

## **Matrix-embedded cells control osteoclast formation**

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## **SUPPLEMENTARY METHODS**

### **Generation of RANKL-flox mice**

To create the RANKL conditional, or floxed, allele, we generated a targeting construct in which a 1.2 kb genomic DNA fragment containing exons 3 and 4 was flanked by loxP sites (**Supplementary Fig. 1**). The 5' homology arm was 3.8 kb in length and the 3' homology arm was 4.9 kb in length. We used the targeting construct, which also contained a neomycin-resistance cassette flanked by Frt sites, to electroporate R1 ES cells, which are derived from 129/Sv hybrid embryos<sup>1</sup>. We identified appropriately targeted ES cell clones by Southern blot using probes flanking the 5' and 3' ends of the targeting construct and used them to generate chimeric mice. We removed the neomycin resistance cassette from the knock-in mice by breeding with mice expressing the FLPe recombinase in their germ cells (The Jackson Laboratory, stock number 003946). Subsequently, we crossed RANKL-flox mice with C57BL/6 mice for 1 to 2 generations before crossing them with mice expressing the Cre recombinase.

### **Cell type-specific deletion of the RANKL-flox allele**

The Cre transgenic mice used in this study have been described previously: Prx1-Cre<sup>2</sup>, Osx1-Cre<sup>3</sup>, Ocn-Cre<sup>4</sup>, Dmp1-Cre<sup>5</sup>, and collagen X (ColX)-Cre<sup>6</sup>. We obtained the experimental animals used in most of the studies described here using a two-step breeding strategy. We crossed hemizygous Cre transgenic mice with heterozygous RANKL-flox mice to generate heterozygous RANKL-flox offspring with and without a Cre allele. We then intercrossed these offspring to generate the following offspring: WT mice, mice hemizygous for a Cre allele, mice homozygous for the RANKL-flox allele, hereafter referred to as RANKLf/f, and RANKLf/f mice that were also hemizygous for a Cre allele. For studies requiring larger numbers of mice, we crossed RANKLf/f mice with RANKLf/f mice that were also hemizygous for a Cre allele. We genotyped offspring by PCR using the following primer sequences: Cre-for, 5'-GCGGTCTGGCAGTAAAACTATC-3', Cre-rev, 5'-GTGAAACAGCATTGCTGTCACCTT-3', product size 102 bp; RANKL-flox-for, 5'-CTGGGAGCGCAGGTTAAATA-3', RANKL-flox-rev, 5'-GCCAATAATTAATACTGCAGGAAA-3', product size 108 bp (WT) and 251 bp (floxed allele).

### **Genetic background of mice used in experiments**

We obtained Prx1-Cre;RANKLf/f mice and littermates by mating RANKL-flox mice (mixture of 129/Sv and C57BL/6) and Prx1-Cre mice (crossed into C57BL/6 for more than 10 generations).

We obtained Osx1-Cre;RANKLf/f mice and littermates by mating RANKL-flox mice (mixture of 129/Sv and C57BL/6) with Osx1-Cre mice (mixed background crossed into C57BL/6 for 5 generations).

We obtained Ocn-Cre;RANKLf/f mice and littermates by mating RANKL-flox mice (mixture of 129/Sv and C57BL/6) with Ocn-Cre mice (generated and maintained in FVB-N).

We obtained Dmp1-Cre;RANKLf/f mice and littermates by mating RANKL-flox mice (mixture of 129/Sv and C57BL/6) with Dmp1-Cre mice (crossed into C57BL/6 for more than 10 generations).

We obtained ColX-Cre;RANKLf/f mice and littermates by mating RANKL-flox mice (mixture of 129/Sv and C57BL/6) with ColX-Cre mice (mixed background crossed into C57BL/6 for 6 generations).

We also mated each of the above strains to R26R mice, which have been crossed into C57BL/6 for more than 10 generations.

### **X-rays**

To examine tooth eruption, we anesthetized mice by i.p. injection of Nembutal (80 mg/kg body weight), placed them on top of radiographic film, and then exposed them to X-rays for 13 seconds at 40 kV in an AXR 110 Minishot X-ray machine (Associated X-Ray Corporation).

### **Bone mineral density (BMD) determination**

We determined BMD using a PIXImus densitometer (GE-Lunar Corp.) running software version 2.0. We measured three sites. We defined the total body window as the whole body image minus the calvarium, mandible, and teeth. Except for the first few caudal vertebrae, we did not include the tail. The spine window was a rectangle depending on animal body length, reaching from just below the skull to the base of the tail. The femoral window captured the right femur. Scan acquisition time was four minutes and analysis time was six minutes. We sedated the mice with isoflurane during scanning to keep the animals motionless for the required four minutes and to facilitate rapid post examination recovery. We monitored the animals by observation of the righting reflex, respiration, and heart rate. Using a proprietary skeletal phantom, we have performed measurements of the total body BMD over the past 4 years and obtained a mean coefficient of variation of 3.1% (n = 285).

