**Supplemental Materials and Methods**

**Animals.**

*Axin2*$^{CreERT2}$ (*B6.129(Cg)-Axin2*$^{tm1(cre/ERT2)Rnu}$*/J*, stock number 018867), *Col2*$^{CreERT}$ (*FVB-Tg(Col2a1-cre/ERT)KA3Smac/J*, stock number 006774), *Aggrecan* $^{CreERT2}$ (*Acan*$^{CreERT2}$, *B6.Cg-Acan*$^{tm1(cre/ERT2)Crm}$*/J*, stock number 019148), *β-catenin*$^{fl/fl}$ (possessing loxP sites in introns 1 and 6 in the *β-catenin* gene, *6.129-Ctnnb1tmKem/KnwJ*, stock number 004152), *Rosa-tdTomato* (*RFP*, *B6;129S6-Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$*/J*, stock number 007905), *RosaZsGreen* (*ZsG*, *B6.Cg-Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$*/J*, stock number 007906) and C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Cathepsin K*$^{Cre}$ (*Ctsk*$^{Cre}$) [1] was a gift from Dr. S. Kato (Tokyo University, Japan).

**Tissue processing.**

For analysis of ZsG-positive cells, the tissues were harvested and immediately fixed in 4% (wt/vol) paraformaldehyde (PFA) at 4°C overnight and decalcified in 10 % EDTA (pH 7.0) for 7–14 days. Decalcified tissue was then embedded in paraffin and sections (4 μm) were made using a Leica CM1850 in 4 μm thickness (Leica Biosystems Inc. Buffalo Grove, IL), mounted onto slide glasses and dried overnight at 40°C. To analyze the RFP-positive cells, the tissues were subjected to frozen sectioning after PFA-fixation, decalcification and cryoprotection in 10, 20 and 30% (wt/vol) sucrose at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc. Torrance, CA). Sections were made using the Leica CM1850 in 4 μm thickness (Leica Biosystems Inc. Buffalo Grove, IL). Fluorescence of the RFP-labeled cells was observed and

captured under a confocal microscope (Leica TCS-LSI, Leica Biosystems Inc. Buffalo Grove, IL) after standard procedure and DAPI (GeneCopia, Rockville MD) staining.

**Immunohistochemistry.**

Immunohistochemistry was performed after antigen retrieval in Tris/EDTA (pH 9.0, 95°C 10 min or 0.2% Triton X-100 in PBS for 2 hours at R.T. Sections were stained with antibodies recognizing Osterix (rabbit polyclonal, 1:500, ab22552, abcam, Cambridge, MA), Sox9 (rabbit polyclonal, 1:500, ab3697, abcam), Runx2 (mouse monoclonal 8G5, 1:500, MBL, Nagoya, Japan), Collagen 1, alpha 1, propeptide (COL1A1, rabbit polyclonal, 1:1000, PhosphoSolutions, Aurora, CO), Ki67 (rabbit monoclonal SP6, 1:50, ab16667, abcam) and β-catenin (mouse monoclonal, 1:50, clone 14/b-catenin, BD Bioscience, San jose, CA). Isotype-matched immunoglobulins for rat and rabbits were used as controls. Antibody localization was visualized by incubation with either Alexa 488-, 594- or 647-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) or biotinylated secondary antibodies followed by development with DAB substrate using the Vectastain ABC system (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. DAPI (GeneCopia, Rockville, MD) was used to visualize the nucleus in immunofluorescent staining. Occasionally, the coverslip of the section was gently removed by soaking in PBS followed by standard Hematoxylin and Eosin staining (HE), Toluidine blue staining, or Safranin O/ fast red staining by standard protocols. Adobe Photoshop software (CS6, Adobe Systems, San Jose, CA) was used to superimpose the histochemical staining image with fluorescence images.

**RNA *In situ* hybridization**

Decalcified tibia of P6 C57BL/6 mice were subjected to frozen sectioning after PFA-fixation and cryoprotection in 10, 20 and 30% (wt/vol) sucrose at 4°C overnight, and then embedded in Tissue-Tek OCT compound (Sakura Finetek USA). Sections were analyzed with RNA scope® assay (Advanced Cell Diagnostics, Hayward, CA) using target probes for *Axin2* (RNAscope® Probe- Mm-Axin2), *Wnt4* (RNAscope® Probe- Mm-Wnt4), *Wnt10b* (RNAscope® Probe- Mm-Wnt10b) and negative control (RNAscope® Negative Control Probe-DapB). The assay was performed according to the manufacturer's instructions. In brief, sections were boiled with pretreatment solution for 10 minutes, incubated with hydrogen peroxide for 10 minutes in room temperature, the sections with targeted probes were then incubated in oven for 2 h at 40°C. Slides were then washed and incubated with the signal amplification solutions as indicated in the manual. Signals were visualized with RNAscope 2.5 HD-Brown detection kit (Advanced Cell Diagnostics) and the slides were counterstained with hematoxylin.

