Runt-Related Transcription Factor 1 is required for murine osteoblast differentiation and bone formation

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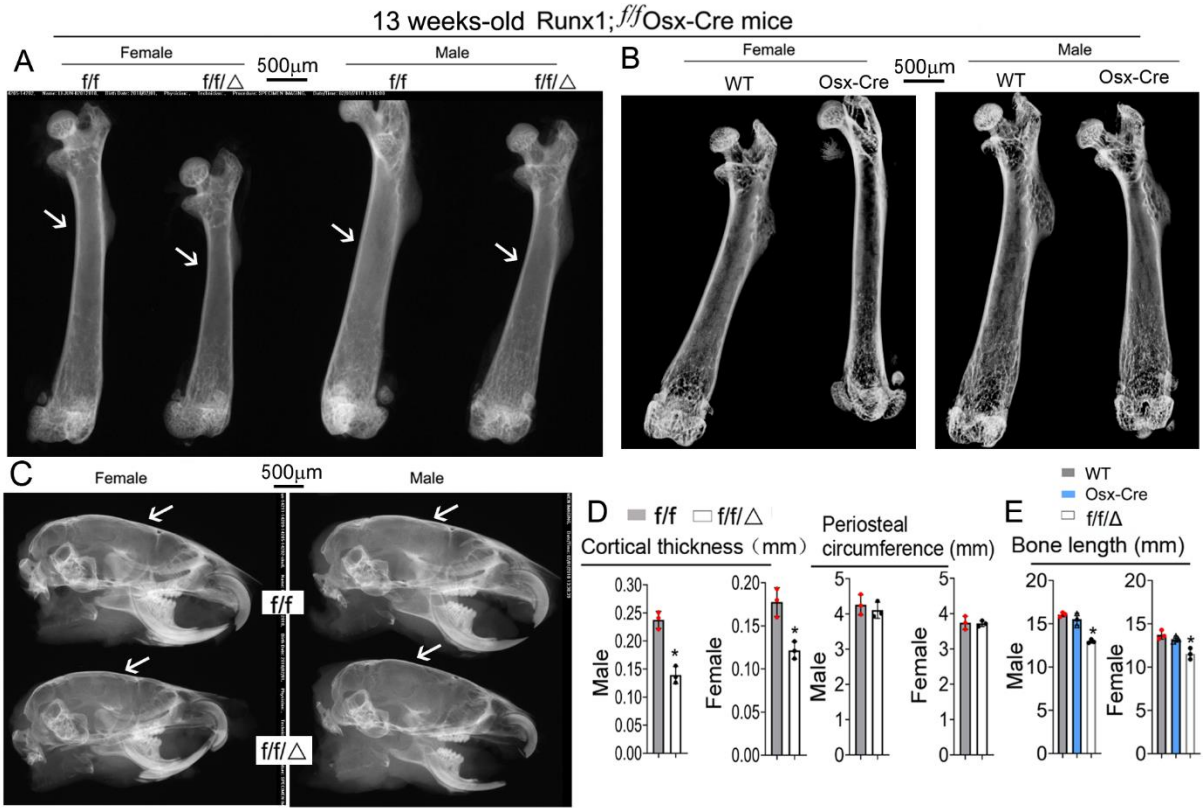
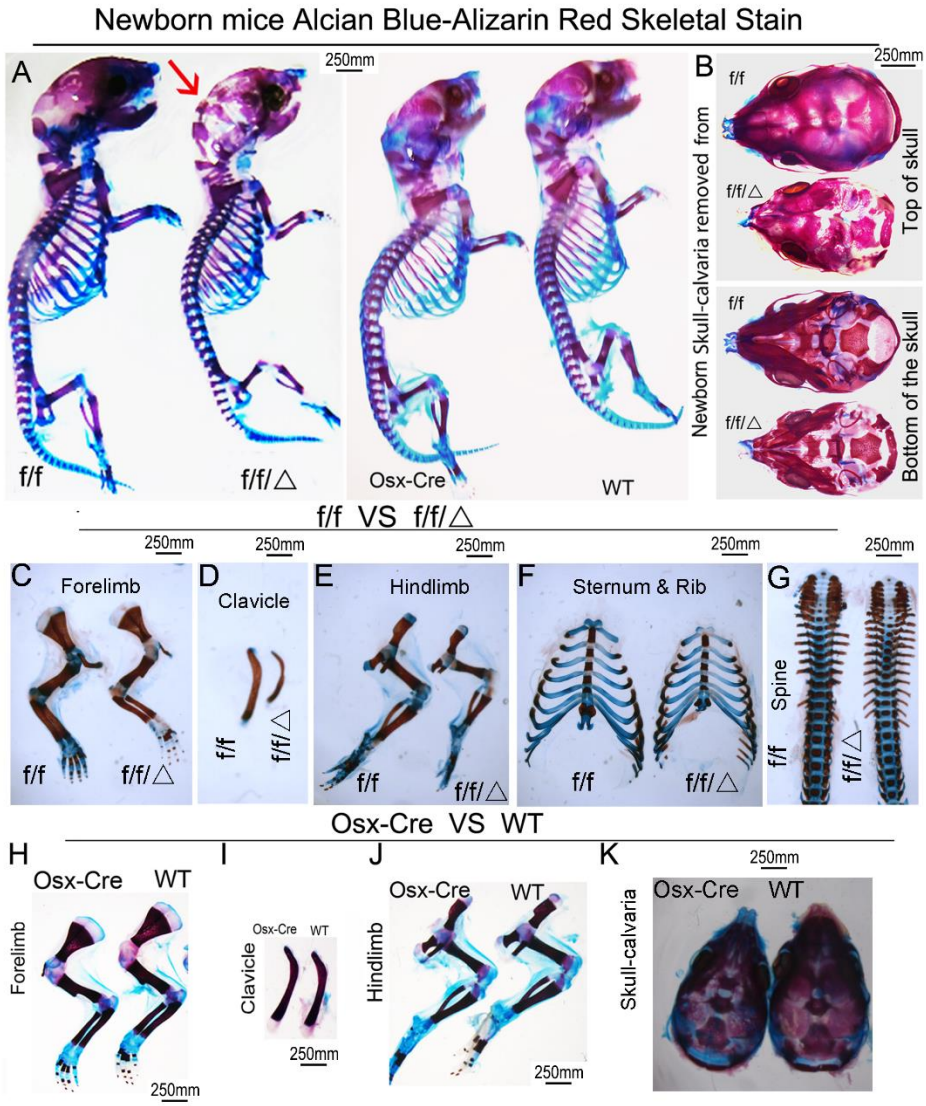
Supporting Materials included:

