Supporting Information

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SI Materials and Methods

Immunohistochemical Analysis and in Situ Hybridization. Bones fixed in 4% paraformaldehyde in PBS (pH 7.4) were decalcified and treated for CryoJane frozen sections as described by Jiang et al. (1). We used the same decalcification procedure for the paraffin sections as for the frozen sections. Both paraffin and frozen sections were used for immunohistochemical analysis. Sections were treated with hyaluronidase [2 mg/mL in PBS (pH 5.0)] at 37 °C for 1 h and incubated with anti-Col X (mouse monoclonal anti-human recombinant Col X generously provided by Klaus von der Mark, Nikolaus Fiebiger Centre of Molecular Medicine, University Erlangen-Nuernberg), anti-eGFP (A11122; Invitrogen), anti-Opg (sc-8468; Santa Cruz), or anti-CD45 (BAM114; R&D Systems). Sections for anti-Runx2 (sc-10758; Santa Cruz) were heated in 10 mM sodium citrate (pH 6.0) at 95 °C for 5 min. Sections for anti-Aggrecan antibody (MAB19310; Chemicon) were treated with chondroitinase ABC. Sections for anti-Col IV (ab19808; Abcam) were treated with 0.1% trypsin.

X-ray, μCT, and Histomorphometry Analyses. X-ray images of mice skeletons were generated by Faxitron. Femurs were fixed in 4% paraformaldehyde overnight and kept in 70% ethanol for μCT scanning (eXplore GE Locus SP; GE Healthcare). We then analyzed the μCT images using MicroView 2.2 software (GE Healthcare). Histomorphometry results were analyzed with the OsteoMeasure histomorphometry system (OsteoMetrics).

RNA Isolation and Quantitative PCR Analyses. RNA was isolated from the bones using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After purification, the total RNA was treated with Turbo DNase (Ambion) and reverse transcribed into first-strand cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). We used 100 ng cDNA per PCR. The TaqMan primer-probe combinations were products of Applied Biosystems.

EMSA and Transfection. Procedures for electrophoretic mobility shift assay (EMSA) and transfections were as described previously (2, 3). The 2-Kb *Sost* promoter was PCR amplified from RP24-131M12 (BACPAC Resources) and cloned into p^{GL3}-Basic vector.

In Vitro Osteoclastogenesis. Bone marrow cells of 2-mo-old mice were flushed out of humeri and femurs with α-MEM and treated with red blood cell lysis buffer (8.3 g/L NH_4Cl, 1 g/L sodium bicarbonate, and 0.4 g/L EDTA). The remaining cells were cultured in complete α-MEM media containing 5% L292 conditional media until confluent to isolate the monocytes. The monocytes were then replated in complete α-MEM media containing 5% L292 conditional media and 50 ng/mL RANKL to induce osteoclastogenesis.

In Vitro Osteoblastogenesis. Primary osteoblasts were isolated from P24 mice. In brief, calvariae were minced, washed by PBS, and subjected to five sequential digestions (for 15, 10, 10, 10, and 10 min, respectively) in α-MEM containing 2 mg/mL Dispase II (Roche) and 1 mg/mL Collagenase P (Roche) at 37 °C. The cells from the second to fifth digestions were combined and cultured in α-MEM supplemented with 10% FBS for 3–5 d. The cells were reseeded in 12-well plates at a density of 5×10^4/well. Differentiation was initiated upon confluence by the addition of 0.1 mg/mL ascorbic acid (Sigma) and 5 mM β-glycerol phosphate (Sigma). Bone marrow stromal cells (BMSC) were isolated from the bone marrow of 2-mo-old mice. To isolate the BMSC, the bone marrow cells were cultured in α-MEM supplemented with 15% FBS for 7 d (half of the media was changed on day 4). The BMSC were seeded in 12-well plates at a density of 1.5×10^5/well. Once confluent, osteogenesis was initiated by the addition of osteogenic media (5 mM β-glycerol phosphate, 0.1 mg/mL ascorbic acid, and 10 nM dexamethasone). The osteogenic media was changed every 2–3 d.