**Tissue clearing and whole imaging.**

To obtain the distribution of ZsGreen-positive cells in three-dimensional manner, wrist of the P9 *Axin2*$^{CreERT2}$*; ZsG*, which received Tx at P6, were cleared with CUBIC method.[2] Distal ulna of the cleared wrist was subjected to two-photon microscopy (Bergamo-scope, Thorlabs, Inc. Newton, NJ) equipped with a femtosecond laser (MaiTai BB, Spectra Physics, Irvine, CA) and a 16x water immersion objective lens (NA=0.80, Nikon). Image-stacks captured at 4 microns step size were visualized with 3D reconstruction software (Volocity 6.3, Perkin-Elmer, Coventry, UK).

**EdU staining for detecting cell proliferation.**

To analyze cell proliferation, EdU (5-ethynyl-20-deoxyuridine, Life Technology, Grand Island, NY) was administered intraperitoneally (50 μg/g body weight)[3] 2 hours prior to harvesting the samples. The knee joints were harvested and processed to prepare longitudinal sections, and stained using Click-iT® EdU Imaging Kits (Life Technology, Grand Island, NY) according to the manufacturer's instructions. Because the EdU staining procedure diminishes the ZsG fluorescence, we imaged the ZsG-positive cells before the EdU staining, then merged the EdU image with the ZsG image.

**Cell isolation and FACS (fluorescence activated cell sorting) analysis.**

Rib cages were dissected from female P9 *Axin2*$^{CreERT2}$*; ZsG* mouse, which received Tx at P6. Costocostal junctions were dissected from the rib cage. Then muscle and rib was removed manually under stereomicroscope (LED2500, Lecia Biosystems Inc.). To obtain the cells from perichondrium and costal growth plate chondrocytes facing the perichondrium, tissue was placed in collagenase digestion buffer (× 0.5 collagenase in DMEM without Fetal Bovine Serum) at 37°C for 10 min under constant agitation. Digested tissue including perichondrium and costal growth plate chondrocytes facing to the perichondrium were then removed under stereomicroscope and placed in collagenase digestion buffer again at 37°C for 30 min. Undigested materials were gently triturated by repeated pipetting. Dissociated cells were filtered through 40 μm cell strainer (Corning, Corning, NY), pelleted at 200 G at 4°C, These isolated cells were stained with following antibodies combined with Propidium Iodide (1.0 mg/mL, Life Technologies) for the

characterization of skeletal stem cells as previously described.[4] Anti-mouse CD45 (Clone 30-F11, PE/Cy7, BioLegend, San Diego, CA, USA), anti-mouse Ter119 (Clone TER-119, PE/Cy7, BioLegend), anti-mouse Tie2 (Clone TEK4, APC, BioLegend, San Diego, CA, USA), anti-mouse AlphaV integrin (Clone RMV-7, PE, eBioscience, San Diego, CA, USA), anti-mouse CD105 (Clone MJ7/18, PerCP/Cy5.5, BioLegend), anti-mouse Thy (CD90.1; Clone OX-7, Pacific Blue, CD90.2; Clone 53-2.1, Pacific Blue, BioLegend), anti-mouse 6C3 (Clone BP-1, BV711, BD Biosciences, San Jose, CA, USA), and anti-mouse CD200 (Clone OX-90, APC-R700, BD Biosciences). Experimental samples along with FMO (fluorescence minus one) controls were examined using a BD LSR II Flow Cytometer (BD Biosciences). The acquired data were analyzed with FlowJo software (Tree Star, Inc. Ashland, OR, USA). We analyzed two samples, and representative data is shown.

RNA extraction and quantitative real-time PCR.

To examine the efficiency of β-catenin ablation in ZsG+ cells of *β-catenin*$^{fl/fl}$*; Axin2*$^{CreERT2}$*;ZsG* mice, ZsG+ cells were corrected from RG of *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*; ZsG* mice (P20) and P20 *β-catenin*$^{fl/wt}$*;Axin2*$^{CreERT2}$*;ZsG* (Control mice) mice (P20), which received a Tx injection at P13. The cells were digested from RG of the tibia as described in Cell isolation and FACS, and separated into ZsG+ cells and ZsG- cells by BD LSR II Flow Cytometer (BD Biosciences). To analyzed the cells by quantitative real-time PCR, Total RNA were extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA was used for cDNA synthesis with random hexamers. Quantitative real-time PCR was carried out using the probe and

primers for *Ctnnb1* (5'-CCAGTCCTT and CACGCAAGAGCAAGT-3', 5'-ACCTCCAAGTCCTTTATGAATG-3' and 5'-CTGAGCCCTAGTCATTGCATAC-3'). Reactions were run in quadruplicate for 2 independent experiments. Expression data were normalized to the mean of housekeeping gene *Gapdh*, *Actb* and *Pgk1* to control the variability in expression levels and were analyzed using the 2 -ΔΔCT method. The primers for GADH, Actb and PgK1 are 5'-CTGGAGAAACCTGCCAAGTA-3' and 5'-TGTTGCTGTAGCCGTATTCA-3' for *Gapdh*; 5'-AAGAGCTATGAGCTGCCTGA-3' and 5'-TACGGATGTCAACGTCACAC-3' for *Actb*; and 5'-GCAGATTGTTTGGAATGGTC-3' and 5'-TGCTCACATGGCTGACTTTA-3' for *Pgk1*.

