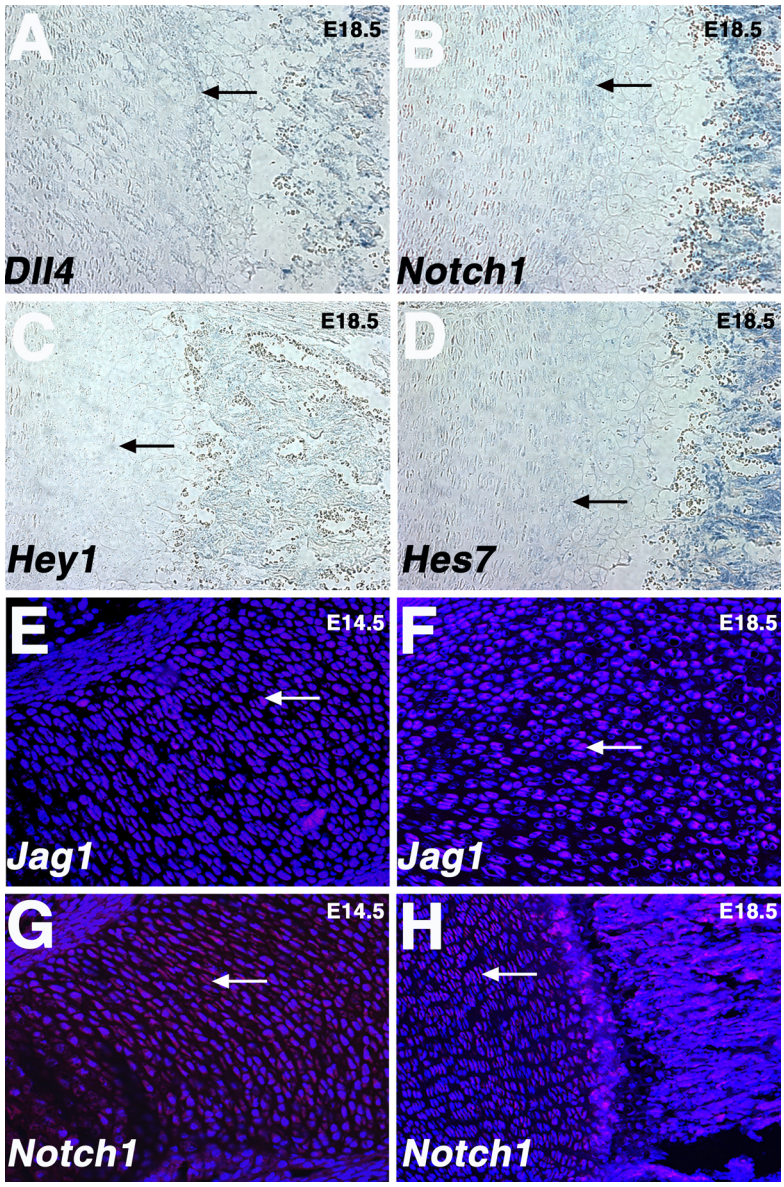

Fig. S3. Notch pathway expression in the chondrogenic lineage. (*A–D*) In situ hybridization of Notch pathway components *Dll4*, *Notch1*, *Hey1*, and *Hes7* illustrate expression in prehypertrophic chondrocytes in E18.5 limb sections, as indicated by arrows. (*E–F*) Antibody staining of Notch ligand *Jag1* shows expression in proliferative chondrocytes, while antibody staining for Notch receptor *Notch1* intracellular domain demonstrates expression in prehypertrophic chondrocytes in E14.5 and E18.5 limb sections (*G* and *H*). Arrows indicate expression.