EM Analysis of Osteocytes. For resin-casted SEM, long bones were dissected and fixed in 4% paraformaldehyde for 24 h. The tissue specimens were dehydrated in ascending concentrations of ethanol (from 70% to 100%), embedded in methyl methacrylate, and then surface polished using 1 μm and 0.3 μm Alpha Micropolish Alumina II (Buehler) in a soft-cloth rotating wheel. The bone surface was acid etched with 37% phosphoric acid for 2–10 s, followed by 5% sodium hypochlorite for 5 min. The samples were then coated with gold and palladium as described previously (4) and examined using an FEI/Philips XL30 Field-Emission Environmental Scanning Electron Microscope.

For back-scattered SEM (BSEM), the long bones were fixed overnight in 2% paraformaldehyde and 2% glutaraldehyde buffered at pH 7.4 with 0.1 M sodium cacodylate. Samples were then rinsed three times (20 min each time) in 0.1 M cacodylate buffer solution followed by secondary fixation (1 h) in a solution of 1% osmium tetroxide in 0.1 M cacodylate buffer. The BSEM samples were then coated with carbon and examined using an FEI/Philips XL30 Field-Emission Environmental Scanning Electron Microscope.

For transmission electron microscopy (TEM), the small bone samples (1–3 mm^3) were fixed in the same way as the BSEM samples. The small bone samples were then dehydrated with an ethanol series and processed for embedding in eEpon resin (Poly/Bed 812; Polysciences Inc.) for 2 h. Complete polymerization was performed in a 60 °C oven for 48 h. Thin sections (100 nm) were then cut using a diamond knife and mounted on copper grids (300 mesh). The sections were stained with uranyl acetate and lead citrate and examined using a Philips CM12 Scanning Transmission Electron Microscope.

Chromatin Immunoprecipitation (ChIP) Assay. Approximately 1.0×10^7 BMP2-treated MC3T3 cells were incubated with 1% formaldehyde in serum-free media for 20 min followed by 125 mM glycine. Cells were washed with cold PBS, resuspended in 10 volumes of cell lysis buffer [25 mM Hepes (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.1% Nonidet P-40 1 mM DTT, 0.5 mM PMSF and protease inhibitor mixture] and homogenized to release the nuclei, which were then lysed with nuclear lysis buffer [50 mM Hepes (pH 7.9), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS, 0.5 mM PMSF, protease inhibitor mixture]. Nuclear extracts were sonicated and cleared by centrifugation. Sonicated chromatin was diluted in lysis buffer without SDS to bring the final concentration of SDS to 0.05%. The antibodies were first conjugated to magnetic-coated protein G beads (magnetic Dynabeads Protein G; Invitrogen), which were previously blocked with 0.5% BSA in PBS. After overnight immunoprecipitation with rabbit anti-Osx and anti-IgG antibodies at 4 °C, the immunoprecipitated complexes were collected using a magnetic stand. Washing, reversal of cross-links, and purification of DNA from immunocomplexes were done using EZ ChIP Kit (Millipore) as per instructions. Quantitative PCR reactions were performed with 500 nM primers, 5 μL 2× SYBR Green PCR Master Mix (Applied Biosystems), 2 μL

DNA in a total 10 μL reaction volume. Results were computed as percent antibody bound per input. Primer sequences are available upon request.

Statistical Analyses. We computed statistical significance with independent-samples *t* test using SPSS 12.0. Data are presented as means ± SD. $P < 0.05$ was considered statistically significant.

1. Jiang X, et al. (2005) Histological analysis of GFP expression in murine bone. *J Histochem Cytochem* 53:593–602.
2. Nakashima K, et al. (2002) The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 108:17–29.
3. Zhang C, et al. (2008) Inhibition of Wnt signaling by the osteoblast-specific transcription factor Osterix. *Proc Natl Acad Sci USA* 105:6936–6941.
4. Feng JQ, et al. (2006) Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* 38:1310–1315.
5. Starnes LM, Downey CM, Boyd SK, Jirik FR (2007) Increased bone mass in male and female mice following tamoxifen administration. *Genesis* 45:229–235.