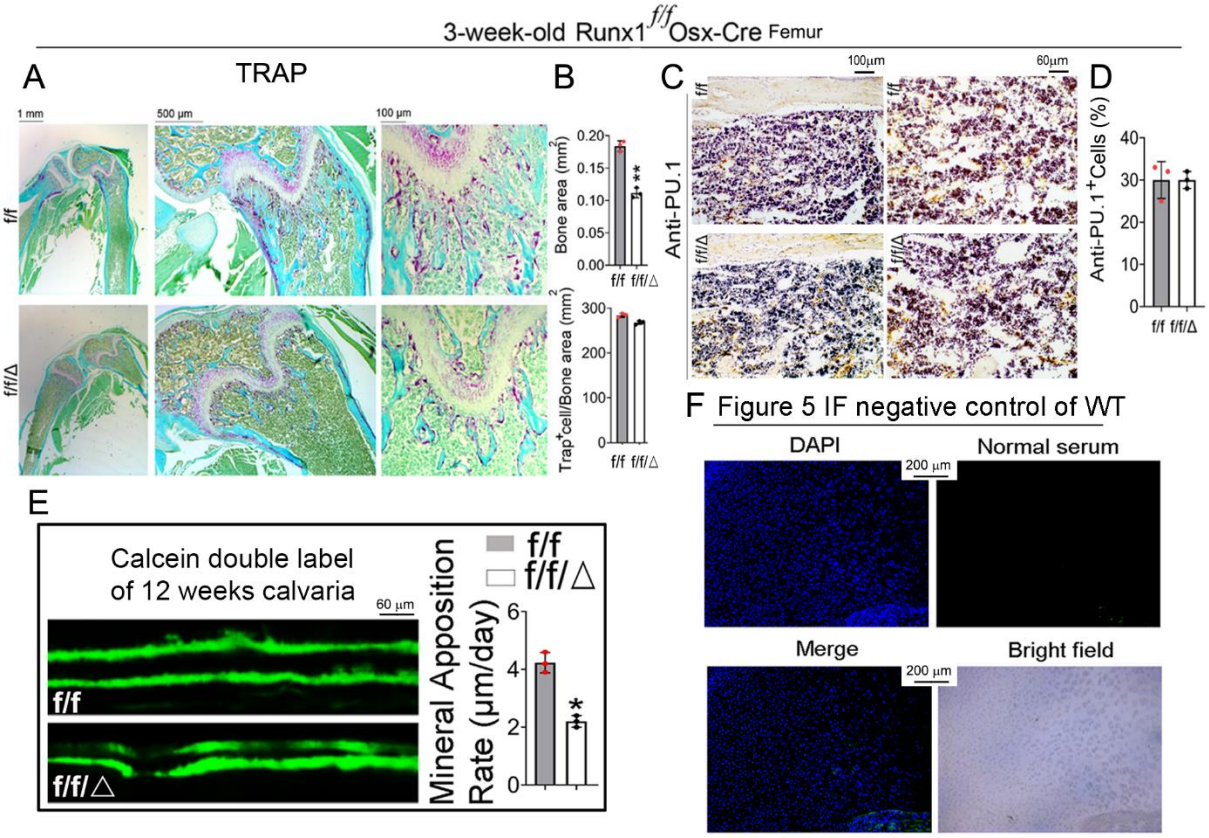
Supplemental Figures and Figure Legends



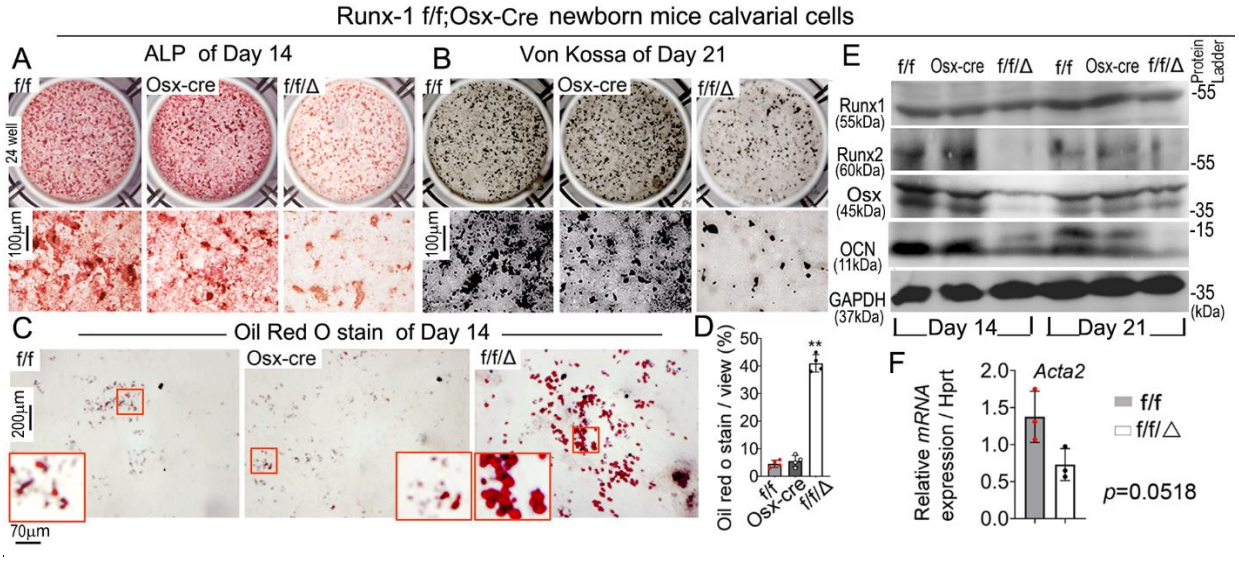
Supplemental Figure 1. *Runx1$^{f/f}$Osx-Cre* mice but not Osx-cre mice have decreased bone mineralization at 13 weeks old. (A) X-ray analysis of 13-week-old female and male Runx1$^{f/f}$*Osx-Cre* (f/f/Δ), and wild-type (f/f) femurs. (B) X-ray analysis of 13-week-old female and male wild-type (f/f) and *Osx-Cre* femurs. (C) X-ray analysis of 13-week-old female and male Runx1$^{f/f}$ *Osx-Cre* (f/f/Δ), and wild-type (f/f) skulls. (D) The cortical thickness and periosteal circumference of female and male Runx1$^{f/f}$*Osx-Cre* (f/f/Δ), and wild-type (f/f) femurs, and the femur length of the female and male Runx1$^{f/f}$*Osx-Cre* (f/f/Δ), *Osx-Cre* and wild-type (f/f). White arrows indicate reduced ossification. Results are presented as mean ± SD with n=6 in each group. *p<0.05.