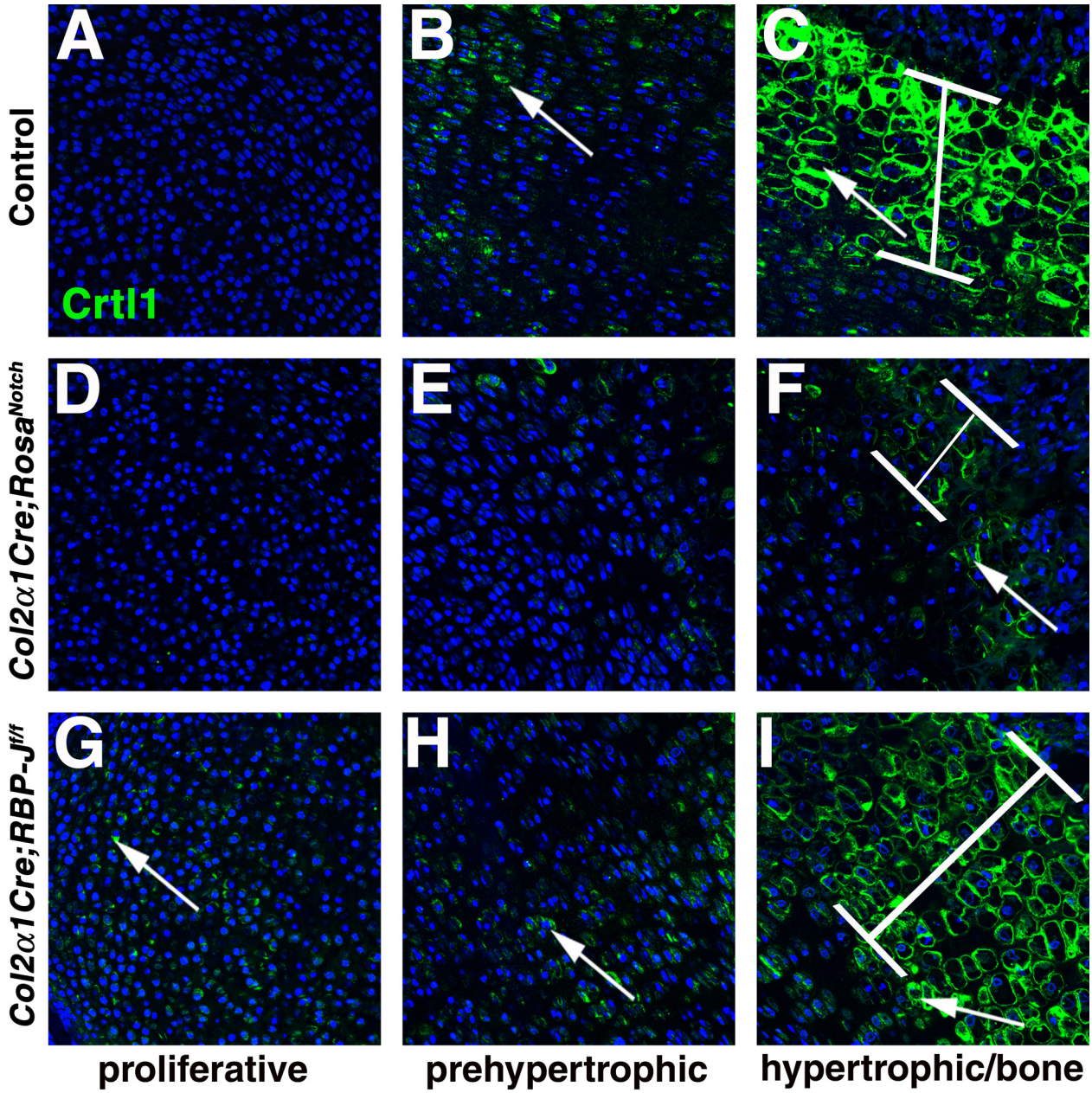

Fig. S4. Notch signaling regulates *Sox9* target gene *Crtl1* in E18.5 limb sections. (*A–C*) Control E18.5 limbs show prehypertrophic and hypertrophic chondrocytes expressing Crtl1. (*D–F*) *Col2α1Cre;Rosa*^{Notch} limb sections show reduced Crtl1 expression in prehypertrophic and hypertrophic chondrocytes. (*G–I*) *Col2α1Cre;RBP-J*$^{f/f}$ limb sections exhibit early induced Crtl1 expression in proliferative chondrocytes, as well as increased expression in prehypertrophic and hypertrophic chondrocytes. Arrows indicate expression of Crtl1. The width of the hypertrophic chondrocyte zone is indicated by brackets and is decreased in *Col2α1Cre;Rosa*^{Notch} mice (*F*) and increased in *Col2α1Cre;RBP-J*$^{f/f}$ mice (*I*) compared with controls (*C*).