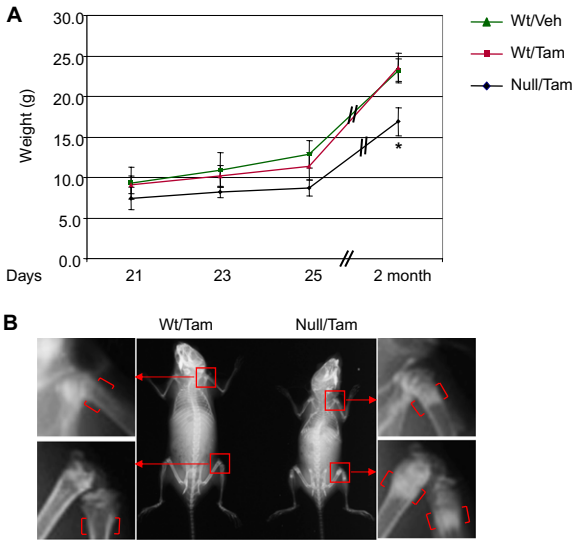

Fig. S1. (*A*) Growth curves of 2-mo-old wild-type controls and *Osx*^{postnatal} mutant mice injected with tamoxifen three times from postnatal day 21 to day 25 (n = 3/genotype group, gender matched, *$P < 0.001$). (*B*) Radiography of 2-mo-old wild-type controls and *Osx*^{postnatal} mutant mice. Red brackets indicate the locations where dense mineralized tissue was found in the mutant but not in the control. For Wt/Veh, Wt/Tam, and Null/Tam, see Fig. 1 legend.