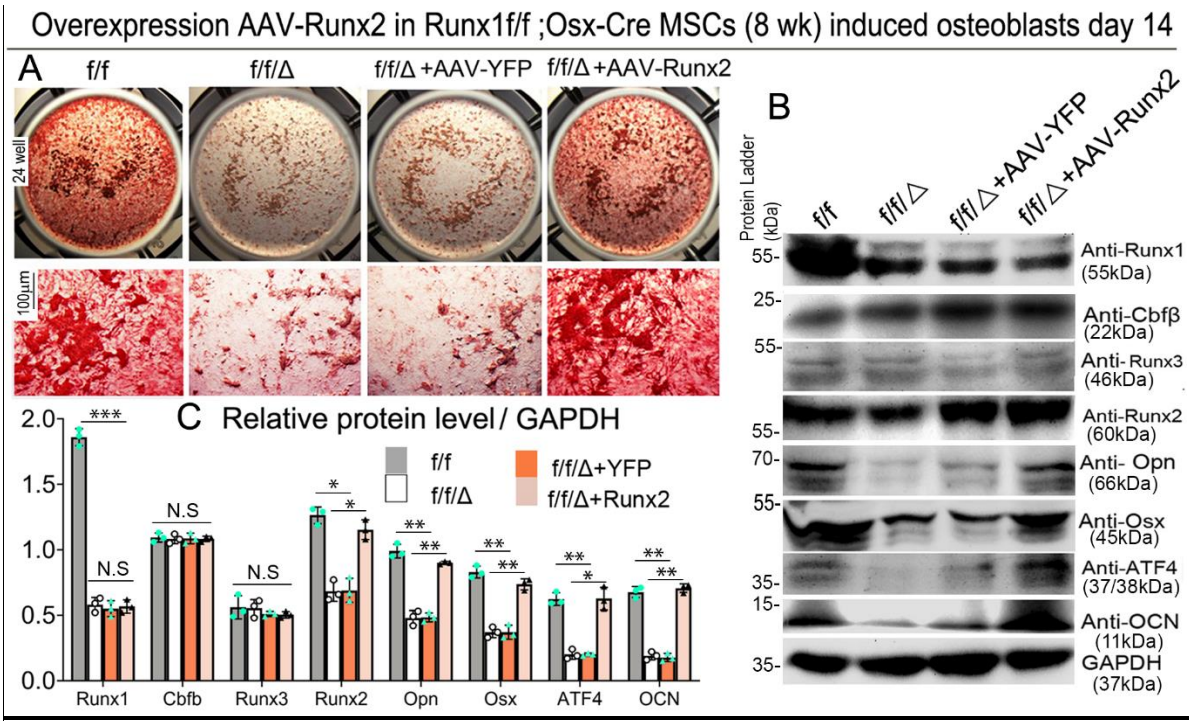
Supplemental Figure 2. *Runx1* depletion in osteoblasts results in decreased ossification. (A) Whole-mount Alizarin red and Alcian blue staining of P0 *Runx1*$^{f/f}$*Osx-Cre* (f/f/Δ), wild-type (f/f), and Osx-cre mice skeletons. (B) Whole-mount Alizarin red and Alcian blue staining of P0 *Runx1*$^{f/f}$*Osx-Cre* (f/f/Δ) and wild-type (f/f) mice cranium (top) and cranial base (bottom). n=3. (C-G) Whole-mount Alizarin red and Alcian blue staining of P0 *Runx1*$^{f/f}$*Osx-Cre* (f/f/Δ) and wild-type (f/f) mice (C) forelimbs, (D) clavicles, (E) hindlimbs, (F) sternum and ribs, and (G) vertebrae. Whole-mount Alizarin red and Alcian blue staining of P0 *Osx-cre* and WT mice (H) forelimbs, (I) clavicles, (J) hindlimbs, and (K) skulls. n=10.