References


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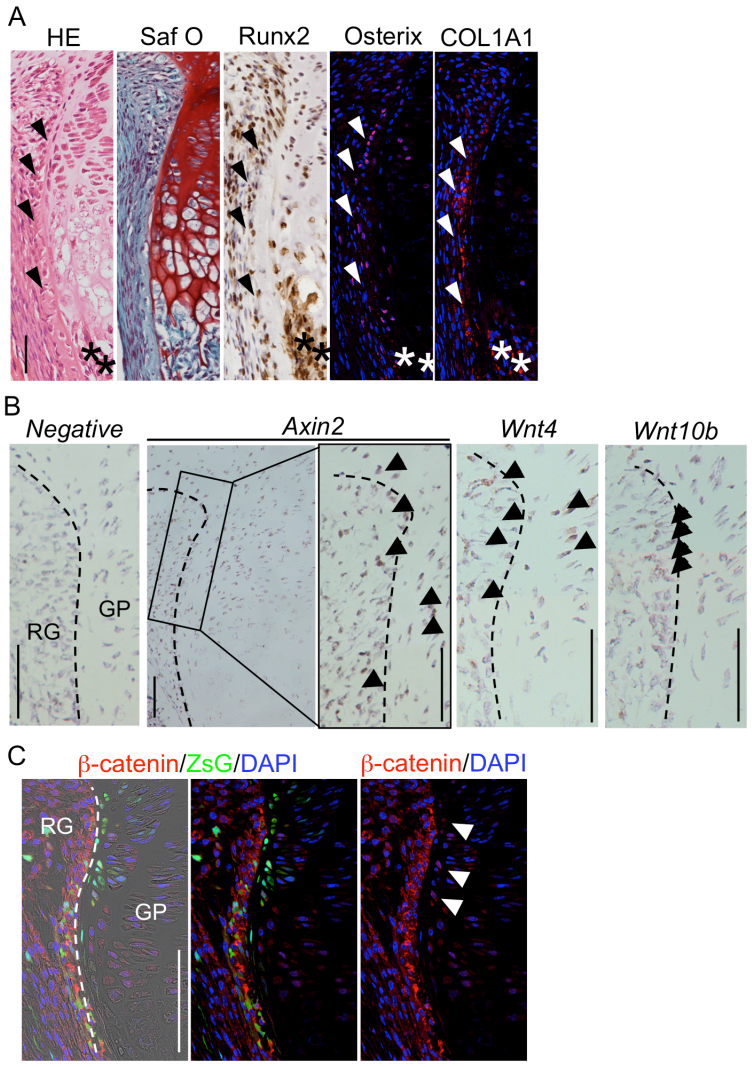
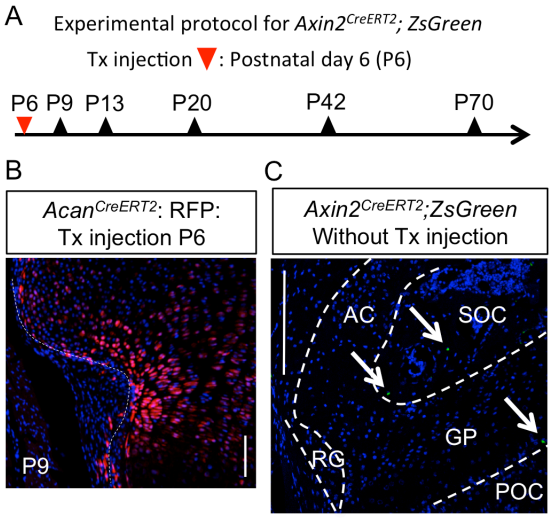
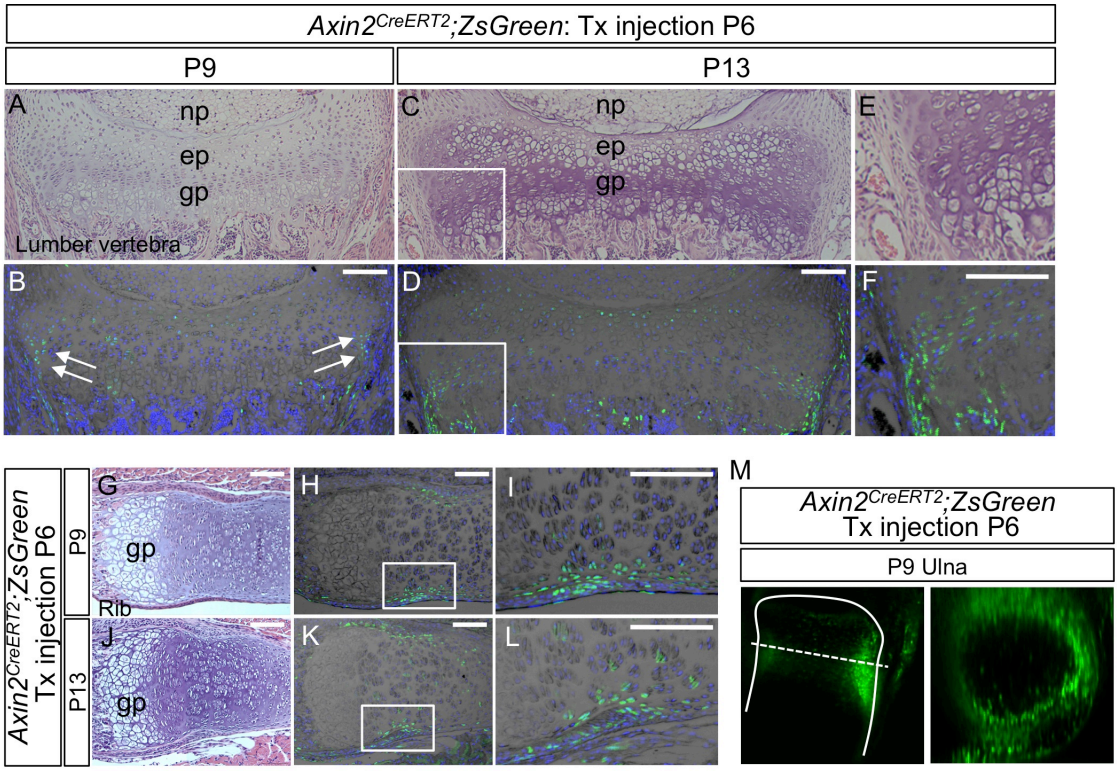
2. Susaki EA, Tainaka K, Perrin D, *et al.* Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. Nat Protoc. 2015;10(11):1709-1729.
3. Zeng C, Pan F, Jones LA, et al. Evaluation of 5-ethynyl-2'-deoxyuridine staining as a sensitive and reliable method for studying cell proliferation in the adult nervous system. Brain Res. 2010; 1319:21-32.
4. Chan CK, Seo EY, Chen JY, *et al*. Identification and specification of the mouse skeletal stem cell. Cell. 2015;160(1-2):285-298.