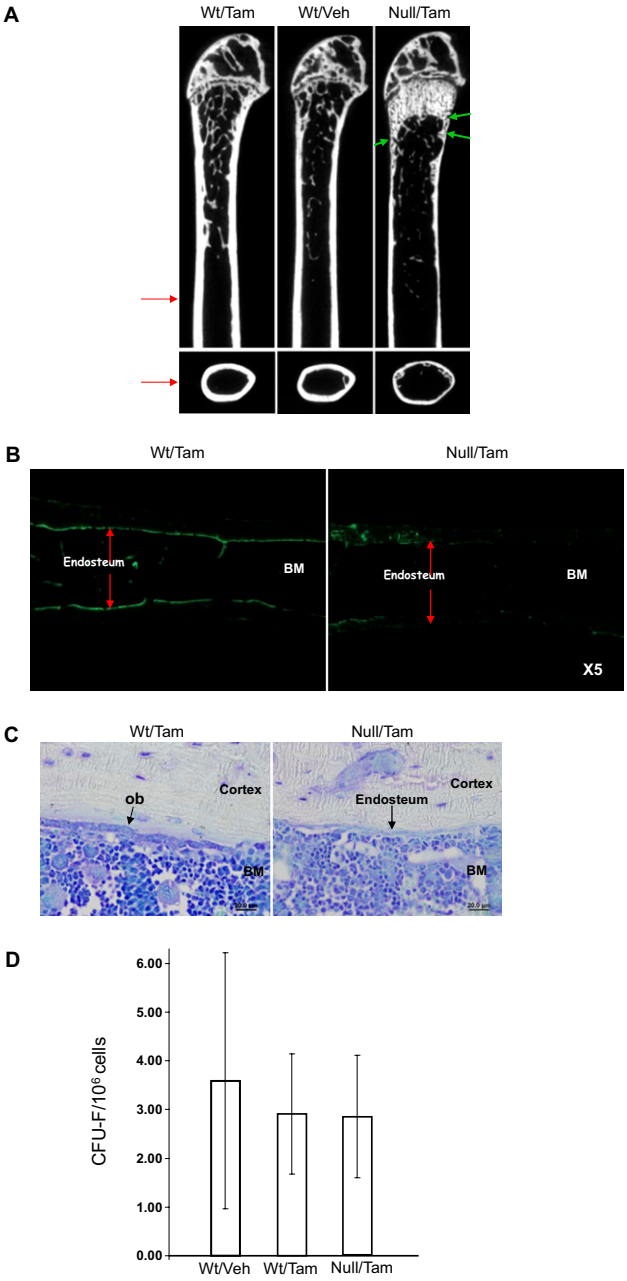

Fig. S2. Inactivation of *Osx* after growth period led to altered bone structure and abolished bone formation. (*A*) The μCT images showed that the cortex of 7-mo-old male $Osx^{postnatal}$ mutant was much thinner than that of both vehicle- and tamoxifen-treated wild-type controls, although the bone mass of tamoxifen-treated wild-type control was slightly higher than the vehicle-treated wild-type control due to tamoxifen injections (5). The red arrows indicate the location of optical cross-sections in the middle of femurs. In addition, right under the growth plate of $Osx^{postnatal}$ mutant femur there was an area of dense mineralized tissue, which was similar but proportionally smaller compared with the $Osx^{postnatal}$ mutants, in which *Osx* was deleted at P20. This finding suggested that longitudinal growth of long bones in mice continues, though at a much slower rate after 4 mo. The green arrows indicate microfractures. (*B*) Calcein incorporation was greatly diminished in the 7-mo-old $Osx^{postnatal}$ mutant. Tamoxifen injections started at 4 mo of age for a total of seven times (1.5 mg/10 g body weight), and mice were killed at 7 mo. For Wt/Veh, Wt/Tam, and Null/Tam, see Fig. 1 legend. (*C*) Toluidine blue staining showed that most of the cells lining the endosteum surface of the mutant femur were flat, unlike the mature osteoblasts (black arrow) found on the endosteum surface of the wild-type control. The plastic sections prepared from 6-wk-old tamoxifen-treated control and $Osx^{postnatal}$ mutant were used for the staining. (*D*) The procedure of CFU-F assay was described in the protocol from STEMCELL Technologies (no. 28374). Bone marrows were isolated from 2-mo-old control and $Osx^{postnatal}$ mutant mice. See *Materials and Methods* for tamoxifen treatment procedure. Three mice of each genotype were used in this experiment.

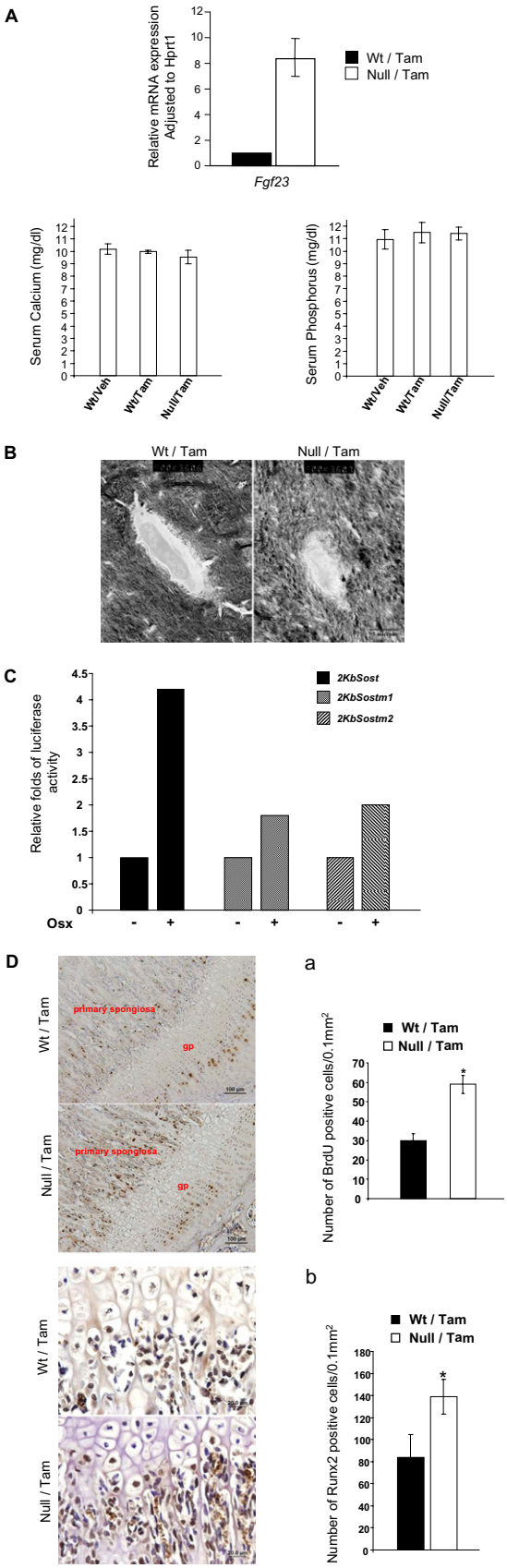

Fig. S3. Osx is required for osteocyte maturation and functions. (*A Upper*) Quantitative PCR analysis of *Fgf23* expression. RNA was extracted from the calvariae of tamoxifen-treated 6-wk-old mice. (*Lower*) Measurements of serum phosphorus and calcium levels. Blood serum was collected from 2-mo-old wild-type control and *Osx*^{postnatal} mutant mice. See *Materials and Methods* for tamoxifen treatment procedure. n = 5 females/genotype. (*B*) TEM images of os-