### **Micro-CT analysis**

We dissected the femur and vertebrae (L4 or L5), cleaned them of soft tissues, fixed them in 10% Millonig's formalin, and gradually dehydrated them into 100% ethanol. We loaded bones into 12.3 mm diameter scanning tubes and imaged them in a  $\mu$ CT (model  $\mu$ CT40, Scanco Medical). We integrated the scans into 3-D voxel images (1024 x 1024 pixel matrices for each individual planar stack) and used a Gaussian filter (sigma = 0.8, support = 1) to reduce signal noise. We applied a threshold of 200 to all scans, at medium resolution (E = 55 kVp, I = 145  $\mu$ A, integration time = 200ms). We scanned whole vertebra with a transverse orientation excluding any bone outside the vertebral body. We manually excluded the cortical bone and the primary spongiosa from the analysis. We made all trabecular measurements by drawing contours every 10 to 20 slices and used voxel counting for bone volume per tissue volume and sphere filling distance transformation indices, without pre-assumptions about the bone shape as a rod or plate for trabecular microarchitecture. We measured cortical thickness at the femoral mid-diaphysis. We performed calibration and quality control weekly using five density standards and verified spatial resolution monthly using a tungsten wire rod. We based beam-hardening correction on the calibration records. We made corrections for 200 mg hydroxyapatite for all energies. Over the past 3 years, the coefficient of variation for the fifth density standard (mean five) was 1.28 (781  $\pm$  10 SD mg HA/cm<sup>3</sup>) and for rod volume was 3.16 (0.0633  $\pm$  0.002 SD cm<sup>3</sup>).

### **Doxycycline or OPG administration to mice**

We fed adult *Osx1-Cre;RANKL<sup>f/f</sup>* and *RANKL<sup>f/f</sup>* mice a diet containing doxycycline (Bio-Serv) beginning 1 week before breeding to suppress expression of the Cre transgene in the resulting offspring. After birth, we maintained the offspring on the doxycycline-containing diet until they were 4 months of age at which time they were switched to a normal rodent chow for 2 months to activate the Cre transgene. We measured BMD and body weight at 4 and 6 months of age.

To reduce osteoblast number, we subcutaneously injected 5-month-old *Dmp1-Cre;RANKL<sup>f/f</sup>* mice and *RANKL<sup>f/f</sup>* littermates with vehicle or OPG-Fc (10 µg/g body weight) (Amgen) three times with a 7-day interval. We then injected these mice i.p. with vehicle or 230 ng/g body weight human PTH-(1-84) (Bachem) 1 hour before harvesting at day 19.

### **Hindlimb unloading by tail suspension**

We performed mechanical unloading of hindlimbs by tail suspension of 6-month-old *Dmp1-Cre;RANKL<sup>f/f</sup>* mice and *RANKL<sup>f/f</sup>* littermates for 3 weeks. We stratified experimental animals by initial hindlimb BMD. We wrapped a 5.5 cm long wire (a straightened paperclip) with porous first-aid tape and then linked it to a fishing swivel by a 2.5 cm long length of hobby chain. We attached the wire to the mouse tail with super glue and reinforced it by wrapping with first-aid tape at both ends. We suspended mice by connecting the fishing swivel to a key ring which was placed on a rod inserted into the top of shoebox mouse cage modified to hold the rod. We adjusted the entire rig to a height that maintained the mouse at a ~30° head-down tilt by adjusting the chain. We housed two tail-suspended animals per cage separated by a plastic divider. We housed fully ambulatory littermate controls in similar cages. We euthanized the animals after 3 weeks of suspension and collected various tissues and cells. We measured BMD and body weight the day before tail-suspension and on the day of euthanasia.

### **Histology**

Femurs for X-gal staining were fixed in fresh 0.2% glutaraldehyde at 4° C for 7 days and then decalcified in 14% EDTA for 3 weeks. After decalcification, femurs were incubated in 30% sucrose for a minimum of 2 days and then embedded in Cryo-Gel (Electron Microscopy Sciences) for frozen sectioning. X-gal staining was performed on 5 µm frozen sections at room temperature for 6 hours in staining solution (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, 0.02% NP40, and 20 mM Tris-HCl, pH 7.4) and counterstained with 1% basic fuchsin.