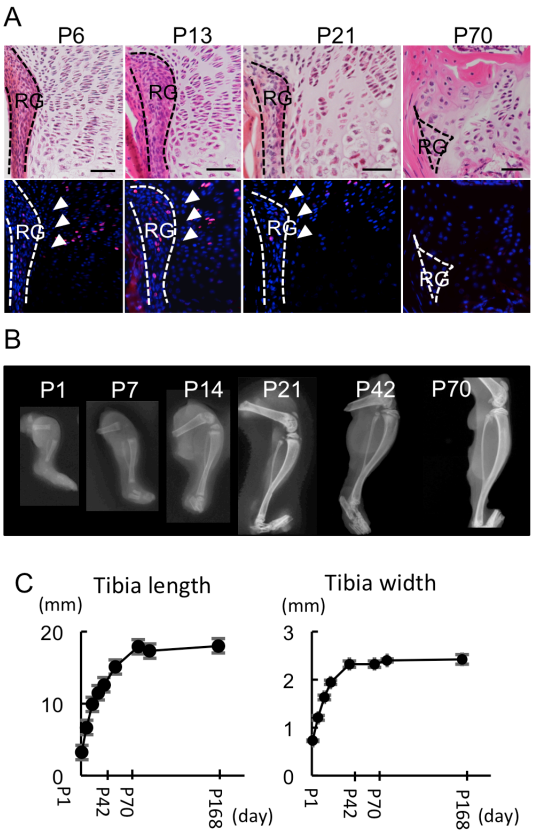

**Supplementary Fig. S1.** Characteristics of Ranvier's groove (RG) and periphery of the growth plate (GP).
(*A*) The cells in the RG adjacent to the growth plate express early osteoblast markers - nuclear Runx2, nuclear Osterix and type 1 collagen pro-peptide- within subcellular matrix (arrows). HE, hematoxylin and eosin staining. Saf O, safranin O staining with Fast green counter staining. Runx2, DAB-immunohistochemistry using the anti-Runx2 antibody (brown). Osterix, immunofluorescence staining with the anti-Osterix antibody (red) with nuclear DAPI staining (blue). COL1A1, immunofluorescence staining with the anti-Type 1 collagen α1 chain propeptide antibody (red) and DAPI (blue). (*B*) *Axin2*, *Wnt4* and *Wnt10b* transcripts in tibia of P6 C57Bl/6 female mice. *Negative*, negative control. *Axin2*, *Axin2* in situ hybridization. *Axin2* expression was found in both most outer layer of the GP and RG (brown, arrowheads). *Wnt4*, *Wnt4* in situ hybridization. *Wnt4* expression was found in both most outer layer of the GP and RG (brown, arrowheads). *Wnt10b*: *Wnt10b* in situ hybridization. *Wnt10b* is predominantly expressed in RG (brown, arrowheads). RG, Ranvier's groove. GP, Growth plate. Bar = 100 μm. (C) β-catenin protein expression in tibia of P9 female $Axin2^{CreERT2}$;*ZsG* mice that had received a Tx injection at P6. Co-localization of ZsG (green) and DAPI (blue) was observed in outermost layer of GP (arrowheads). RG, Ranvier's groove. GP, Growth plate. Bar = 100 μm