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terocytes. The samples were prepared from the humeri of tamoxifen-treated 2-mo-old mice. (*C*) 293T cells were cotransfected with β-galactosides expression plasmid (2), *Osx* expression construct (2), or control vector and 2 Kb wild-type or mutated *Sost* promoter-reporters. The sequences in the mutated 2-Kb *Sost* promoters (*2KbSostm1* and *2KbSostm2*) are the same as in the corresponding EMSA oligos m1 and m2. Luciferase values were normalized by β-galactosides values. Oligis for EMSA: *Sost*: 5′-ggg tca cct ggg agg tgc cag cag caa ttt gg-3′ (−216 to −186); m1: 5′-ggg tca act ggg aga tgc cag cag caa ttt gg-3′; m2: 5′-ggg tca cct ggg agg taa aag cag caa ttt gg-3′. (*D*) (*a*) BrdU staining showed increased proliferation of cells in the primary spongiosa of $Osx^{postnatal}$ mutant mice (n = 3/genotype group, gender matched; $*P < 0.001$). gp, growth plate. (*b*) IHC showed that there were more Runx2-positive cells in the primary spongiosa of the mutant femur than in the wild-type control. The decalcified tibia paraffin sections of tamoxifen-treated 6-wk-old mice were used for BrdU staining, and the frozen sections were used for anti-Runx2 IHC.