Supplemental Figure 3. Osteoclast formation is not affected in 3-week-old *Runx1$^{f/f}$Osx-Cre* femurs. (A) 3-week-old *Runx1$^{f/f}$Osx-Cre* (f/f/Δ) and wild-type (f/f) mice femurs were stained with TRAP stain to detect osteoclast formation. (B) Quantification of A. (C) IHC staining PU.1 antibody of femur paraffin sections from 3-week-old Runx1f/fOsx-Cre (f/f/Δ), and wild-type (f/f) mice. (D) Quantification of C. (E) Calcein double label of 3-month-old mice and Mineral apposition rate. (F) Immunofluorescence (IF) stain of Anti-Sox9 negative control from Figure 5A. Results are expressed as mean ± SD. n=3 in each group. N.S, not significant; ***p*<0.01.



Supplemental Figure 4. Runx1-deficiency in primary calvarial cells cultured from *Runx1$^{f/f}$Osx-Cre* mice inhibits osteoblastogenesis and promotes adipogenesis. (A-C) Calvarial cells from *Runx1$^{f/f}$Osx-Cre* (f/f/Δ), *Osx-Cre*, and wild-type (f/f) newborn mice were cultured in osteogenic medium for 14 days and 21 days, followed by ALP, Von Kossa stain, or Oil red O stain. (D) Quantification of C. (E) Protein levels of Runx1, Runx2, Osx, and Ocn were analyzed by Western blot analysis on day 7 and day 14. GAPDH is shown as a control. (F) qPCR analysis of mRNA expression levels of *Acta2* in calvaria-derived osteoblasts from *Runx1$^{f/f}$Osx-Cre* (f/f/Δ) and wild-type (f/f) mice. $^{**}p<0.01$.



Supplemental Figure 5. Overexpression of Runx2 rescues osteoblast differentiation in Runx1-deficient cells. (A) Runx1 f/f MSCs, Runx1f/f Osx-Cre MSCs, and Runx1f/f Osx-Cre MSCs overexpressing Runx2 mediated by AAV were cultured in osteogenic medium for 14 d, followed by ALP staining to detect osteoblast formation. (B) Protein levels of Runx1, Runx2, Runx3, Cbfβ, Osx, Atf4, Opn and Ocn were analyzed by Western blot analysis on day 14. GAPDH is shown as a control. (C) Quantification of B. n=3 in each group.

Supplementary materials and methods

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Generation of Runx1$^{f/f}$Osx-Cre mice.