For TRAP and safranin-O staining, femurs were fixed for 24 hours in 10% Millonig's formalin and decalcified in 14% EDTA for 1 week. Femurs were then embedded in paraffin, and 5 µm longitudinal sections were obtained. After de-paraffinization and rehydration, sections were incubated with 0.2 M acetate buffer containing 10% (vol/vol) naphthol-AS-BI-phosphate at 37°C for 30 minutes. The sections were then incubated with pararosaniline chloride (1.5 mg/ml) for 5 minutes and counterstained with 0.1% fast green. For safranin-O staining, sections were de-paraffinized, rehydrated, and stained with 0.1% fast green solution for 2 minutes followed by a

quick rinse with 1% acetic acid solution for no more than 10 seconds. Sections were stained in 0.1% safranin-O solution for 5 minutes, and then developed in 95% ethanol.

### **Histomorphometry**

TRAP-stained sections, counter-stained with toluidine blue, were used for osteoclast enumeration. Osteoclast counts were performed using a computer and digitizer tablet (OsteoMetrics) interfaced to a Zeiss Axioscope (Carl Zeiss) with a drawing tube attachment. The numbers of TRAP-positive multinucleated cells on the cancellous perimeter (osteoclast number) were measured directly. Terminology recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research was used in this study<sup>7</sup>.

### **RANKL Immunostaining**

Immunohistochemistry was performed using 5 µm frozen sections from decalcified femurs fixed in Millonig's formalin. The sections were rinsed in PBS for 5 minutes followed by quenching in 0.3% H<sub>2</sub>O<sub>2</sub> for 30 minutes. Sections were washed in PBS for 5 minutes and blocked in 2.5% normal goat serum for 20 minutes. Anti-RANKL antibody (R&D Systems) was diluted to 5 µg/ml in PBS and incubated with the sections for 1 hr at 20°C followed by rinsing and additional incubation for 20 min with biotinylated anti-goat IgG. Non-immune goat IgG was used as a negative control. Elite ABC reagent (VectaStain, Vector Laboratories) was applied for 30 min, washed thoroughly, and developed with AEC (Vector Laboratories). Sections were counterstained with 2% Mayer's hematoxylin for 30 sec and mounted with aqueous mounting medium.

### **Blood chemistry**

Blood was collected by retro-orbital bleeding into heparinized tubes and was centrifuged at 1500 x g for 10 minutes to separate plasma from cells. Soluble RANKL in blood plasma was measured using a Quantikine mouse RANKL kit (R&D Systems) according to the manual provided by the manufacturer. Carboxy-terminal crosslinked telopeptide of type I collagen was measured using the RatLaps EIA CTX kit from Immunodiagnostic Systems Inc. and osteocalcin was measured using an EIA kit from Biomedical Technologies, Inc., both according to the manufacturer's instructions.

### **Cell culture**

Bone marrow cells harvested from long bones were plated in 12-well plates at 5×10<sup>6</sup> cells/well and cultured in α-MEM containing 10% FBS, 1% penicillin/streptomycin/glutamine, and 1% ascorbic acid. One-half of the culture medium was changed at days 3 and 6. Medium was completely changed at days 8 and 10 and at day 10, the cells were treated with vehicle or 10<sup>-7</sup> M human PTH (1–34) for 4 hours before RNA extraction.

### **RNA purification and gene expression analysis**

Tissues were dissected from animals and were stored immediately in liquid nitrogen. Total RNA was purified from tissues or cultured cells using Ultraspec reagent (Biotecx Laboratories), according to the manufacturer's instructions. Three µg total RNA was reverse-transcribed into

cDNA as using the High Capacity Reverse Transcriptase kit from Applied Biosystems. Taqman quantitative RT-PCR was performed using the following Taqman assays from Applied Biosystems: RANKL (Mm0041908-m1); IL-6 (Mm00446190-m1); Cathepsin K (Mm01255862-g1); TRAP (Mm00475698\_m1); Calcitonin receptor (Mm00432271\_m1); and ribosomal protein S2 (for, 5'-CCCAGGATGGCGACGAT-3', rev, 5'-CCGAATGCTGTAATGGCGTAT-3', probe, 5'-FAM-TCCAGAGCAGGATCC-NFQ-3'). Relative mRNA levels were calculated by normalizing to the house-keeping gene ribosomal protein S2 using the  $\Delta$ Ct method<sup>8</sup>.

