Supplemental Information

Supplemental Figure legends

Supplemental Figure 1. Analysis of pStat3 (active Stat3) expression, proliferation and cell death of chondrocytes in developing long bones. (A) Immunofluorescent staining of pStat3 in the humerus sections of P0 littermate pups of the indicated genotypes. Higher magnification images are shown on the right side. Stat3 phosphorylation was upregulated in the differentiating osteoblasts (arrows). (B, C) The gross appearance of *Prrx1Cre: Stat3*$^{C/C}$ (*Stat3* KO) mice at P10 and P21, respectively. The limbs were bowed and shortened. Forelimbs showed more severe defects as the *Prrx1Cre* induces more complete deletion in forelimbs. (D) Cell proliferation was assayed by BrdU incorporation at E16.5 and P0. Boxed regions are shown with higher magnification on the right side. Cell death was determined by immunofluorescent staining of Cleaved Caspase (Cl-Casp) in the humerus sections of P0 littermate pups of the indicated genotypes. Quantification of BrdU^{+} cells and Cl-Casp^{+} cells in the marrow area are shown in the right panel. Scale bars: 100 μm. p values ***P < 0.005. Data are presented as mean ± SD. (E) Stat3 protein depletion was confirmed by Western blotting analyses in the lysates of E15.5 limbs with indicated genotypes, β-actin was loading control. Numbers above the gel picture indicate fold changes.

Supplemental Figure 2. Limb specific *Stat3* deletion did not affect embryonic cartilage development. (A) Sections of humerus from E16.5 littermate embryos and P0 pups of the indicated genotypes were analyzed by H&E staining, *in situ* hybridization with *Col2a1* probes, and immunofluorescent staining of Col10a1. Scale bars: 200μm. (B) Higher magnification images of humerus sections from P0 littermate pups.

Scale bars: 100 μm. (C) Quantification of the hypertrophic regions in (B). (D) Representative images of alcian blue stained micromass cultures of limb bud cells isolated from $Stat3^{C/C}$ embryos, which were transduced with indicated adenoviruses.

Supplemental Figure 3. *Stat3* deletion in hypertrophic chondrocyte doesnot affect osteoblast differentiation. (A) Whole mount skeletal preparation of *Col10a1Cre; Stat3$^{c/c}$* and control littermates at E18.5 and P0. Scale bars, 1 mm. (B) H&E staining of humerus sections from the indicated litter mate mice. Scale bars, 100 μm. (C) von Kossa staining of humerus sections from the indicated litter mate mice. Scale bars: 100 μm. (D) Immunofluorescent staining of Col10a1, Sp7, and Opn. Scale bars, 100 μm. (E) qRT-PCR analysis of indicated gene expression (n=5) in the *Col10a1Cre; Stat3$^{c/c}$* mice and control littermates. ns, not significant.

Supplemental Figure 4. Chondrocyte specific deletion of *Stat3* disrupts chondrocyte column length & reduces the number of chondrocytes trans-differentiated into osteoblasts. Immunofluorescent staining (A-C and A'-C') and quantification (A"-C") of marker expression as indicated in tdTomato (Td-tm) labeled mutant (*Col2a1CreER; Stat3$^{C/C}$; Rosa26Tm*), in littermate control (*Col2a1CreER; Stat3$^{C/+}$; Rosa26Tm*) cells. Tamoxifen (Tam) was injected at P2 and humerus was cryo-sectioned at P21 for further analysis. Scale bars 100μm. p values ** < 0.005, ***P < 0.001. Data are presented as mean ± SD. ColX: Col10a1, Osx: Sp7 and Opn: Osteopontin.

Supplemental Figure 5. Loss of Stat3 in limb mesenchymal cells led to increased osteoclast numbers. (A) Representative images of TRAP staining (black arrows), counter stained with hematoxylin, on the humerus sections of littermate pups with indicated genotypes at P0. Red arrows point to the dead osteoblasts. (B) Quantification of osteoclast surface/bone surface (Oc.S/BS) in field of view (FOV). (C) qRT-PCR analysis of the indicated gene expression from RNA isolated from the humerus bone at P0. **p values < 0.05, ***p values 0.005. data are presented as mean ± SD. (D-E) Representative images of F4/80 and MPO (myeloperoxidase antibodies) immunofluorescent staining of the humerus sections of P0 littermate pups of indicated genotypes. Scale bars 100 μm. (D'-E') Quantification of the IF results in (D-E).

Supplemental Figure 6. Stat3 deletion leads to reduction of Wnt/β-catenin signaling and increase in *Sost* expression. (A) Stat3 did not interact with β-catenin *in vitro*. Immunoprecipitation (IP) assays of cell lysates prepared from limb bud cells from E12.5 embryos (Left panel), and P0 bone (right panel) with the indicated antibodies, and IgG was used as negative control. (B) Representative images of in situ hybridization with anti-sense probes of *Sost*, *Dmp1* and *Phex*. (C) Immunofluorescent staining of Sost in the humerus sections of P5 and P21 littermate pups of the indicated genotypes. Scale bars: 100μm. (D) Western blotting analyses of Sost expression of the tissue lysates prepared from P0 bone tissues of the indicated genotypes, GAPDH was loading control. (D') Quantification of the Western Blotting results in (D). (E) Representative images of Alkaline phosphatase (ALP) staining of *Stat3*$^{c/c}$ BMSCs cultured with osteogenic medium 7 days after Ad-GFP or Ad-Cre infection. N=3 biological replicates. (F) qRT-PCR analysis of

osteoblast marker expression (upper panel), Wnt signaling target gene and *Sost* expression in *Stat3*$^{c/c}$ BMSCs, 7 days after Ad-Cre or Ad-GFP infection. N=3 biological replicates. **p<0.01 one-way ANOVA followed by Tukey's multiple comparisons tests.

Supplemental Figure 7. Stat3 may not regulate Sost expression directly. (A) (A) The ATAC-seq signal tracks of the *Sost* and *Socs3* gene loci in mouse limb tissue at E14.5 (39). Processed ATAC data was downloaded from the website of the Ren Lab (http://renlab.sdsc.edu/renlab_website//download/encode3-mouse-histone-atac/) and visualized by IGV software (http://software.broadinstitute.org/software/igv/). Stat3 binding site was found in the boxed regions (red). (B) DNA sequences of Stat3 binding. (C) ChIP-qPCR confirmation of Stat3 and H3K27Ac binding of the boxed regions in the *Sost* and *Socs3* genes as shown in (A). *Stat3*$^{c/c}$ BMSCs, 7 days after Ad-Cre or Ad-GFP infection were used for the ChIP-qPCR assay. Data represent mean ± SD. *p<0.05; **p < 0.01; ****p < 0.0001; P values were in 1-way ANOVA with Sidak's test. NS: not significant. (D) The DNA sequences of Lef/Tcf binding. The ATAC-seq signal tracks of the *Axin2, Tcf7 (Tcf1)* and *Lef1* gene loci in mouse limb tissue at E14.5. The Lef/Tcf binding elements (BEs) are shown as red bars and Stat3 binding BEs are shown as blue bars, which are boxed in red.

Supplemental Figure 8. Pharmacological or genetic activation of Wnt/β-catenin signaling rescued the bone phenotypes of *OsxCre; Stat3*$^{c/c}$ mice. (A) Masson's trichrome staining of the humerus sections from P0, P15 and P21 mice. Scale bars: 100 μm. (B) Western blotting analyses of the Osx (Sp7) in the tissue lysates from the bone of

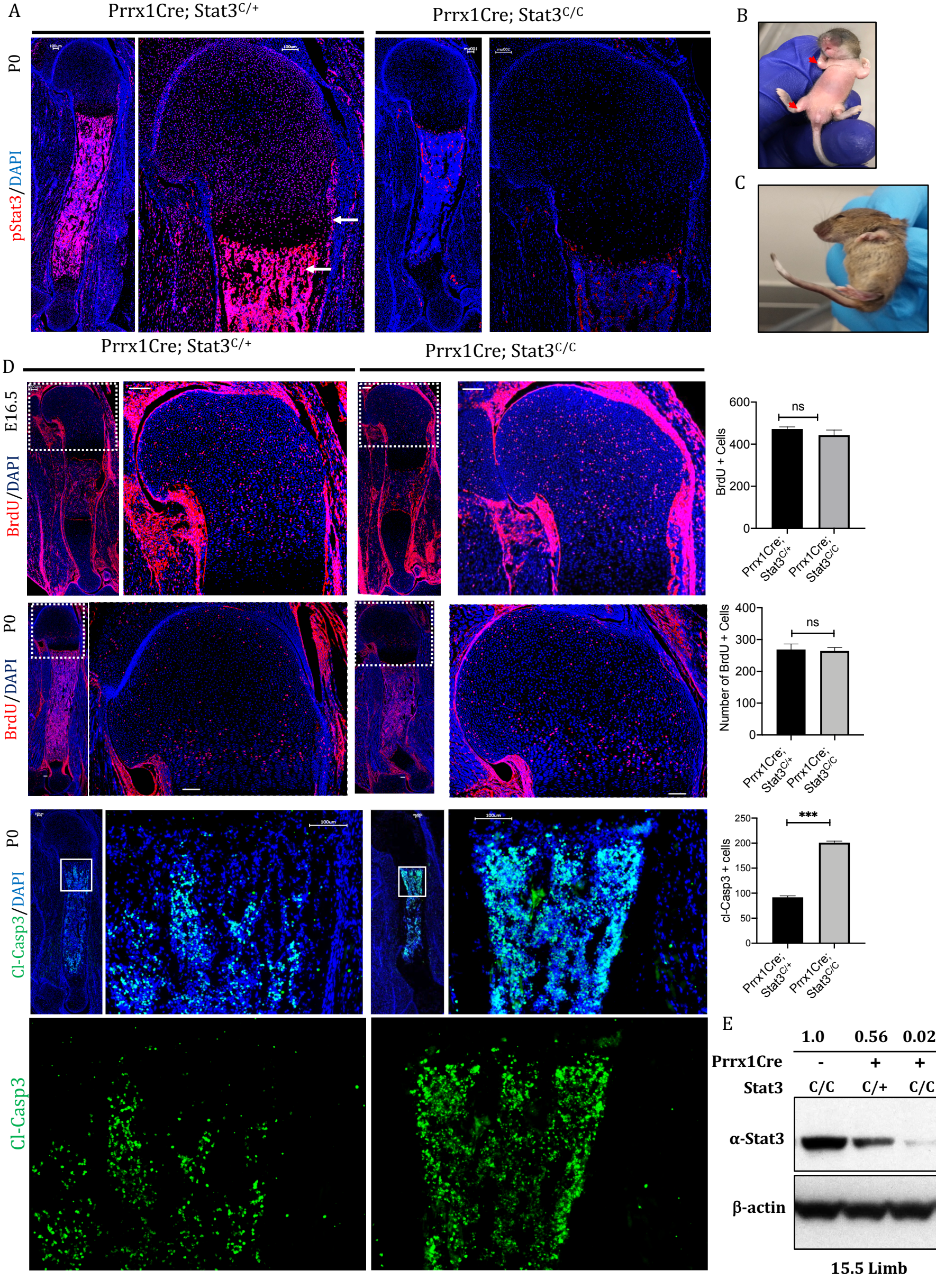
P0 pups with indicated genotypes/treatments. (C) Whole mount alizarin red and alcian blue staining of littermate mice of the indicated genotypes at P0. The forelimb (FL) and hindlimb (HL) are shown in the lower panel. Wnt/β-catenin signaling activation by BIO treatment rescued the *OsxCre; Stat3*$^{C/C}$ mice. The humerus and skull (arrow) of the rescued mice showed more bone formation compared to the untreated *OsxCre; Stat3*$^{C/C}$ mice. Scale bars: 1 mm. (D) Restoration of Wnt/β-catenin signaling either using BIO or expressing *Lrp5*^{A} improved skeletal growth and viability. Whole mount skeletal preparations of littermates of indicated genotypes at P28. Scale bars: 1 mm. (E) Representative μCT images sand quantification of humerus and cross sections of the cortical, trabecular distal femur bones from 5 weeks old littermate mice of the indicated genotypes as described in Fig. 5B-E. Scale bars: 1 mm in the whole bone image, 100 μm in cross sections. *p values < 0.05 and p** < 0.005. Data are shown as means ± SD. NS: not significant. (F) Representative von Kossa stained images of humerus sections from the P0 mice of the indicated mice. Scale bar, 100 μm. (G) Representative images of fluorescent staining of β-catenin or Mmp13 in the humerus sections of P0 littermate pups with indicated genotypes. Boxed regions were shown in higher magnification in the lower panel. DAPI stained the nucleus. Scale bars: 50 μm.