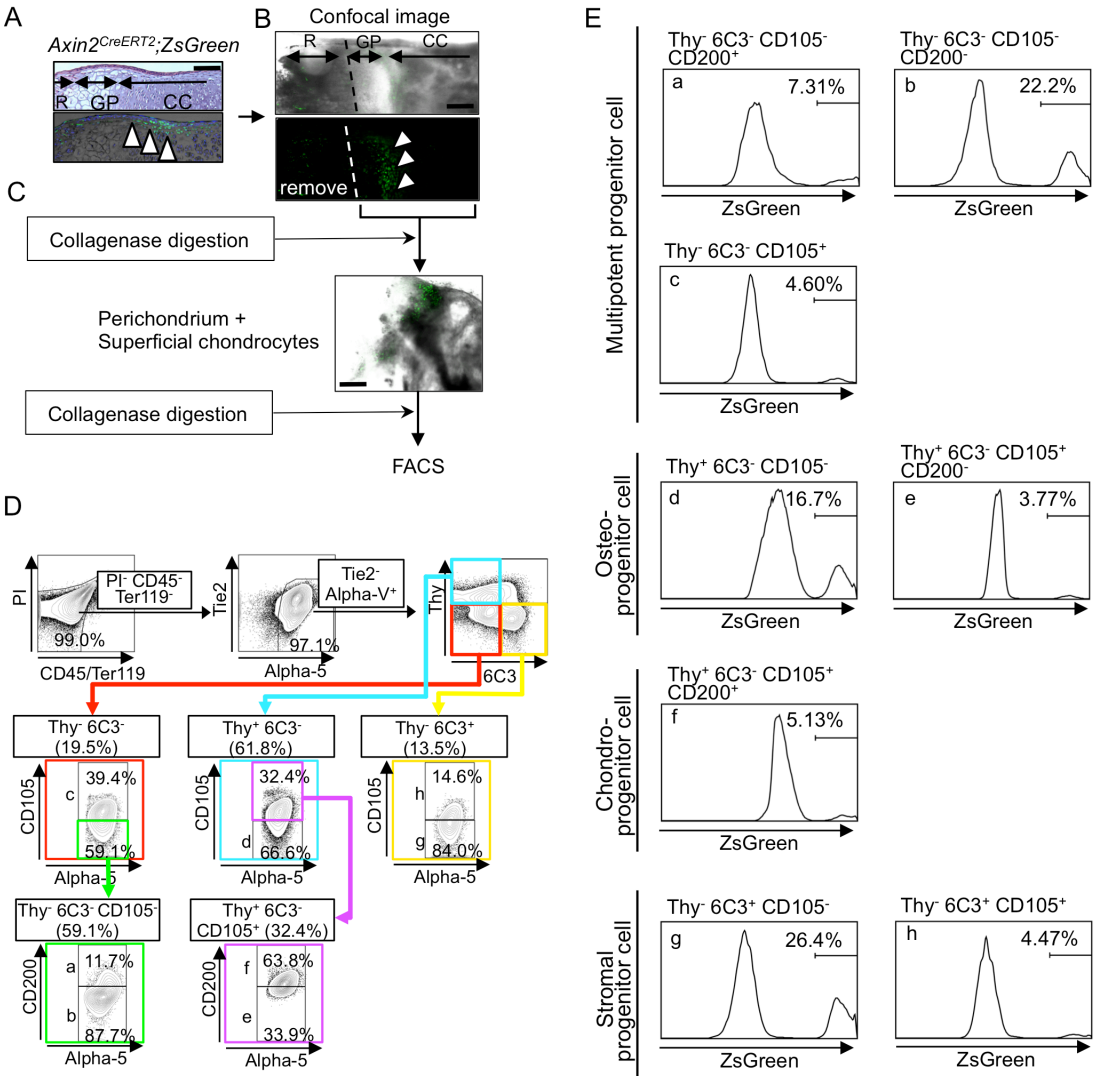

**Supplementary Fig. S2.** Fate mapping protocol and the results from *Acan*$^{CreERT2}$*;RFP* mice after the tamoxifen injection and *Axin2*$^{CreERT2}$*;ZsG* mice without tamoxifen injection.
(*A*) Schematic representation of the experimental protocol for the lineage tracing using *Axin2*$^{CreERT2}$*;RosaZsGreen* (*Axin2*$^{CreERT2}$*;ZsG*) mice. Female mice carrying *Axin2*$^{CreERT2}$*; ZsG* received a tamoxifen injection (Tx, 40 mg/ kg) at P6. Tibiae were harvested 3, 7, 14, 35 and 64 days post-injection, corresponding to P9, P13, P20, P42 and P70, and histologically examined. (*B*) Distribution of reporter cells in *Aggrecan*$^{CreERT2}$*;Rosa-tdTomaton* (*Acan*$^{CreERT2}$*;RFP*) mice. Female *Acan*$^{CreERT2}$*;RFP* mice received a Tx injection at P6 and tibiae were harvested 3 days post-injection (P9). The RFP image in the posterior region of the proximal tibia shows massive RFP-positive cells inside the growth plate. Bar = 100 μm. (*C*) ZsG+ cells without Tx injection in *Axin2*$^{CreERT2}$*;ZsG* mice. The tibia of P42 female *Axin2*$^{CreERT2}$*;ZsG* mice were harvested without Tx injection. A few positive cells were detected (arrows). AC, articular cartilage. RG, Ranvier's groove. GP, Growth plate. SOC, secondary ossification center. POC, primary ossification center. Bar = 500 μm.




**Supplementary Fig. S3**. Fate mapping of Wnt-responsive cells in the perichondrial groove in the vertebras and ribs.
Female mice carrying *Axin2*$^{CreERT2}$*;RosaZsGreen* (*Axin2*$^{CreERT2}$*;ZsG*) received Tx injection at P6. Lumber vertebra (*A-F*) and ribs (*G-L*) were examined 3 and 7 days post injection. (*A and B*) At P9, only a small number of ZsG+ cells were found in perichondrium and the adjacent growth plate (arrows). (*C and D*) At P13, number of ZsG+ cells increased in the outer growth plate. (*E and F*) Magnified view of the white box in *C* and *D*. *A, C and E*, HE. *B, D and F*, ZsG and DIC. Np, nucleus pulposus. ep, endplate. Gp, growth plate. (*G-I*) At P9, a small number of ZsG+ cell was found in grooved perichondrium and the adjacent growth plate of the ribs. (*J, K and L*) At P13, number of ZsG+ cells increased in the growth plate. (*I and L*) Magnified view of the white box in H and K. *G and J*, HE. *H, I, K and L*, ZsG/DIC. Gp, growth plate. Bars = 500 μm for *B-L*. (M) The 3D image of ZsG+ Wnt-responsive cells. Female *Axin2*$^{CreERT2}$*;ZsG* mice were injected with Tx at P6, and the wrists were harvested at P9. ZsG+ cells were observed by two-photon microscopy after clearing the tissue by CUBIC method. The longitudinal (left) and transverse (right) images were reconstructed.