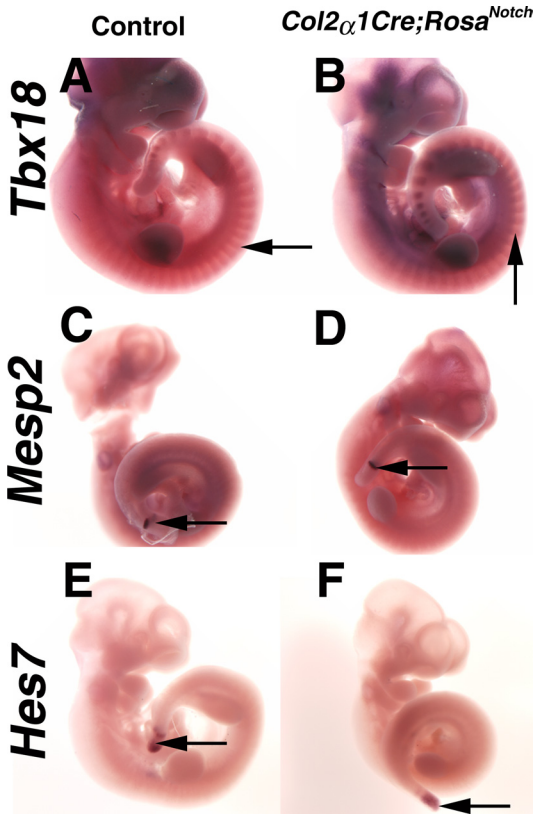

Fig. S5. Whole-mount in situ hybridization studies demonstrate that somite segmentation is unaffected in *Col2α1Cre;Rosa*^{Notch} mice. The normal *Tbx18* expression in E10.5 *Col2α1Cre;Rosa*^{Notch} embryos (*A* and *B*) suggests proper somite segmentation, whereas appropriate *Mesp2* expression (*C* and *D*) and *Hes7* expression (*E* and *F*) suggest normal Notch pathway expression during the somite segmentation clock (arrows).

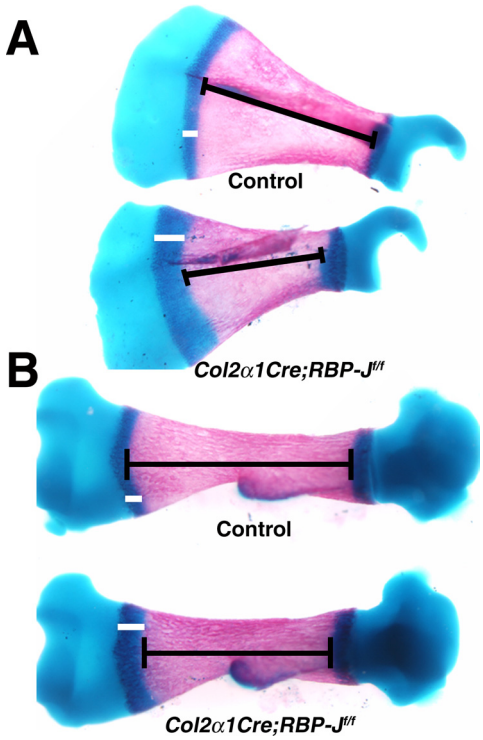

Fig. S6. *Col2α1Cre;RBP-J$^{f/f}$* mice exhibit an increased hypertrophic chondrocyte zone with an accompanying decrease in osteoblasts. *Col2α1Cre;RBP-J$^{f/f}$* scapulae (*A*) and humerus (*B*) have an increased hypertrophic chondrocyte zone (white bars) and an accompanying decrease in the length of the mineralized region (black bars) while maintaining similar overall bone length compared with controls.