Supplemental Figure 9. BIO treatment of the wild type mice did not have obvious effect. (A) images of von-Kossa stained humerus sections from P0 mice. (B) images of Masson's Trichrome stained humerus sections from P5 mice. (C) Whole mount skeletal preparations of littermates of indicated P5 mice. Limbs were shown below. Scale bars: 200 μm.

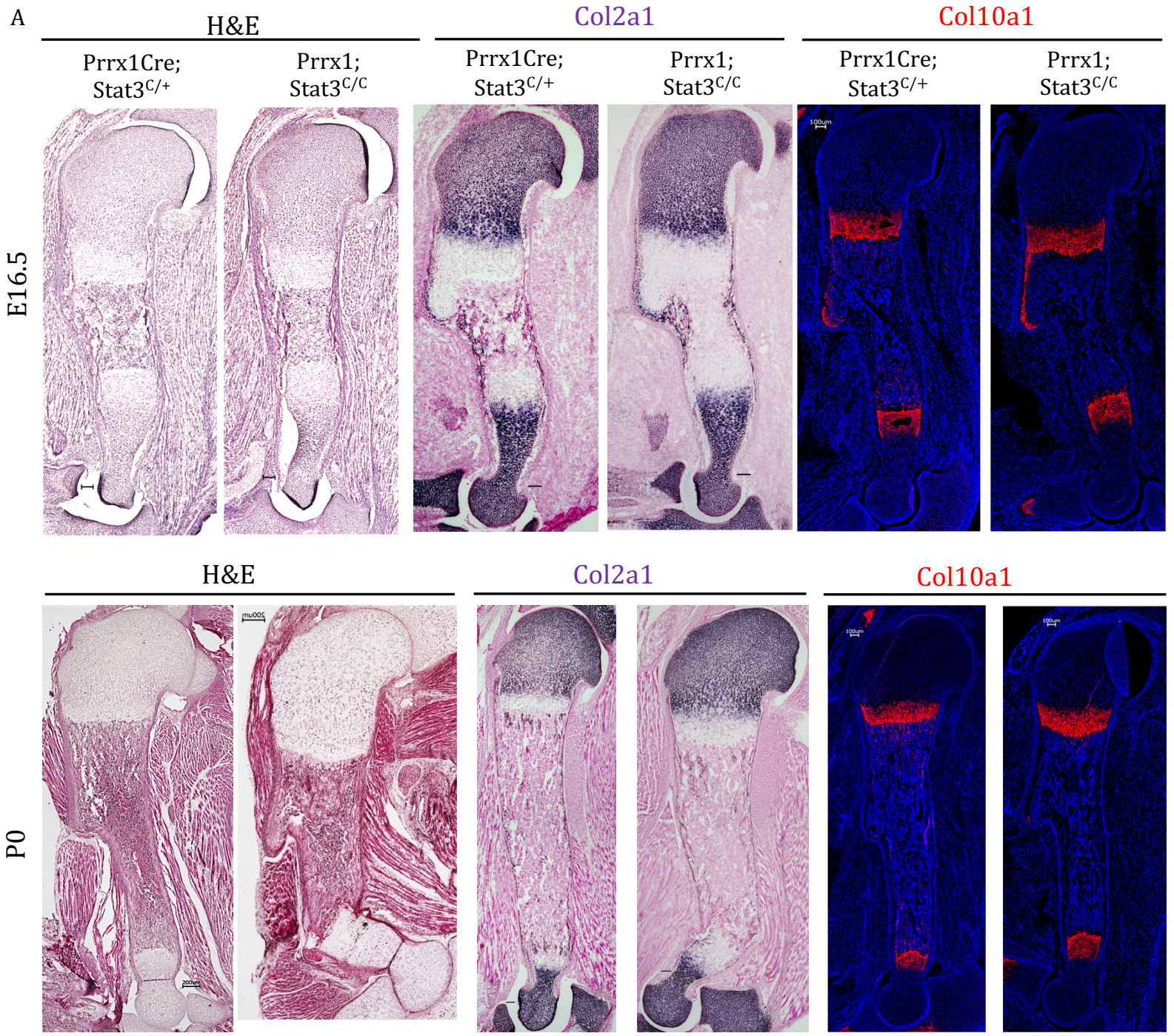
Supplemental Table 1: Antibodies use in the study. The name, source and application condition of the antibodies used in the study are summarized.

Supplemental Table 2: Oligo Primers used in the study. The forward and reverse sequences of the oligo primers are shown.

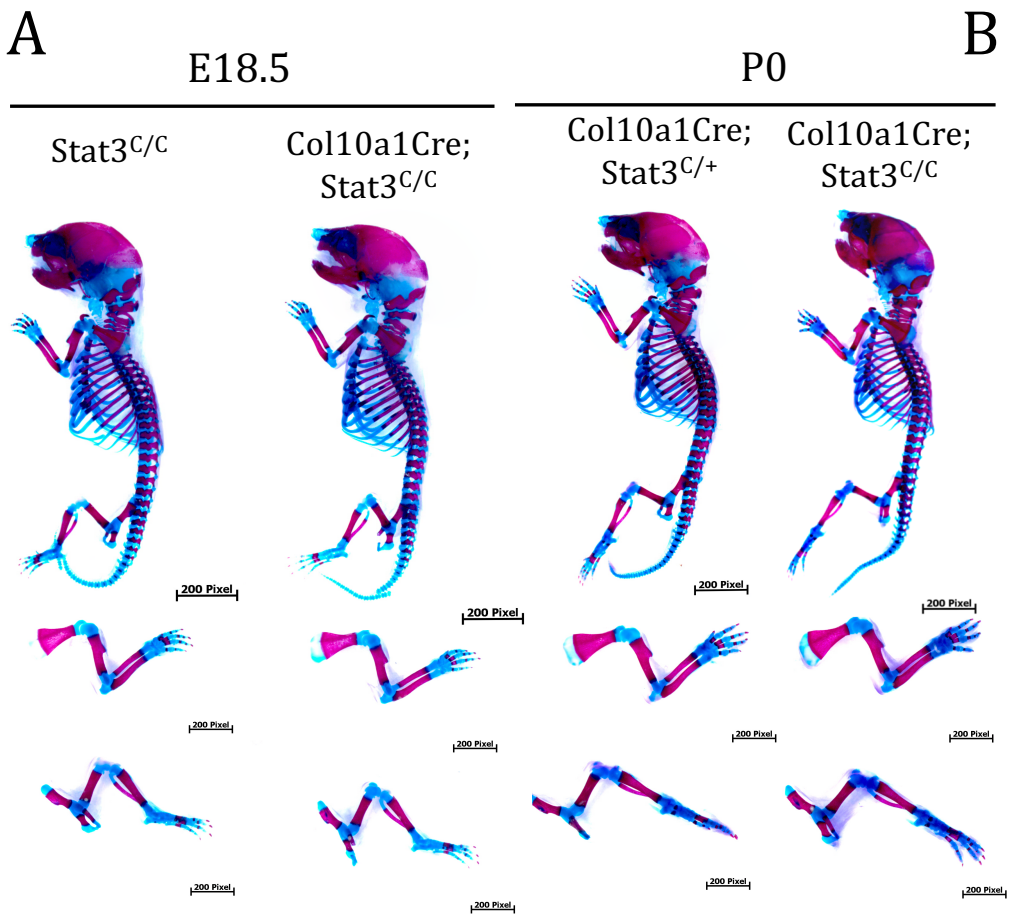
Supplemental Figure 1. Expression analysis of pStat3 (active) and cell proliferation and cell death analysis in developing long bones