All animal experimentation was carried out according to the legal requirements of the Association for Assessment and Accreditation of the Laboratory Animal Care International and the University of Alabama at Birmingham Institutional Animal Care and Use Committee. Jackson Laboratory, strain name B6.129P2-Runx1tm1Tani/J, JAX no. 008772 were crossed with skeletal tissue cell (including osteoblast precursors, osteoblasts, chondrocytes, and odontoblasts)-specific *Osx-cre* mice (1) (*Tg(Sp7-tTA,tetO-EGFP/cre)1Amc* - Mouse Genome Informatics) Their progeny were crossed with *Runx1$^{f/f}$* mice to obtain *Runx1$^{f/f}$Osx-Cre* mice. In our study, we only use one copy of *Osx-Cre* (*Runx1$^{f/f}$ Osx-Cre/+*) in the CKO mutation. Given one functional allele, heterozygous *Osx -Cre* had no detectable bone phenotype, and served as control groups along with *Runx1$^{f/f}$* mice. Mice were bred in-house and euthanatized by CO_2 asphyxiation. All mice were maintained under a 12 h light–dark cycle with ad libitum access to regular food and water at the UAB Animal Facility. Both male and female mice of each strain were randomly selected into group of five animals each. The investigators were not blinded during allocation, animal handling, and endpoint measurements. Genotyping by PCR was carried out as described (2).

Radiographic Procedures.

For X-ray analysis, radiography was performed using the Faxitron Model MX-20 at 26 kV by the University of Alabama at Birmingham (UAB) Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease. The microcomputed tomography analysis was

performed to determine the bone mass of fixed femurs by the UAB Small Animal Bone Phenotyping Core associated with the Center for Metabolic Bone Disease.

uCT Analysis

Excised mouse humerus and femurs were scanned using the Scanco CT40 desktop cone-beam micro–CT (mCT) scanner (Scanco Medical AG, Bruttisellen, Switzerland). The trabecular bone scanning was performed from the growth plate (310 slices at 12mm per slice) analyzed using the CT Evaluation Program (v5.0A; Scanco Medical). The scanning and analysis of the cortical bone were performed at the midshaft of the femur and consisted of 25 slices (12 mm per slice).

Skeletal Analysis.

For skeletal preparations, mice were skinned, eviscerated, fixed in 95% (vol/vol) ethanol, cleared in acetone, stained with Alizarin red and/or Alcian blue stains, and sequentially cleared in 1% KOH. Cartilage and mineralized bone were characterized by different colors (blue and red, respectively) after the stain, according to standard protocols (3).

Tissue Preparation

Femurs and tibiae of mice were harvested, skinned, and fixed in 4% (wt/vol) paraformaldehyde overnight. Samples were then dehydrated in ethanol solution and decalcified in 10% (wt/vol) EDTA for 1–4 wk. For paraffin sections, samples were dehydrated in ethanol, cleared in xylene, embedded in paraffin, and sectioned at 6 μm with Leica microtome and mounted on Superfrost Plus slides (Fisher). For frozen sections, samples were infiltrated in 30% (wt/vol) sucrose, embedded in optimal cutting temperature compound, sectioned at 8 μm with a freezing microtome,

and affixed to Superfrost Plus Gold slides (Fisher). Histological analysis was performed including staining with Alcian blue, safranin O, ALP and hematoxylin/eosin (H&E) stains using paraffin sections.

TRAP Staining.

Paraffin sections were stained using the Acid Phosphatase, Leukocyte [tartrate-resistant acid phosphatase (TRAP)] Kit (387A-1KT, Sigma) following the manufacturer's instructions, counterstained with hematoxylin, dehydrated, and mounted. Data are included as graphs of osteoclasts per millimeter of bone perimeter.

Von Kossa Staining.

Von Kossa staining was performed as follows. Cells were washed with Ca2+/Mg2+–free PBS and then fixed on slides in 10% (vol/vol) cold Neutral Formalin solution. We then added 2.5% (wt/vol) silver nitrate solution, and the slides were incubated under UV light for 5–10 min. After incubation, the unincorporated silver nitrate was removed by washing with 5% (wt/vol) sodium thiosulfate. Slides were then mounted. Stain surface per bone surface (BS) area and 5 sections measured using ImageJ software.

ALP staining.