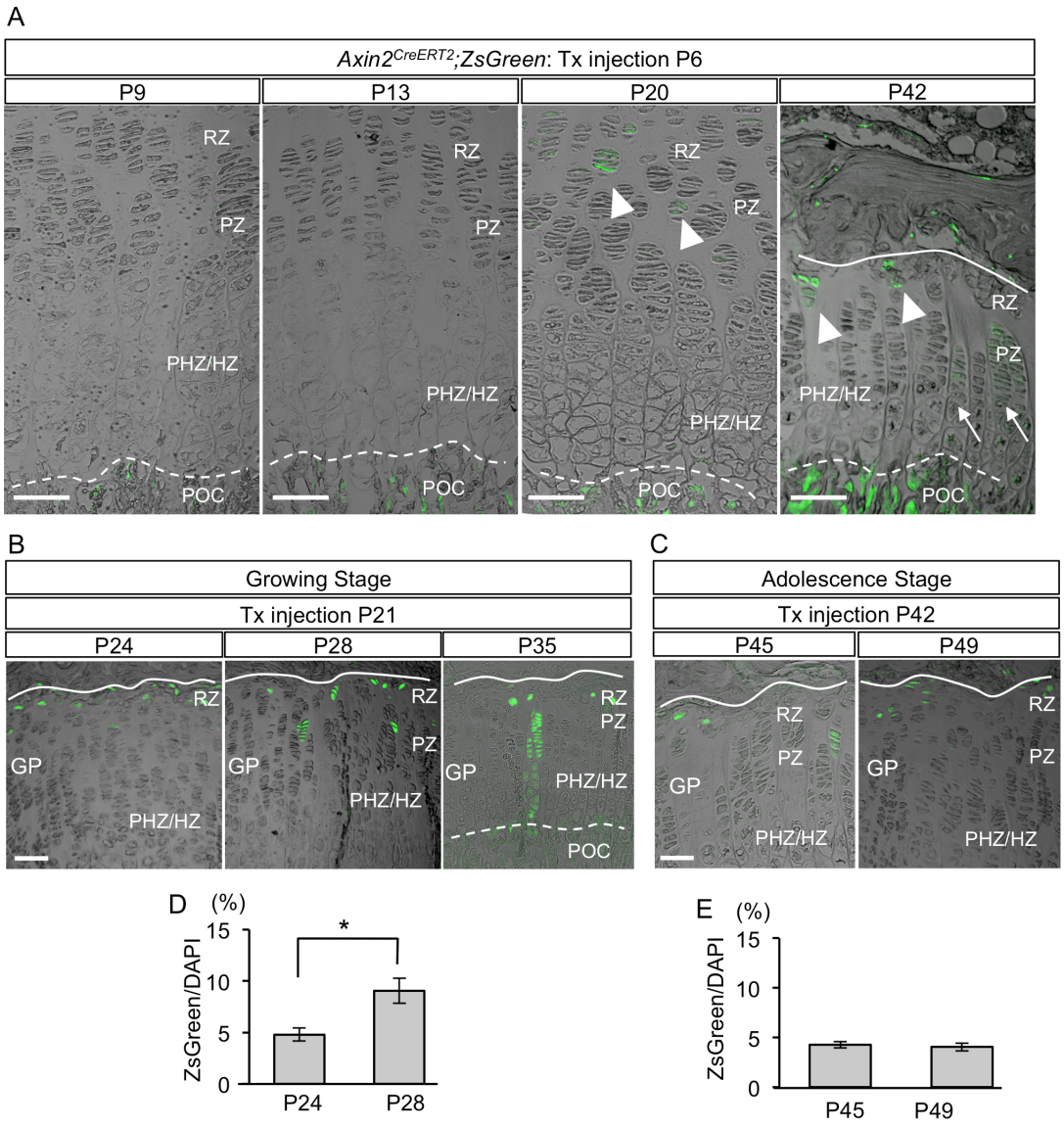



**Supplementary Fig. S4.** Changes in histology of the RG and the growth of the tibia during skeletal growth.
(*A*) HE and EdU staining around the RG. C57Bl/6j mice received an EdU injection (50μg/Kg) 2h prior to sample collection at P6, P13, P21, P42 and P70. Longitudinal tibiae sections were stained for EdU (bottom) followed by HE staining (top). EdU-labelled cells were found in the RG and the proliferative zone of the GP excluding the cells in the outermost of the growth plate. (*B*) Radiological images of tibiae during skeletal growth. Tibiae were harvested from C57Bl/6j female mice at P1 to P70 and subjected to radiological inspection. (*C*) Graphs showing tibia length (left) of calcified bone and width of chondro-osseous junction in the proximal tibia growth plate (right) from P1 to P168. n = 3 in each age. The values and error bars represent average and standard deviations.



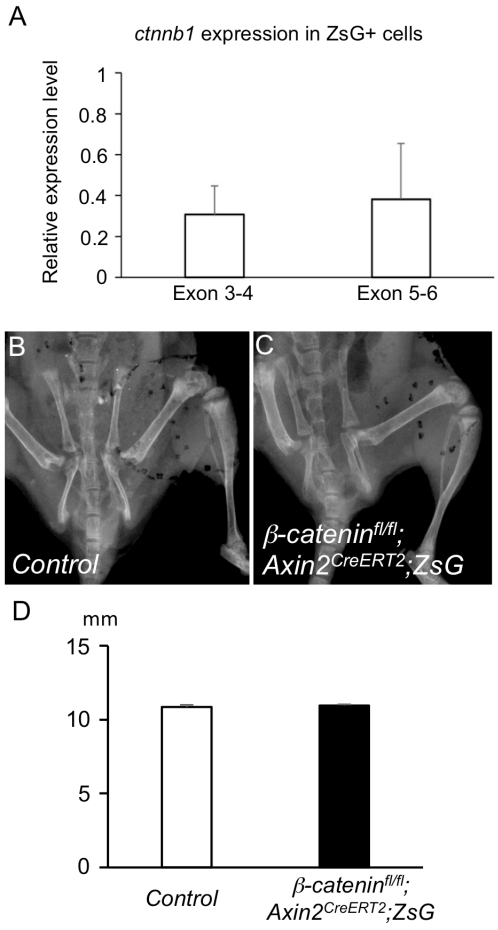

**Supplementary Fig. S5.** Characterization of the chondroprogenitors in Ranvier's groove and the outermost layer of the growth plate.
(*A)* Histology of the costchondral junction of the ribs. The Axin2$^{CreERT2}$;ZsG female mice received a Tx injection at P6, and the ribs were dissected at P9. Upper panel, HE staining. Lower panel, ZsG/ differential interference contrast (DIC). ZsGreen positive (ZsG+) cells were distributed in the grooved perichondrium and neighboring growth plate (arrowheads). (*B*) The micro-dissected costchondral junction of the ribs under fluorescent stereomicroscope. R, rib. GP, growth plate. CC, costal cartilage. (*C*) The perichondrium and costal growth plate chondrocytes facing to the perichondrium were micro-dissected after collagenase digestion (30 min at 37°C). The ZsG image was superimposed with the bright field image. The dissected tissues were digested by additional collagenase incubation (30 min at 37°C). Dissociated cells were filtered and subjected to the