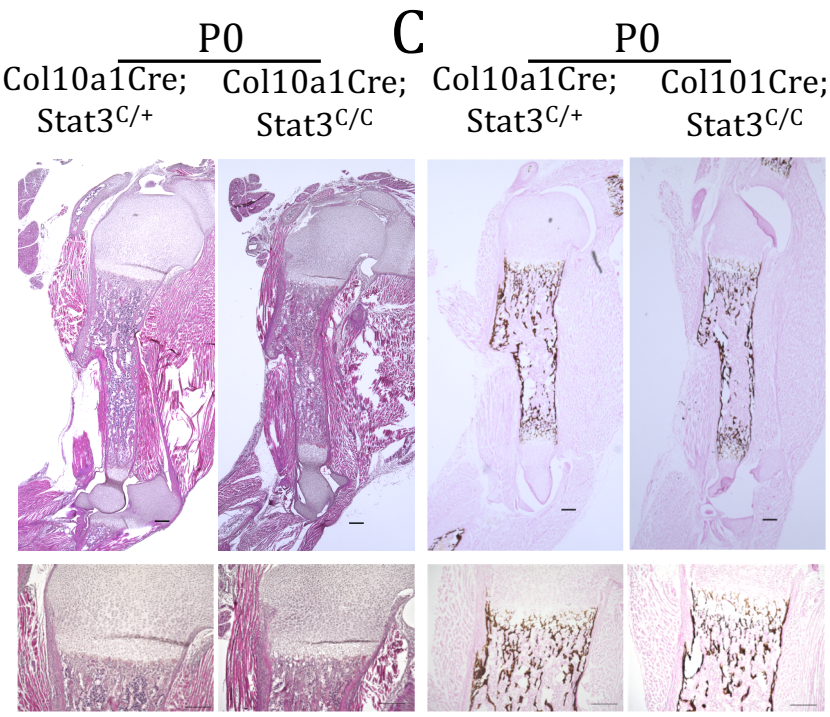


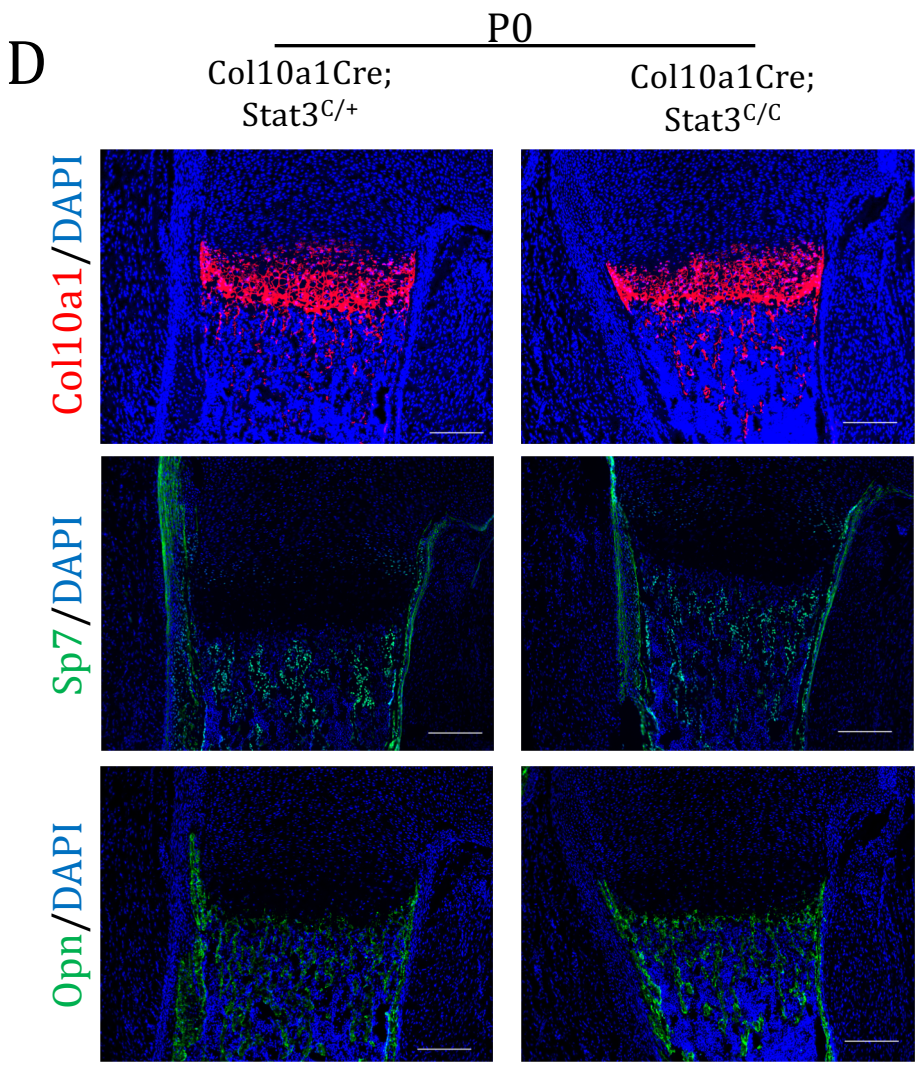
Supplemental Figure 2. Limb specific Stat3 deletion did not affect embryonic cartilage development

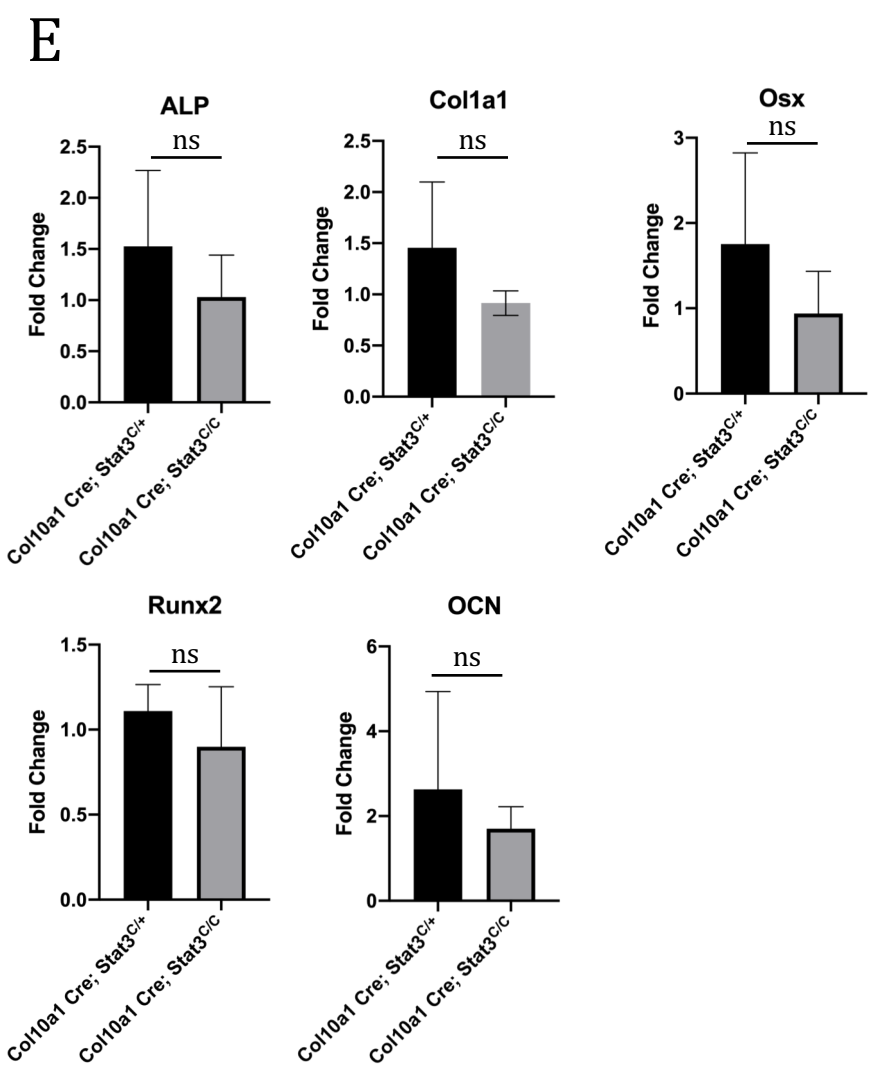


Supplemental Figure 3 Stat3 deletion in hypertrophic chondrocyte does not affect osteoblast differentiation.