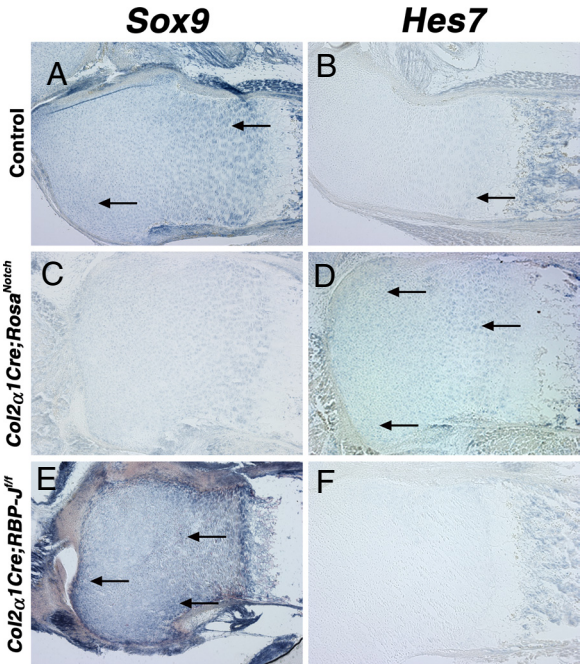

Fig. S7. Notch pathway regulation of *Hes7* and *Sox9* expression in chondrogenesis. *Sox9* expression is decreased in *Col2α1Cre;Rosa*^{Notch} limbs but increased in *Col2α1Cre;RBP-J*$^{f/f}$ E18.5 limb sections (*A*, *C*, and *E*). *Hes7* is expressed in an earlier proliferative chondrocyte lineage in E18.5 *Col2α1Cre;Rosa*^{Notch} limb sections (*D*) compared with controls, in which it is expressed only in the prehypertrophic lineage (*B*) (arrows). *Hes7* is not detected in E18.5 *Col2α1Cre;RBP-J*$^{f/f}$ limb sections in the chondrogenic lineage (*F*). Arrows indicate expression.

Table S1. Primers for generation of antisense RNA probes

Gene	Accession number	Product size (bp)	Primer sequences
Sox9	NM_011448	778	5′-CGGCGGAGGAAGTCGGTGAAG-3′ 5′-AGCGGGTGATGGGCGGGTAG-3′
Aggrecan	NM_007424	702	5′-GGCAACCTCCTGGGTGTAAG-3′ 5′-TGGGGTTCGTGGGCTCACAA-3′
Ihh	NM_010544	943	5′-CCACTTCCGGGCCACATTTG-3′ 5′-CACATTGCCCGGATCAGAGACT-3′
Col10α1	NM_009925	883	5′-AGGGGGTGGTGTCTGTTATTCC-3′ 5′-TGTAGAGTCACCCCCGCTTCAC-3′
Runx2	NM_009820	727	5′-AGGACAGAGTCAGATTACAG-3′ 5′-TCAATATGGCCGCCAAACAG-3′
BSP	NM_009263	898	5′-CATGAGATTGGCAGTGATTTG-3′ 5′-TTTGCCTCTTCTTTAGTTGACC-3′
MMP9	NM_013599	960	5′-CCTGAAAACCTCCAACCTCA-3′ 5′-AGGAAGACGAAGGGGAAGAC-3′
Uncx4.1	NM_013702	667	5′-GGCCAAATGGAGAAAGAAGGAGAA-3′ 5′-CGTGATGGGGTAGAGCAAGAAGTG-3′
Mesp2	NM_008589	745	5′-CTCTCACCGATGCCCTCAAT-3′ 5′-GGCCATAGCTAAGCAGACCTCA-3′
Hes7	NM_033041	649	5′-CCCGGGAGCGAGCTGAGAATAG-3′ 5′-GCGGGCGGTGGGAGTGAC-3′
Lfng	NM_008494	759	5′-TCGCGCCACAAGGAGATGACG-3′ 5′-GGGCGCCCAGGGACAGAGTTT-3′
Dll4	NM_019454	762	5′-GGAGGCCCGATGACGAGAGC-3′ 5′-ACAGCAGCGCCAAGACCCACTA-3′
MMP13	NM_008607	885	5′-AGCGCTGCGGTTCACTTTG-3′ 5′-GGCCACCTCCACTGTCCTTGTA-3′
Hey1	NM_010423	900	5′-ACCTCGGCCTCCAAACTGTCTC-3′ 5′-TGCTGCCAACGCTCCAAATG-3′