ALP staining was performed as previously described method(4). Slides were deparaffinized and hydrated through a xylene and graded ethanol series, stained with , and counterstained with fast green solution. Slides were then mounted. Stain surface per bone surface (BS) area and 5 sections measured using ImageJ software.

Safranin O staining

Safranin O staining was performed as follows. Slides were deparaffinized and hydrated through a xylene and graded ethanol series, stained with Weigert's iron hematoxylin, rinsed in tap water, and counterstained with fast green solution. Slides were then stained in 0.1% Safranin O solution, dehydrated and mounted.

Proliferation Assay.

To detect proliferating cells in culture, immunochemistry staining was performed according to the manufacturer's instructions. Horseradish peroxidase-conjugated proliferating cell nuclear antigen (PCNA) antibodies (cat. no. 93–1143; Zymed Laboratories Inc.) and Vector DAB (3,3′-diaminobenzidine) kits (cat. no. SK-4100; Vector Laboratories) were used.

Immunohistochemistry.

For immunohistochemistry, samples were embedded in paraffin and sectioned as described previously. The Vector DAB substrate kit (cat. no. SK-4100; Vector Laboratories) was used along with secondary staining kits for mouse (on mouse) and rabbit (cat. no. BMK-2202 and PK6101, respectively; Vector Laboratories) and primary antibodies for Runx1 (sc-365644; Santa Cruz), col2(sc-52658; Santa Cruz), colx (ab58632; Abcam), Runx2 (ab23981; Abcam). For the

quantification of immunohistochemistry, we used NIH ImageJ to perform counts, and the percent of positive cells represents the number of positive cells expressed the targeted gene.

Immunofluorescence.

Samples were embedded in tissue freezing medium, and sections were cut at a thickness of 8 μm with a cryotome. Pictures were taken by Leica confocal microscopes (SP1) and a Zeiss fluorescent microscope (Zeiss Axio Imager). The following antibodies were used: Osterix (ab22552; Abcam), Ocn (ab10911, Abcam), Ihh (MABF23; citeab), PTC1(sc-6149; Santa Cruz), cyclinD1(sc-753; Santa Cruz), PPR(sc-12722; Santa Cruz), Sox9(sc-20095; Santa Cruz). For the quantification of immunofluorescence, we used NIH ImageJ to perform counts, and the percent of positive cells represents the number of positive cells expressed the targeted gene.

Serum P1NP assay.

14-week old mouse serum was collected after 6-hour fasting, and the serum P1NP activity was detected and quantified using the Human Pro-Collagen I alpha Duo Set ELISA (DY6220-05) according to the manufacturer's instructions.

In-vivo calcein labelling.

Calcein labelling was previously described (5). Briefly, 3-month-old mice were intraperitoneally injected with 20mg/kg of calcein in a 2% sodium bicarbonate solution, 8 days and 2 days before killing of mice. Calvarias were fixed in 4% PFA, soaked in 30% glucose in PB, embedded in OCT and frozen sectioned. Mineral apposition rate is the distance between the midpoints of the two labels divided by the time between the midpoints of the interval.

Quantitative Real-Time PCR Analysis.

mRNA was extracted from cultured cells on day14 using TRIzol (Invitrogen) and then reverse-transcribed into cDNA according to the manufacturer's manual (qScript cDNA Synthesis Kit, Quanta Biosciences Inc.). Expressions of osteoblastic marker genes were analyzed by quantitative real-time PCR (qRT-PCR) using the StepOne Real-Time PCR System (Life Technologies). Expression of Atf4, Col1α1, Spp1, Runx2, Sox9, OPG, RANKL, and OCN was analyzed. The primer sequences are available upon request.

Western Blot Analyses.