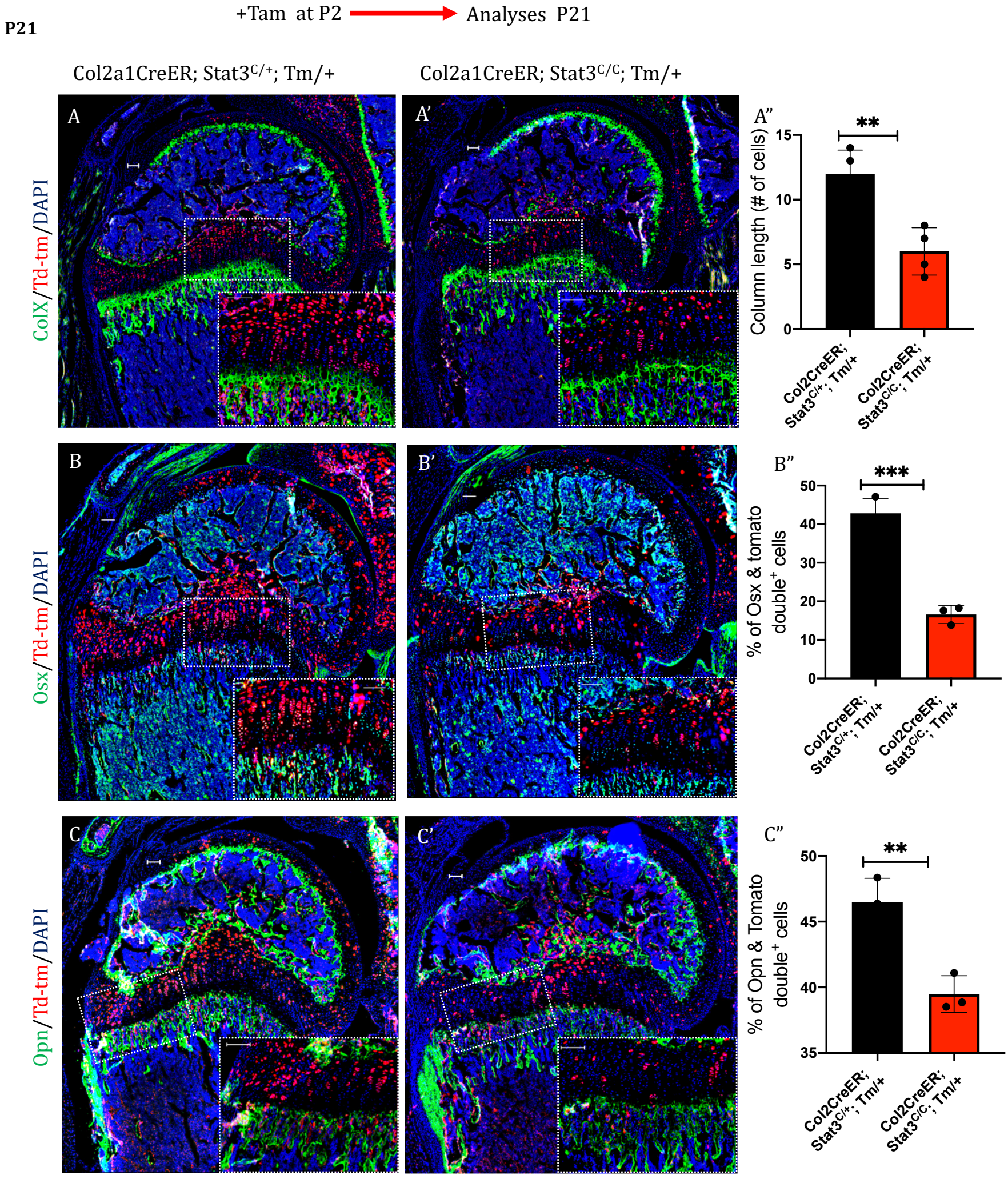




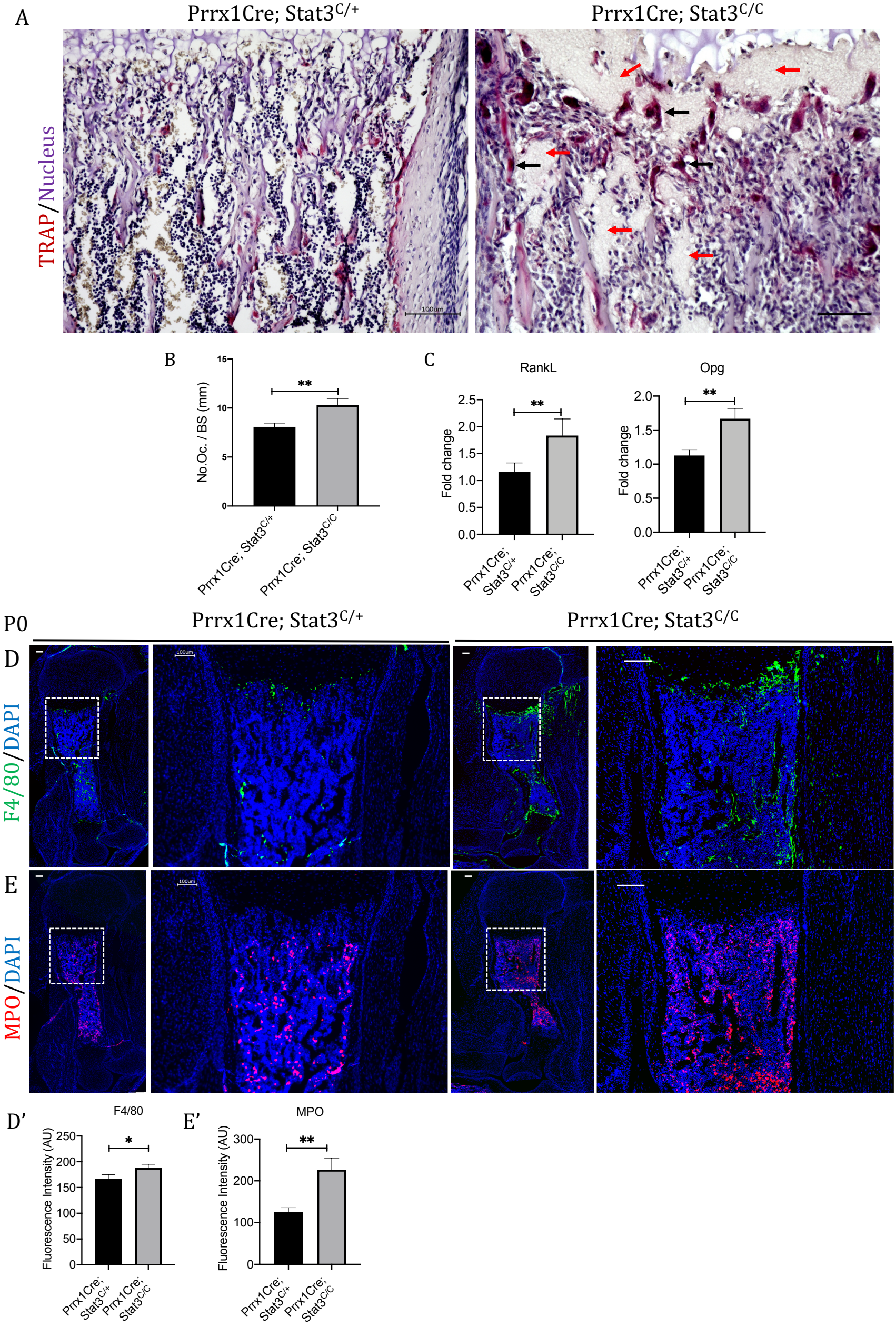




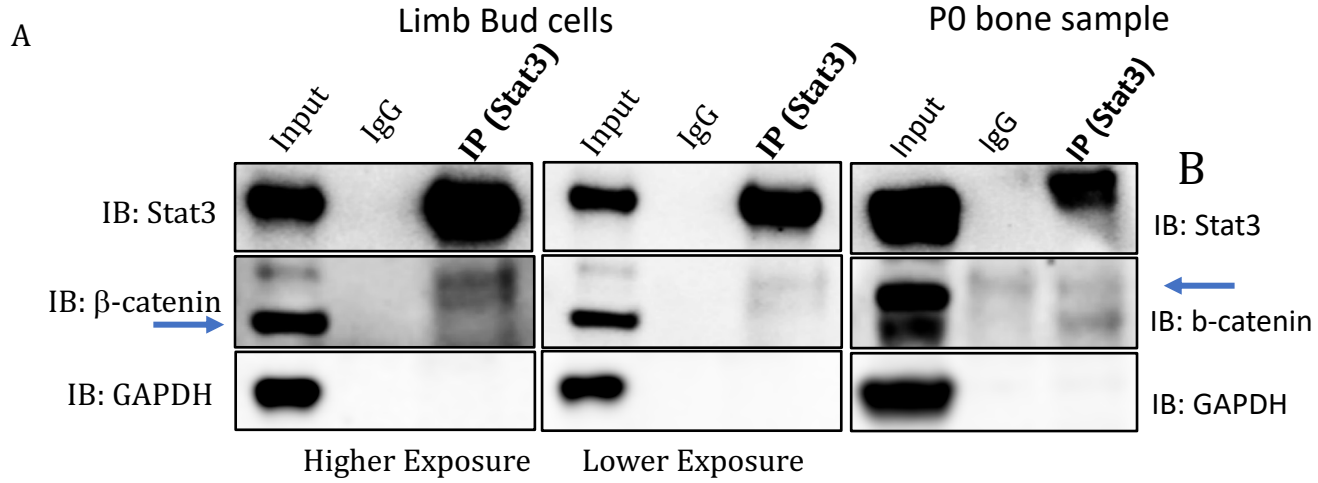
Supplemental Figure 4. Chondrocyte specific deletion of Stat3 disrupts chondrocyte column length & reduces the trans-differentiation of chondrocytes into osteoblasts.