fluorescence activated cell sorting (FACS) analysis. (*A*-C) Bar = 100 μm. (*D*) FACS gating strategy for isolation of skeletal progenitor subpopulation from CD45-, Ter-119-, Tie2-, Alpha-V+ (skeletal progenitor markers in Reference (4)). The CD45-, Ter-119-, Tie2-, Alpha-V+ skeletal progenitor population was separated into multipotent progenitor cells (Thy- 6C3-, red square and arrow), osteo- and chondro-progenitor cells (Thy+ 6C3-, blue square and arrow) and stromal progenitor cells (Thy- 6C3+, yellow square and arrow) according to Thy and 6C3 expression. These populations were subdivided according to CD105 and/or CD200 expression; the multipotent progenitor cell population was subdivided into (a) skeletal stem cell subpopulation, (b) pre-bone, cartilage and stromal progenitor subpopulation and (c) bone, cartilage and stromal progenitor subpopulation, the osteo- and chondro-progenitor cell population was subdivided into (d and e) two osteoprogenitor cell subpopulation and (f) chondroprogenitor cell subpopulation, the stromal progenitor cell population was subdivided into (g and h) two stromal progenitor cell subpopulation. (*E*) ZsG+ cells were included in the skeletal progenitor population. Each subpopulation (a-h) was sorted with endogenous ZsG expression, and plotted in histogram plot. (a-c) Multipotent progenitor cell population, (d-e) Osteoprogenitor cell subpopulation, (f) chondroprogenitor cell subpopulation, (g and h) stromal progenitor cell subpopulation. X axis indicates ZsG expression, Y axis indicates cell number. Percentages of the ZsG+ cell in each subpopulation are shown in histogram plot.




**Supplementary Fig. S6**. Wnt-responsive cells in center portion of the growth plate. (*A*) Fate mapping of Wnt-responsive cells in center portion of the growth plate (GP). Female mice carrying *Axin2*$^{CreERT2}$*;RosaZsGreen* (*Axin2*$^{CreERT2}$*;ZsG*) received tamoxifen (Tx) injection at P6. Center portion of the GP was histologically evaluated on P9, P13, P20 and P42, corresponding to 3, 7, 14 and 36 days after Tx injection, respectively. Arrowheads represent the ZsGreen positive (ZsG+) cells. The arrows indicate the GP column consisting of ZsG+ cells. The broken lines indicate the border between the bottom of growth plate and primary ossification center (POC). The solid line indicates the top of the growth plate. RZ, resting zone. PZ, proliferative zone. PHZ/HZ, prehypertrophic and hypertrophic zone. Bar = 100 μm. (*B* and *C*) Growth stage dependent contribution of Wnt/β-catenin responsive cells in center portion of the growth plate. Female mice carrying *Axin2*$^{CreERT2}$*;ZsG* received Tx injection at P21 (growing stage, *B*) or P42 (adolescence stage, *C*). The distribution of ZsG+ cells were

histologically examined in the center portion of growth plate after 3 days (P24 or P45), 7 days (P28 or P49) or 14 days (P35) post injection. The broken line indicates the border between the bottom of growth plate and primary ossification center (POC). The solid lines indicate the top of the growth plate. GP, growth plate. RZ, resting zone. PZ, proliferative zone. PHZ/HZ, prehypertrophic and hypertrophic zone. POC, primary ossification center. Bar = 100 μm.
(*D and E*) Changes in the percentage of number of ZsG+ cells to number of DAPI positive cells at the growing stage (B) and the adolescent stage (C) were examined, respectively. The values and error bars represent average and standard deviations. Note the significant increase of ZsG+ cells in the growth plate was only seen in the growing stage (*D*). *, $p < 0.05$.




**Supplementary Fig. S7.** Efficiency of the *β-catenin* ablation in Wnt-responsive cells and the radiological views of the *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*;ZsGreen* (*ZsG*) mice after TX injection. (*A*) *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*;ZsGreen* (*ZsG*) mice received a TX injection at postnatal day 13 (P13) and the epiphyseal chondrocytes were isolated 3 days after tamoxifen injection. The ZsG+ and ZsG- cells were sorted by FACS and subjected to the quantitative real-time PCR to examine RNA expression of *ctnnb1* with the primers for Exon 3-4 and Exon 5-6. The average of *β-actin*, *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) and *Phosphoglycerate kinase 1* (*Pgk1*) Ct values was served as internal control. The relative ratios of the ZsG+ group values to the ZsG- group values were calculated. Values and error bars represent average and standard deviations. (*B-D*) X-ray analysis of *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*; ZsG* mice and control mice. The *β-catenin*$^{fl/wt}$*;Axin2*$^{CreERT2}$*;ZsGreen* (*ZsG*) (Control) and the *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*; ZsGreen* (*ZsG*) mice received a Tx injection at postnatal day 13 (P13), and the tibiae were radiographically examined at P20 (B and C). Representative image is shown. Note there is no significantly change between two groups. (*D*) Length of the tibia in *β-catenin*$^{fl/fl}$*;Axin2*$^{CreERT2}$*;ZsG* mice and *β-catenin*$^{fl/wt}$*;Axin2*$^{CreERT2}$*;ZsG* (Control) mice. The values and error bars represent average and standard deviations.