Protein samples extracted from calvaria derived osteoblasts were prepared in protein lysis buffer, resolved on SDS/PAGE, and electrotransferred onto nitrocellulose membranes. Immunoblotting was performed according to the manufacturer's instructions. Osteoblast- regulators and marker genes including Cbfβ, Runx2, Osterix, Atf4, and Ocn were detected using primary antibodies as follows: rabbit anti-Cbfβ (1:1,000; ab72696, Abcam), rabbit anti-Runx2 (1:2,000; ab23981, Abcam), rabbit anti-Sp7/Osterix (1:1,000; ab22552, Abcam), mouse anti-ATF4 (1:1,000; ab50546, Abcam),rabbit anti-Ocn (1:1,000; ab10911, Abcam); sc-20095, Santa Cruz Biotechnology).Horseradish peroxidase-linked anti-rabbit IgG and horseradish peroxidase-linked anti-mouse IgG were purchased from Cell Signaling (nos. 7074 and 7076).

Data availability

The RNA-Seq data are available upon request. Contact: Yi-Ping Li, Department of Pathology, University of Alabama at Birmingham, E-mail: yipingli@uabmc.edu. All other data are contained within the manuscript.

Supplemental Tables

Table S1. Primers used for qPCR

Gene	Forward primer	Reverse primer
RUNX1	GATGGCACTCTGGTCACCG	GCCGCTCGGAAAAGGACAA
RUNX2	AGAGACCACAATAACCAGCACG	GGCGGCCATATGACTACAAAG
OSX	ATGGCGTCCTCTCTGCTTG	TGAAAGGTCAGCGTATGGCTT
ATF4	ATGGCGCTCTTCACGAAATC	ACTGGTCGAAGGGGTCATCAA
COL1A	CTTGGTGGTTTTGTATTCGATGAC	GCGAAGGCAACAGTCGCT
ALP	AGTTACTGGCGACAGCAAGC	GAGTGGTGTTGCATCGCG
OCN	GAACAGACAAGTCCCACACAG	GAGCTGCTGTGACATCCATAC
RANKL	CAGCATCGCTCTGTTCCTGTA	CTGCGTTTTCATGGAGTCTCA
OPG	TGTCCAGATGGGTTCTTCTCA	CGTTGTCATGTGTTGCATTTCC
HPRT	GGTGGAGATGATCTCTCAACTTTAA	AGGAAAGCAAAGTCTGCATTGTT

Table S2. Primers used for ChIP assay

Gene	Forward primer	Reverse primer
OCN 1	TTGTGCCTCACAACTACCCG	TACACCAGAAGAGGGCGTCA
OCN 2	AGTGCTTGGTCTTTGCTCCA	GCTCTTACCTGCTGAGCCAT
OCN 3	CTTGGGAGTCAGGATGTGTTGAG	ATTCTGCAGTTGTTCCCCAAGT
OCN 4	CAGAGCTGCCCTGAACTGG	CAGGGAGGGAGTGGTCAGTA
OCN 5	CTGAGCACATGACCCCCAAT	ATTGGGGGTCATGTGCTCAG
RUNX2 1	ATGTTCTCTCTGGGCATCCAATC	TGGCAGTGGTCTTTCTAAGTGT
RUNX2 2	GGGTACGTGGACAATGAATGC	TAGTAGAGGTTGCTGAACGTGG
RUNX2 3	TCCTCTGCATGAATAATGACCCTAA	GAGGCTAGACTCATGTTTTACTGT
RUNX2 4	AGTAACCATGGGATGATGGCA	ACGTGGCGGCTCTTACAATA
RUNX2 5	CTAGCCAAATCCTCATGAGTCACAA	GTAAGGCCTTCCTGGCATT

Table S3. Primers used for subcloning

Gene	Primer
RUNX2 F1	CGGGGTACCAGATCACACTGGCACACTTTA
RUNX2 F2	CGGGGTACCCCCTCTTACCTCCACTGTGC
RUNX2 F3	CGGGGTACCTTACAGTTTCTGTTAACCCCCTC
RUNX2 F4	CGGGGTACCAAGCTTGTGATACAATCCCAAGATGCGA
RUNX2 F5	CGGGGTACCAAGCTTGTCAGAGGGAGAAAGGGAGAGAG
RUNX2 R	CCGCTCGAGGCACTATTACTGGAGAGACAGAA

References

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