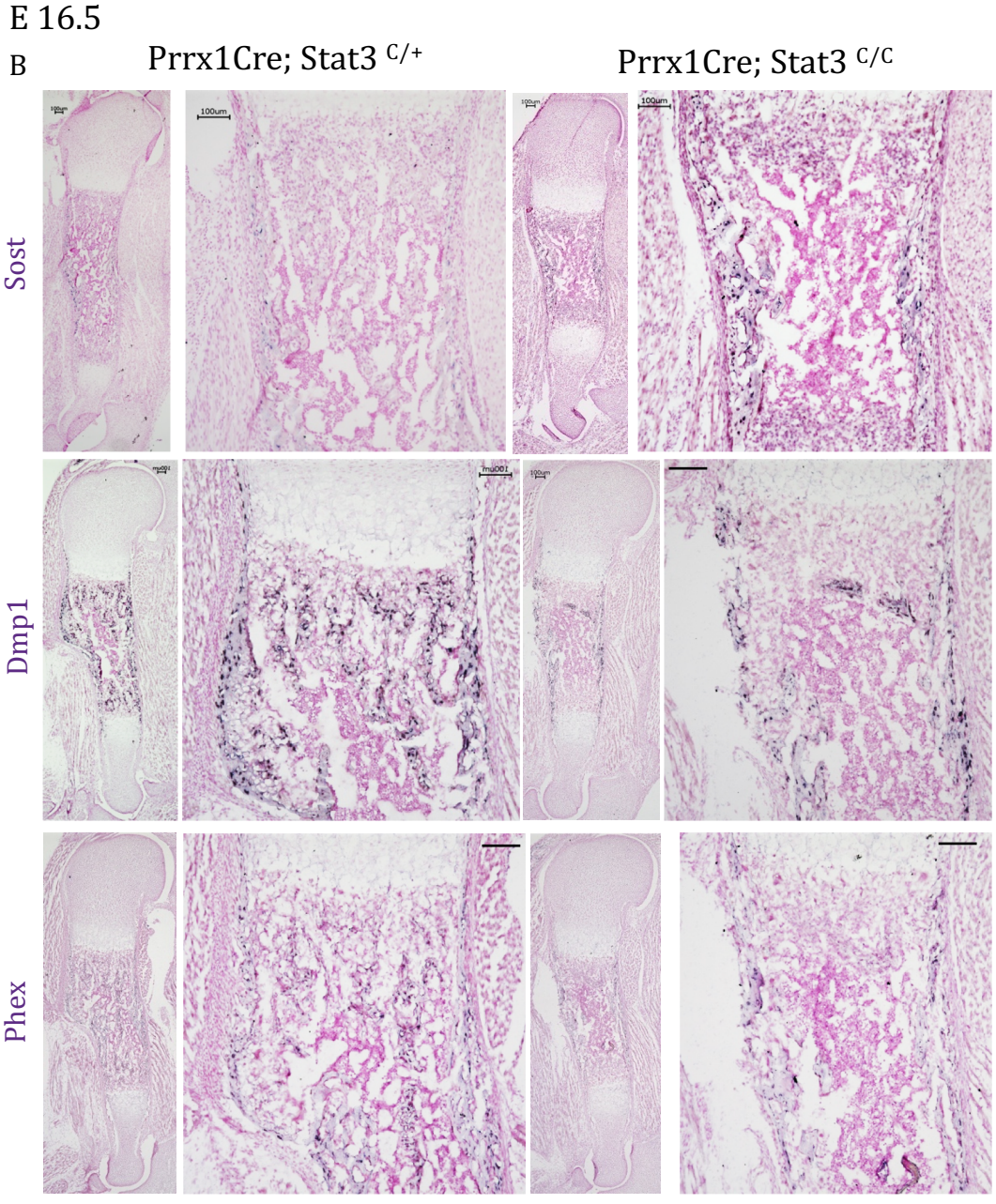


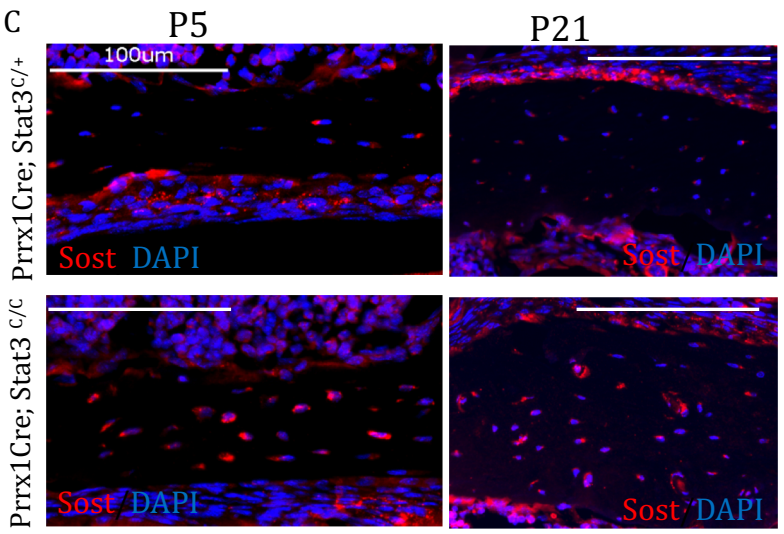
Supplemental Figure 5. Loss of Stat3 in limb mesenchymal cells led to increased number of osteoclasts.

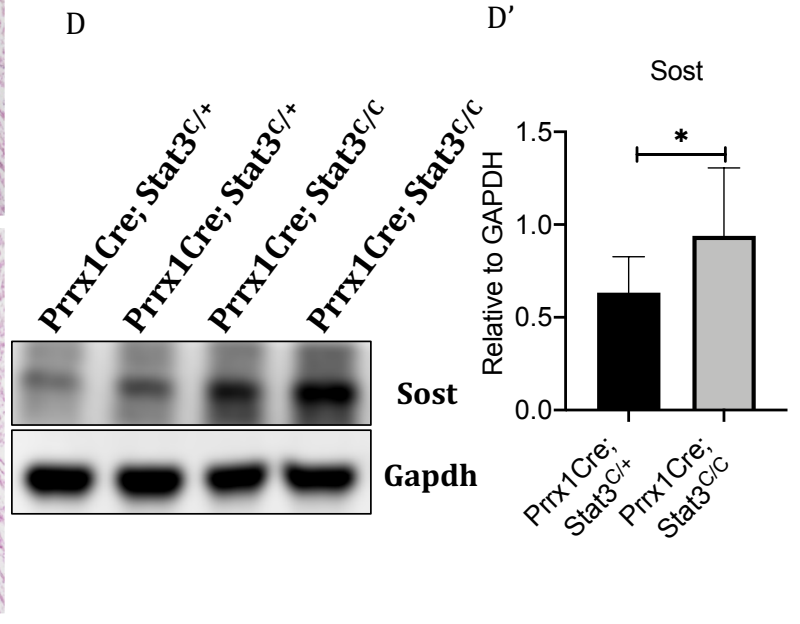


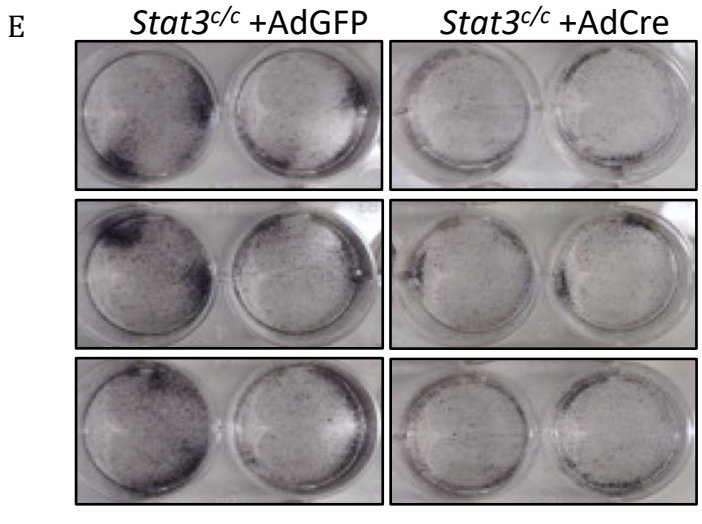
Supplemental Figure 6. Stat3 deletion leads to reduction of Wnt/β-catenin signaling and increase in Sost expression.

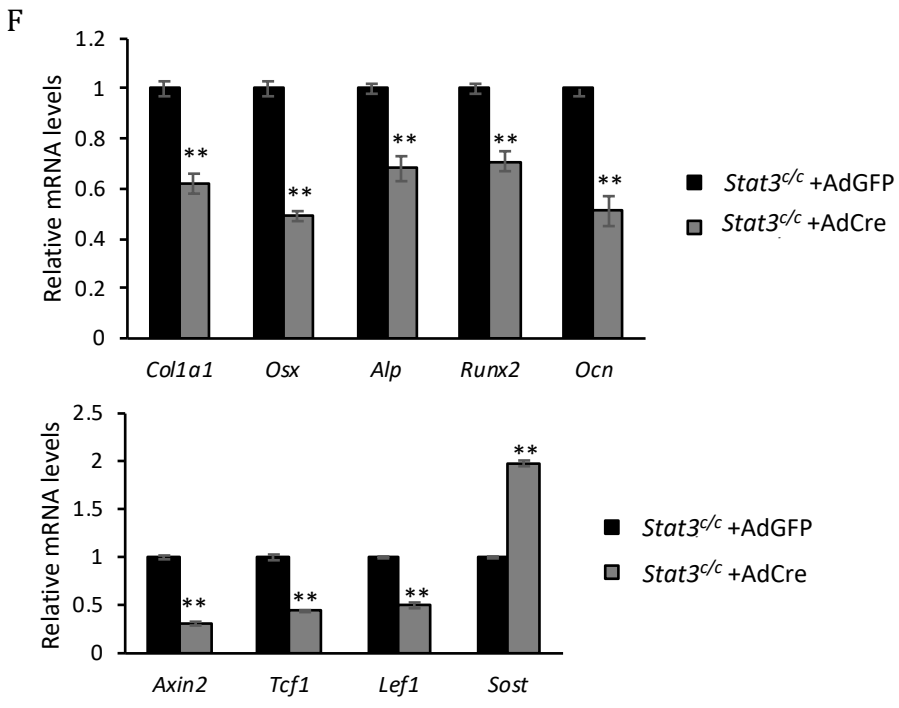






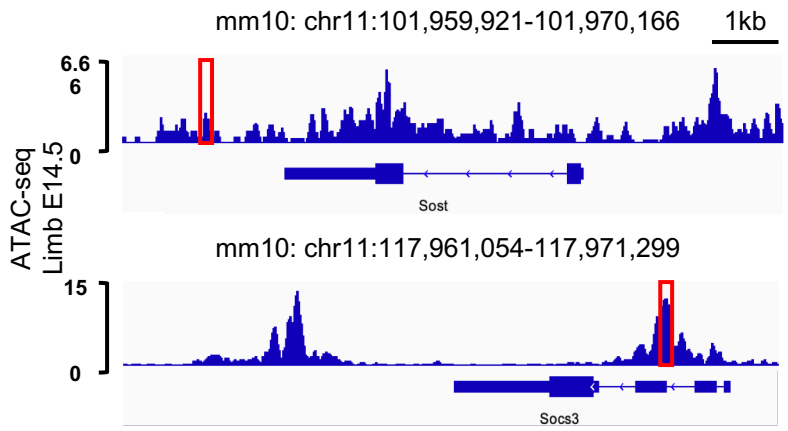






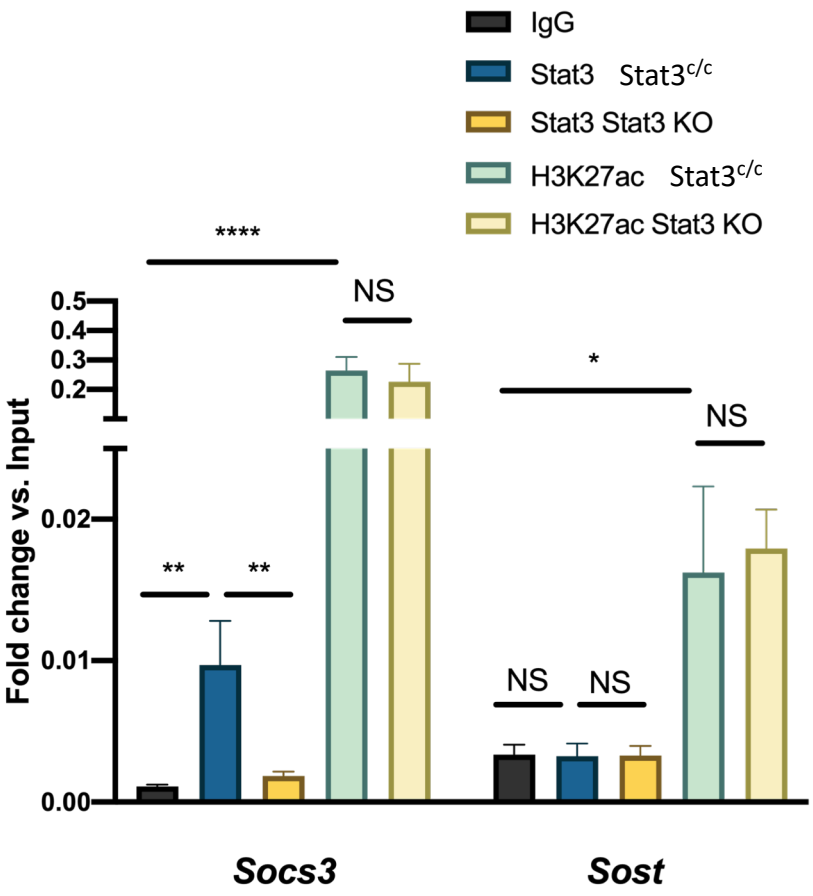
Supplemental Figure 7. Stat3 may not regulate Sost expression directly