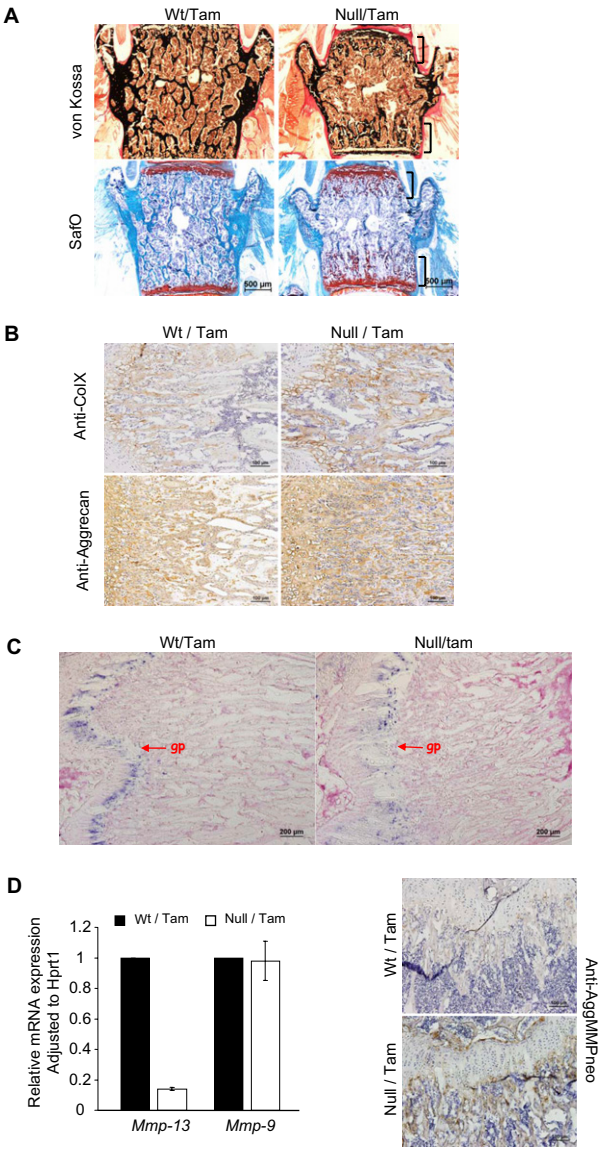

Fig. S4. Inactivation of *Osx* led to massive accumulation of calcified cartilage templates. (*A*) von Kossa. (*B*) Safranin O (SafO) staining on the plastic sections of lumbar vertebrae of tamoxifen-treated 2-mo-old mice. Brackets indicate the area where cartilage matrix accumulates in the lumbar vertebrae of $Osx^{postnatal}$ mutants. In the area between the two brackets, there were less trabeculae compared with the wild-type controls. (*B*) IHC analyses were performed with Collagen X and Aggrecan antibodies on the decalcified paraffin sections of humeri of tamoxifen-treated 6-wk-old mice. (*C*) In situ hybridization of *Col10a1* RNA. Decalcified frozen sections were prepared from the humeri of tamoxifen-treated 6-wk-old mice. gp, growth plate. (*D Left*) Quantitative PCR analysis of *Mmp13* and *Mmp9* expression. RNA was isolated from the femurs of tamoxifen-treated 6-wk-old mice. (*Right*) IHC analysis with anti-Aggrecan MMPneo antibody was performed on the decalcified paraffin sections of tibias from tamoxifen-treated 2-mo-old mice.

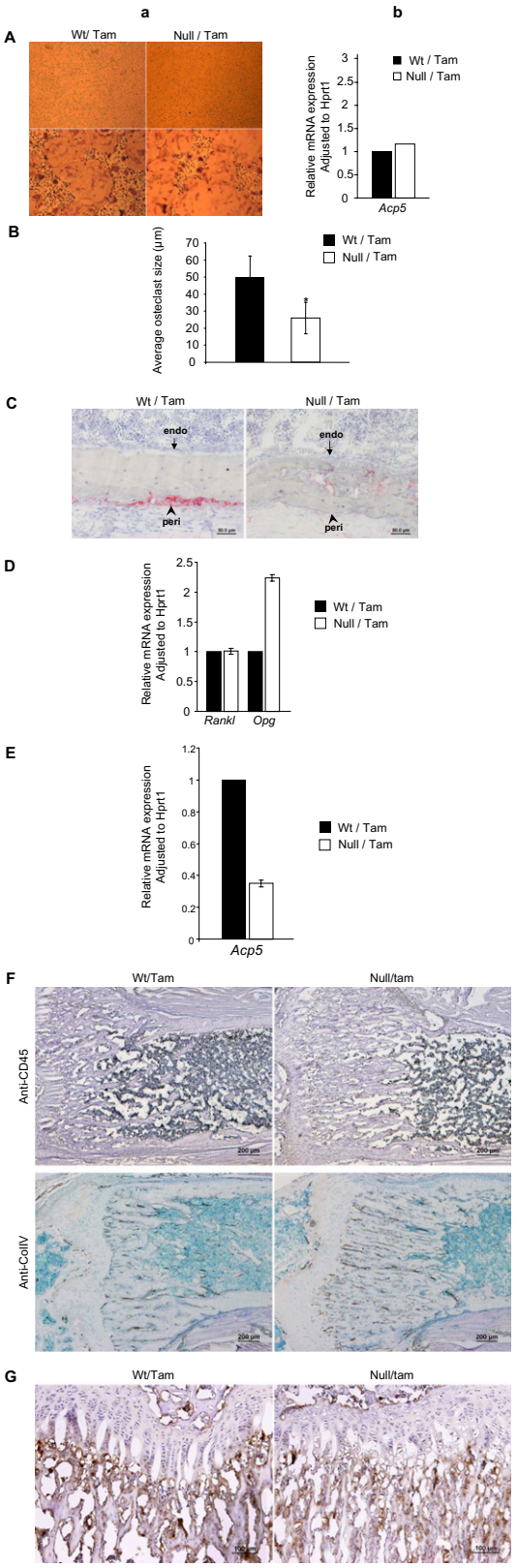

Fig. S5. Absence of bone marrow cells and reduced density of osteoclasts in the region of ectopic mineralized cartilage. (*A*) In vitro osteoclastogenesis assay. Monocytes were isolated from tamoxifen-treated 2-mo-old mice to induce osteoclastogenesis in vitro. (*a*) TRAP assay. (*Upper*) A similar number of monocytes of Wt/Tam and Null/Tam were seeded in media containing 5% L292. (*Lower*) Cells in upper panels further treated with RANKL to form multinucleated osteoclasts. (*b*) quantitative PCR measurement of *Acp5* RNA from cells cultured in media containing 5% L292 and RANKL for 5 d. (*B*) Comparison of the size of osteoclasts of 6-wk-old tamoxifen-treated wild-type and $Osx^{postnatal}$ mutant mice. The average size of osteoclasts found in the $Osx^{postnatal}$ mutant was reduced about 50% compared with the ones in the tamoxifen-treated control mice. $*P < 0.05$, $n = 3$/genotype group. (*C*) TRAP-positive osteoclasts were found mostly lining the periosteum of the wild-type cortex, whereas in the $Osx^{postnatal}$ mutant many more TRAP-positive osteoclasts were found within the cortex. (*D*)