A



B

C



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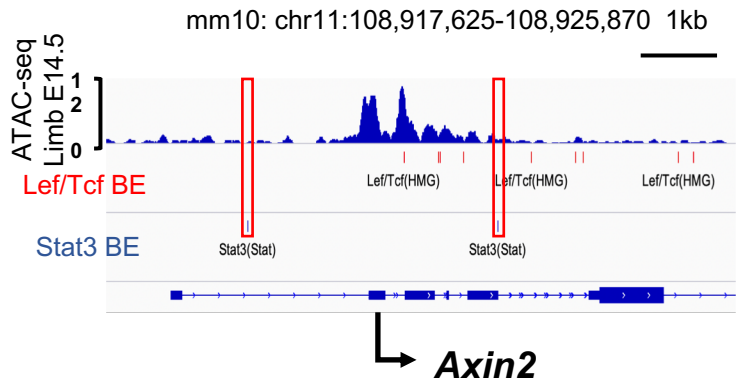
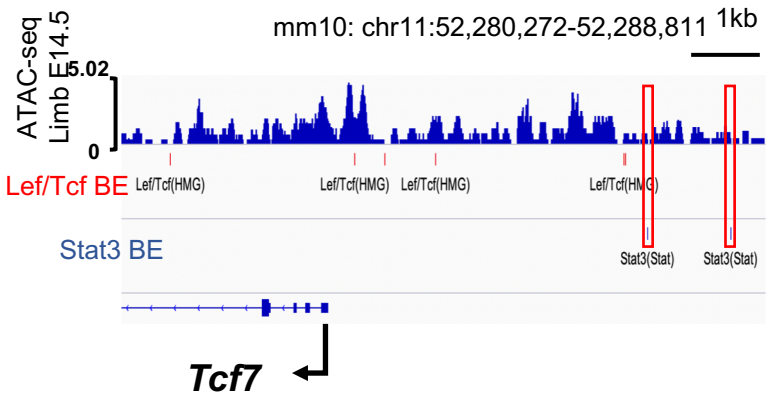
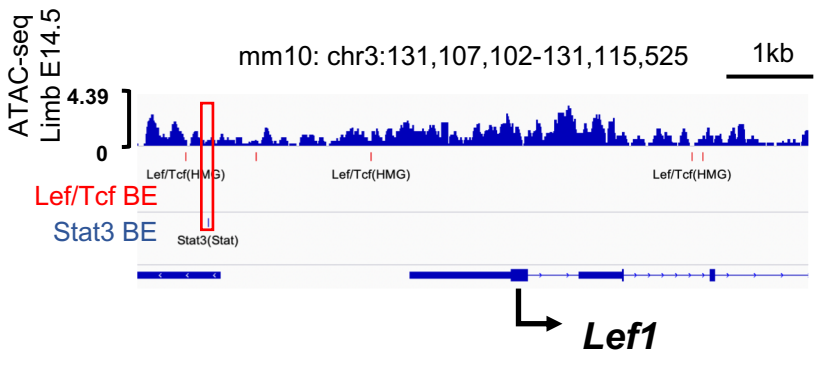
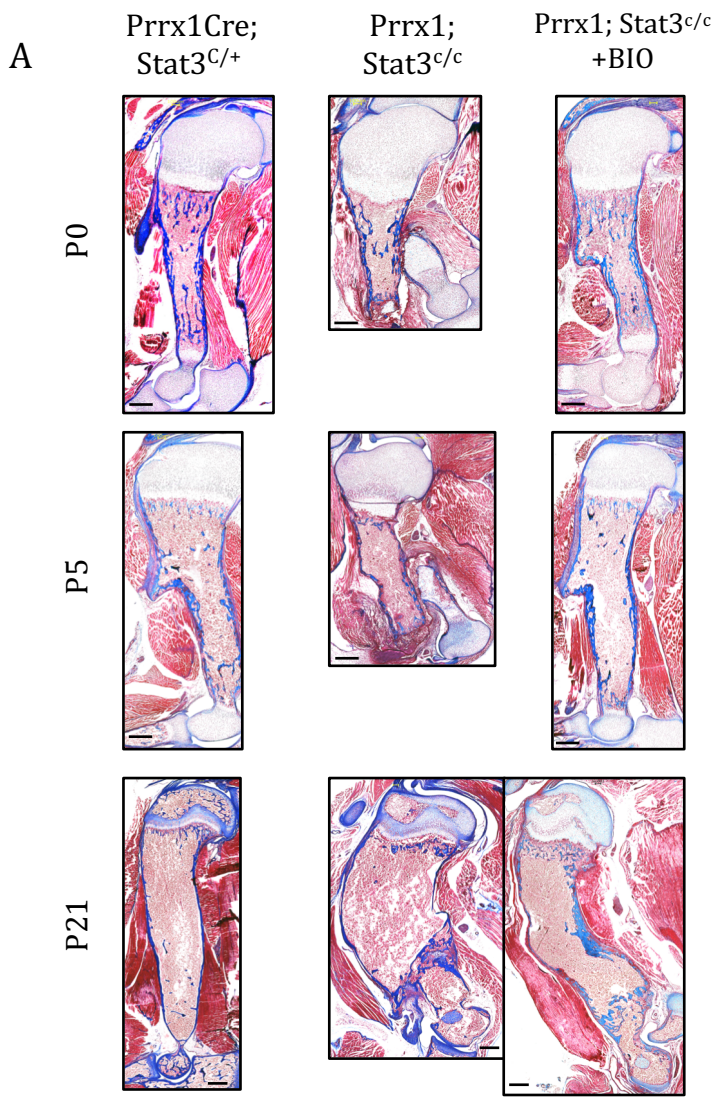
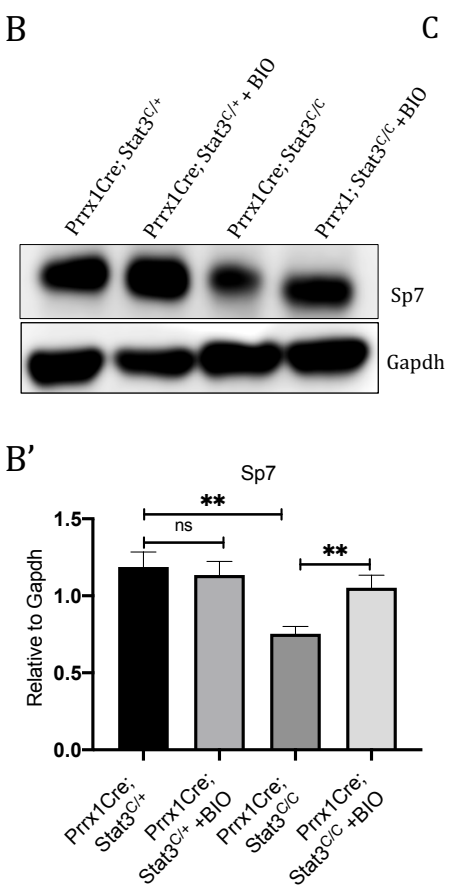


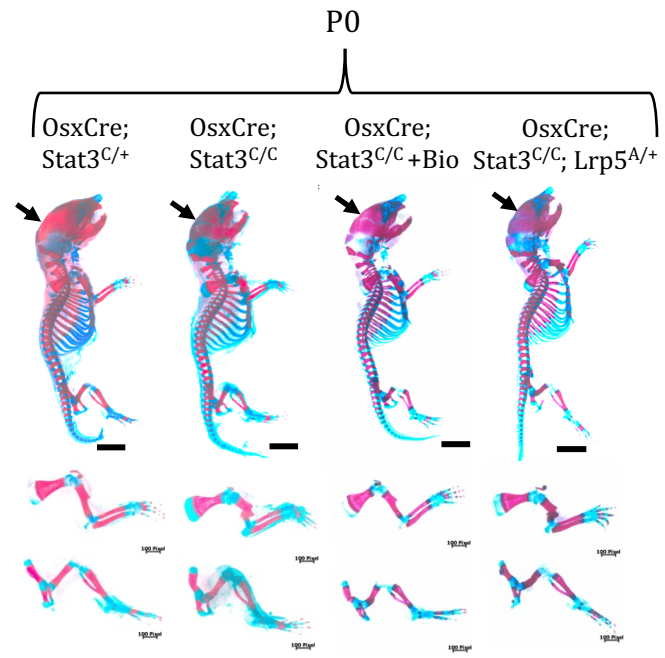


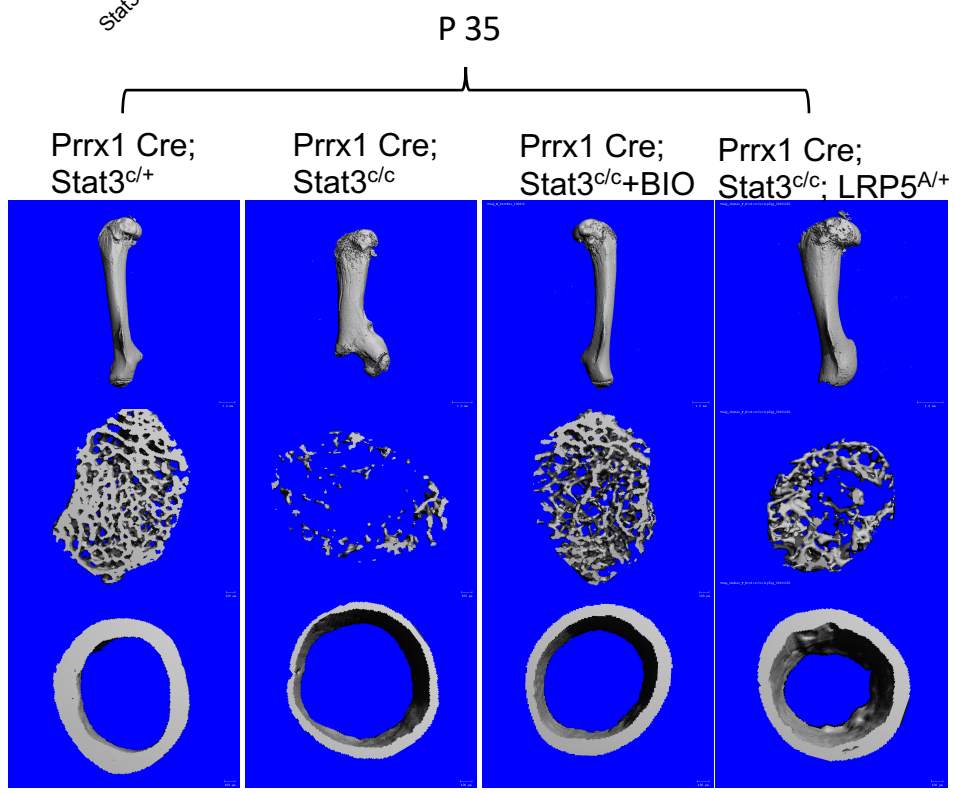


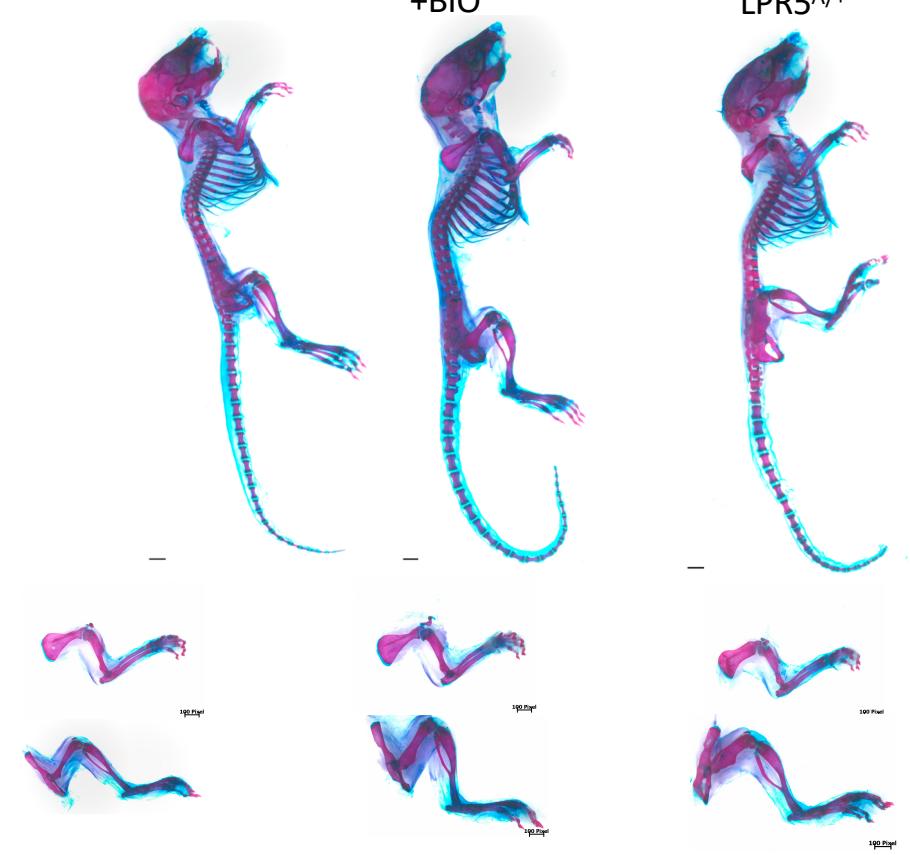
Supplemental Figure 8. Pharmacological or genetic activation of Wnt/β-catenin signaling partially rescued the bone phenotypes of OsxCre; Stat3$^{c/c}$ mice.

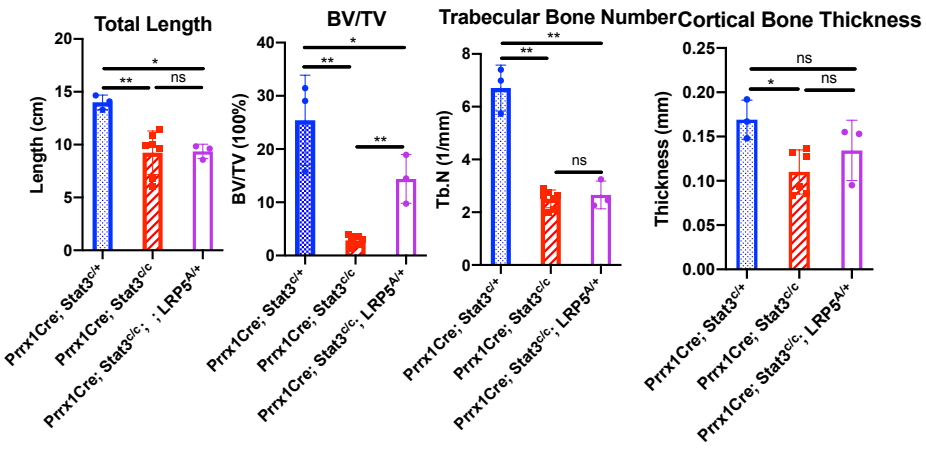












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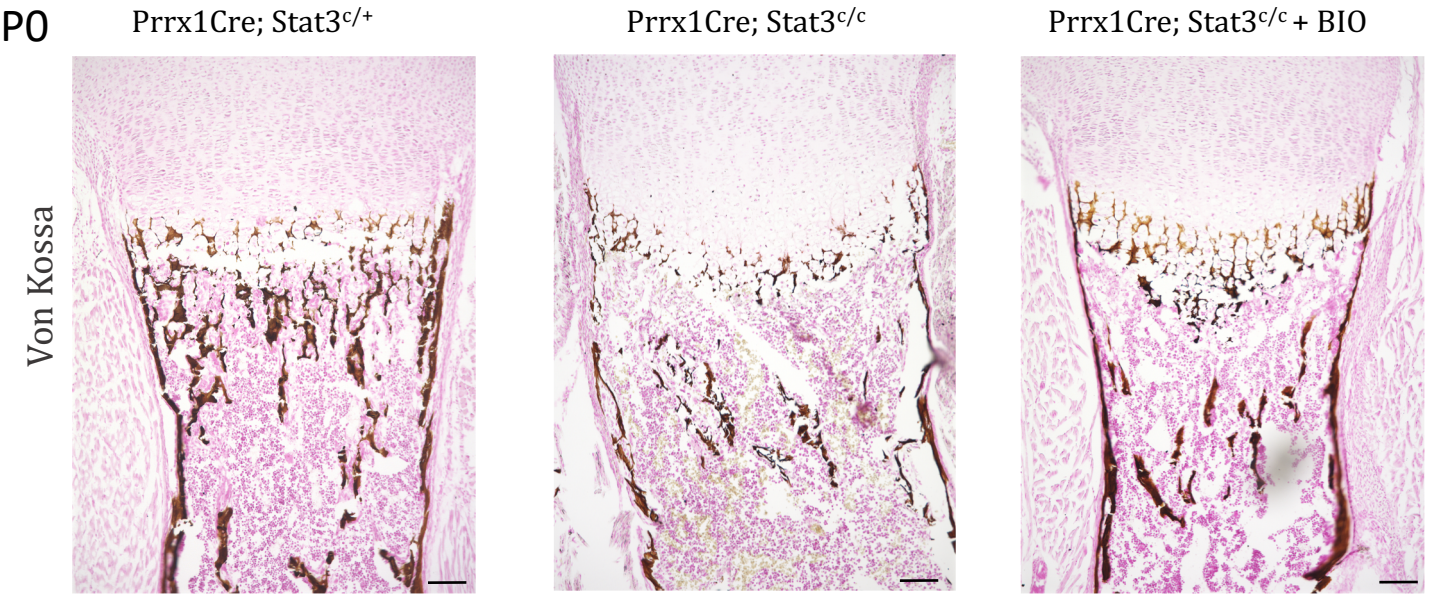
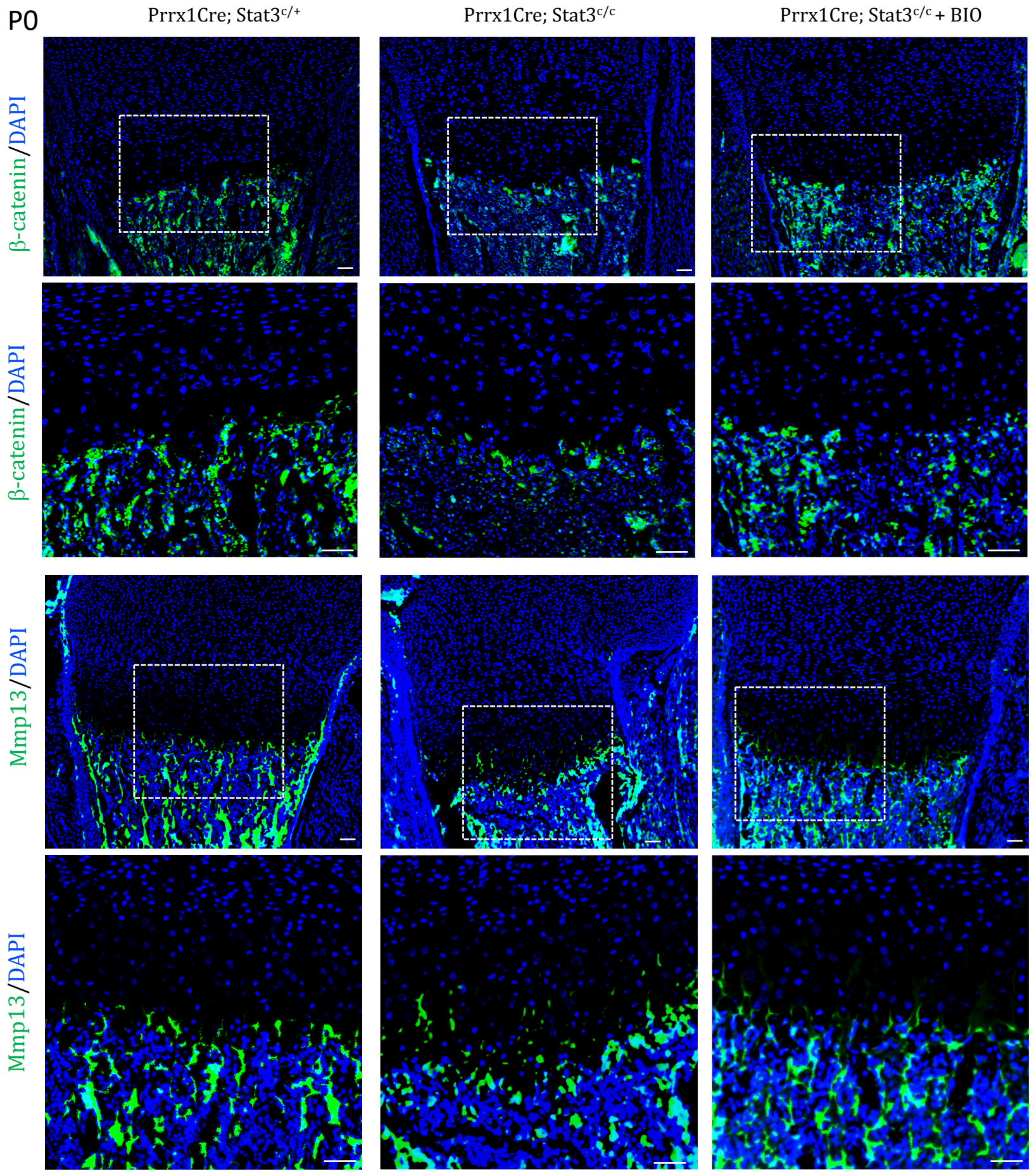