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Quantitative PCR analysis. RNA was isolated from the humeri of tamoxifen-treated 6-wk-old wild-type and $Osx^{postnatal}$ mutant mice. (*E*) Quantitative PCR analysis. RNA was isolated from the humeri of P24 tamoxifen-treated mice. (*F*) IHC of anti-CD45 and anti-type IV on the humerus sections of tamoxifen-treated 6-wk-old wild-type and $Osx^{postnatal}$ mutant mice. (*G*) IHC of anti-OPG on the humerus sections of tamoxifen-treated 6-wk-old wild-type and $Osx^{postnatal}$ mutant mice.

Table S1. Histomorphometry analysis of bone and bone formation parameters in lumbar vertebrate of 6-wk-old vehicle and tamoxifen-treated controls and tamoxifen-treated $Osx^{postnatal}$ mutant mice

	BV/TV% ± SD	Tb.Th (mcm) ± SD	Tb.N (mm-1) ± SD	Tb.Sp (mcm) ± SD
Wt/Veh	18.55 ± 2.57	28.78 ± 0.19	6.44 ± 0.85	128.25 ± 20.89
Wt/Tam	23.79 ± 0.82*	30.07 ± 1.77	7.92 ± 0.37**	96.36 ± 4.43**
Null/Tam	16.85 ± 2.37	28.97 ± 1.75	5.80 ± 0.46	144.20 ± 15.00
	Ob.S/BS% ± SD	N. Ob/B. Pm (Ob/mm) ± SD	N. Ob/ T. Ar (Ob/mm2 ± SD)	BFR/BS (mcm/d) ± SD
Wt/Veh	25.80 ± 1.78**	17.05 ± 1.12**	219.17 ± 27.47**	0.56 ± 0.031**
Wt/Tam	43.98 ± 3.94**	26.85 ± 2.58**	424.71 ± 35.30**	0.44 ± 0.04**
Null/Tam	11.02 ± 2.49	7.48 ± 2.39	85.63 ± 23.73	0.07 ± 0.021
	OS/BS% ± SD	OS/T.Ar ± SD	MS/BS% ± SD	MAR (mcm/d) ± SD
Wt/Veh	7.29 ± 1.61*	0.96 ± 0.33*	24.01 ± 0.75**	2.34 ± 0.18**
Wt/Tam	22.68 ± 3.90**	3.58 ± 0.51**	20.28 ± 1.83**	2.19 ± 0.06**
Null/Tam	2.11 ± 1.18	0.24 ± 0.13	6.99 ± 1.89	1.02 ± 0.04

n = 3/genotype group.
*$P < 0.05$, **$P < 0.001$.

Table S2. Histomorphometry analysis of osteoclasts in cortex

	Oc. S. endo/endo S% ± SD	Oc. S. peri/peri S % ± SD	Oc. S. inner/inner % ± SD	Oc. S. total/total S % ± SD
Wt/Tam	3.46 ± 1.12**	22.22 ± 3.86*	2.70 ± 2.61*	11.0 ± 0.7
Null/Tam	15.03 ± 1.46	4.47 ± 1.97	10.28 ± 3.07	9.97 ± 1.30
	Oc. endo/endo (Oc/mm) ± SD	Oc. peri/peri (Oc/mm) ± SD	Oc. N inner/inner S (Oc/mm) ± SD	Total Oc. N/total BS (Oc/mm) ± SD
Wt/Tam	1.61 ± 0.43**	7.81 ± 1.88*	1.42 ± 1.31*	4.10 ± 0.67
Null/Tam	6.16 ± 0.55	1.91 ± 0.30	4.89 ± 1.22	4.38 ± 0.63

Endo, endosteum; peri, periosteum; inner, the area between endosteum and periosteum; total, the sum of peri, endo, and inner. *n* = 3/genotype group.
*$P < 0.05$, **$P < 0.001$.