P0
Prrx1Cre; Stat3c/+
Prrx1Cre; Stat3c/c
Prrx1Cre; Stat3c/c + BIO
Von Kossa

G

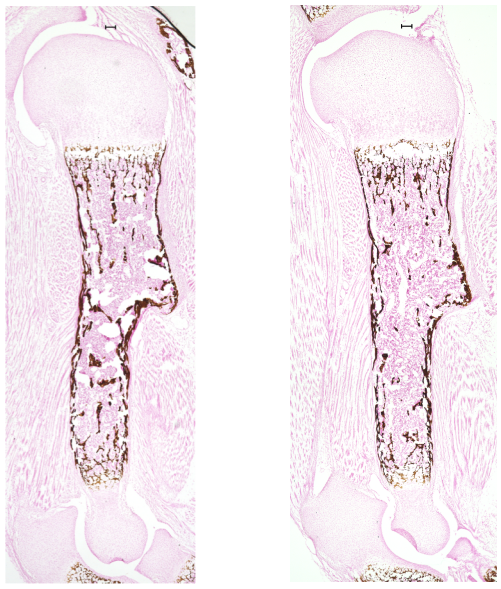

P0
Prrx1Cre; Stat3c/+
Prrx1Cre; Stat3c/c
Prrx1Cre; Stat3c/c + BIO
β-catenin/DAPI
Mmp13/DAPI
Mmp13/DAPI

Supplemental Figure 9: BIO administration to the control mice does not have any obvious effect.

A

Prrx1Cre; Stat3$^{C/+}$ Prrx1Cre; Stat3$^{C/+}$ +BIO

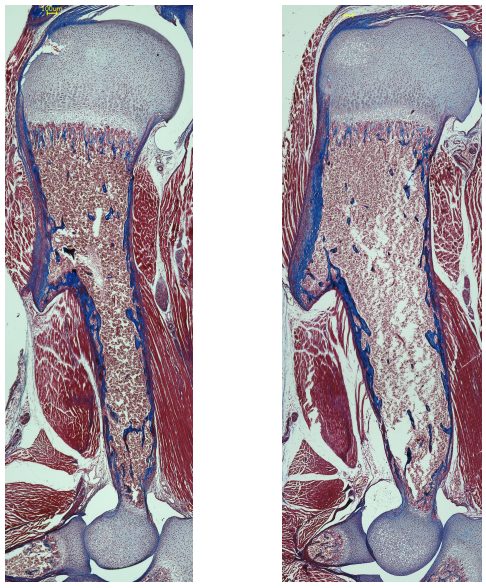
P0



B

Prrx1Cre; Stat3$^{C/+}$ Prrx1Cre; Stat3$^{C/+}$ +BIO

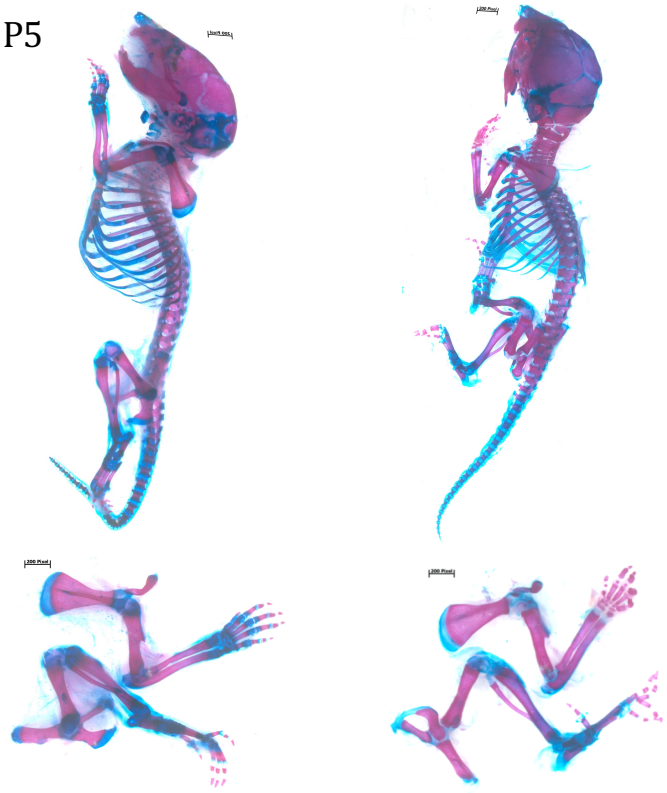
P5



C

Prrx1Cre; Stat3$^{C/+}$ Prrx1Cre; Stat3$^{C/+}$ +BIO

P5



Supplemental Table 1

	Antibodies	Sources	Cat. No.	Dilutions	Applications
1	Anti-Stat3	CST	#4904	1:500	WB
2	Anti-pStat3	ABclonal	RK04099	1:200	IF
3	Anti-Sp7	Abcam	ab22552	1:600 1:2000	IF WB
4	Anti-Opn	R&D System	AF808	1:200	IF
5	Anti-Col10a1	ABclonal	A6889	1:200	IF
6	Anti-BrdU	BioLegend	#317902	1:100	IF
7	Anti-β-catenin	BD Biosciences	#610154	1:500 1:2000	IF WB
8	Anti-β-catenin	CST	#8814	1:100	IP
9	Anti-Col1a1	ABclonal	A16891	1:500	WB
10	Anti-GAPDH	CST	# 5174	1:3000	WB
11	Anti-DIG-AP	Roche	11093274910	1:3000	ISH
12	Anti-Mmp13	ABclonal	A1606	1:500	WB
13	Anti-b-actin	CST	#3700	1:3000	WB
14	Anti-Sost	Abcam	ab63097	1:100 1:500	IF WB
15	Anti-Stat3	ABclonal	A11216	1:500	WB
16	Anti-Stat3	CST	#9139	1:150	IP
17	Anti-Cl-Caspase3	CST	#9664	1:300	IF
18	ECL Donkey anti-rabbit	GE Healthcare Life Science	NA9340-1m	1:5000	WB
19	ECL Sheep anti-mouse	GE Healthcare Life Science	NA9310-1ml	1:5000	WB
20	Alexa Fluor 488 donkey anti-mouse	Life Technologies	A-21202	1:500	IF
21	Alexa Fluor 488 donkey anti-rabbit	Life Technologies	A-21206	1:500	IF
22	Alexa Fluor 568 donkey anti-mouse	Life Technologies	A-10037	1:500	IF
23	Alexa Fluor 568 donkey anti-rabbit	Life Technologies	A-10042,	1:500	IF

Supplemental Table 2

S.No.	Gene name (*Mus musculus*)	Direction	Sequence
1	*RankL*	Forward primer	5′-CACAGCGCTTCTCAGGAGCTC-3’
		Reverse primer	5’-GAGATCTTGGCCCAGCCTCGA-3’
2	*Opg*	Forward primer	5’-AGTCCGTGAAGCAGGAGTGCA-3’
		Reverse primer	5’-AAGTCTCACCTGAGAAGAACC-3’
3	*Alpl*	Forward primer	5′-CTTGACTGTGGTTACTGCTGAT-3′
		Reverse primer	5′-GGAATGTAGTTCTGCTCATGGA-3′
4	*Sp7/Osx*	Forward primer	5′-CCCACTGGCTCCTCGGTTCTCTCC-3′
		Reverse primer	5′-GCTBGAAAGGTCAGCGTATGGCTTC-3′
5	*Col1a1*	Forward primer	5′-CACCCTCAAGAGCCTGAGTC-3′
		Reverse primer	5′-GTTCGGGCTGATGTACCAGT-3′
6	*Runx2*	Forward primer	5’-AACCCACGGCCCTCCCTGAACTCT-3’
		Reverse primer	5’-ACTGGCGGGGTGTAGGTAAAGGTG-3’
7	*Lef1*	Forward primer	5′-CTTCGCCGAGATCAGTCATCC-3′
		Reverse primer	5′-ACGGGTCGCTGTTCATATTGG-3′
8	*Tcf1*	Forward primer	5′-ACATGAAGGAGATGAGAGCCA-3′
		Reverse primer	5′-CTTCTTCTTTCCGTAGTTATC-3′
9	*Axin2*	Forward primer	5′- GAAGCTGGAGTTGGAGAGCCGCC-3′
		Reverse primer	5′- TCTCTCTTCATCCTCTCGGATCTGC-3′
10	*Ocn*	Forward primer	5′- ACCCTGGCTGCGCTCTGTCTCT-3′
		Reverse primer	5′-GATGCGTTTGTAGGCGGTCTTCA-3′
11	*β-Actin*	Forward primer	5’-TGAGCGCGGCTACAGCTT-3’
		Reverse primer	5’-TCCTTAATGTCACGCACGATTT-3’
12	*Gapdh*	Forward primer	5′-AGGTCGGTGTGAACGGATTTG-3′
		Reverse primer	5′- TGTAGACCATGTAGTTGAGGTCA-3′
13	*Socs-CHIP*	Forward primer	5’-CGGAGAGACAGCGGTCGTAA-3’
		Reverse primer	5′- GGGCAGTTCCAGGAATCGGG-3′
14	*Sost-CHIP*	Forward primer	5’-CCATGGCCTCCTGCTCTAAG-3’
		Reverse primer	5’-CTAAACCCAGCCCTCACTGG-3’