### **RNA and genomic DNA isolation from enriched osteocytes**

The distal and proximal ends of the femur or tibia were removed and bone marrow cells were flushed out completely with PBS. The surfaces of the bone shafts were scraped with a scalpel to remove the periosteum and then cut into a few small pieces. Bone pieces were then digested with 1 ml of Hank's solution containing 0.1 % bovine serum albumin, 1 mM CaCl<sub>2</sub>, and 1 mg/ml of collagenase (type I:II, ratio 1:3) (Worthington Biochemical Corporation) in a 12-well-plate. A total of 6 digestions for 15 minutes each were performed at 37°C in water bath shaker to remove the cells on the bone surface. After the final digestion, bone pieces were washed with PBS and frozen in liquid nitrogen for RNA isolation. For genomic DNA isolation, bone pieces were decalcified in 14% EDTA for 1 week after collagenase digestion. Decalcified bone was then digested with proteinase K (0.5 mg/ml in 10 mM Tris, pH 8.0, 100 mM NaCl, 20 mM EDTA, and 1% SDS) at 55° C overnight. Genomic DNA was then isolated by phenol/chloroform extraction and ethanol precipitation.

### **Taqman assay to quantify gene deletion**

Two custom Taqman assays were obtained from Applied Biosystems for quantifying RANKL deletion efficiency: one specific for sequences between the loxP sites (for, 5'-GCCAGTGGACTTACTCAAACCTT-3'; rev, 5'-GGTAGGGTTCAACTGAAGGGTTTA-3'; probe, 5'-FAM-CCTCCTCCTCATGGTTTAGT-NFQ-3') and the other specific for sequences downstream from the 3' loxP site (for, 5'-GGTGCCGTGCATTATCCTAGAC-3'; reverse, 5'-AAGTAATGTGACCCTTGAGAACTG-3'; probe, 5'-FAM-CTAGCACACGTGCCTGCT-NFQ-3'). The relative genomic DNA amount was calculated using the  $\Delta$ Ct method<sup>8</sup>.

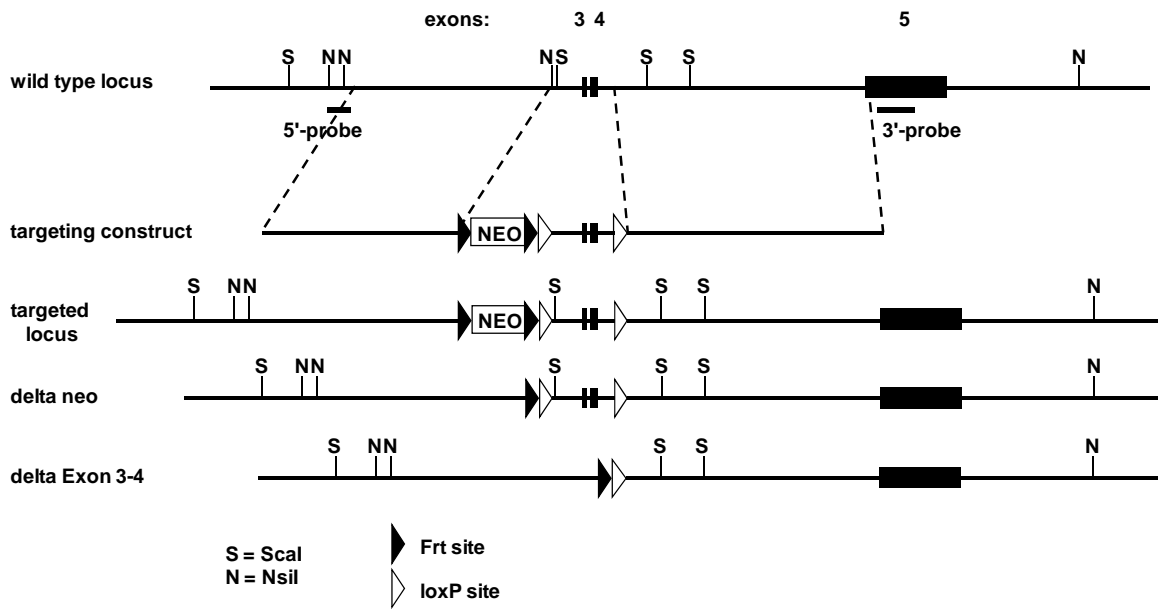
**Statistics.** Two-way ANOVA or Student's t-test was used to detect statistically significant treatment effects, after determining that the data were normally distributed and exhibited equivalent variances. In some cases, log transformation was used to obtain normally-distributed data. All t-tests were two-sided. Bonferroni or Holm-Sidak corrections were used for multiple comparisons. *P*-values less than 0.05 were considered significant.

## REFERENCES TO THE SUPPLEMENTARY INFORMATION

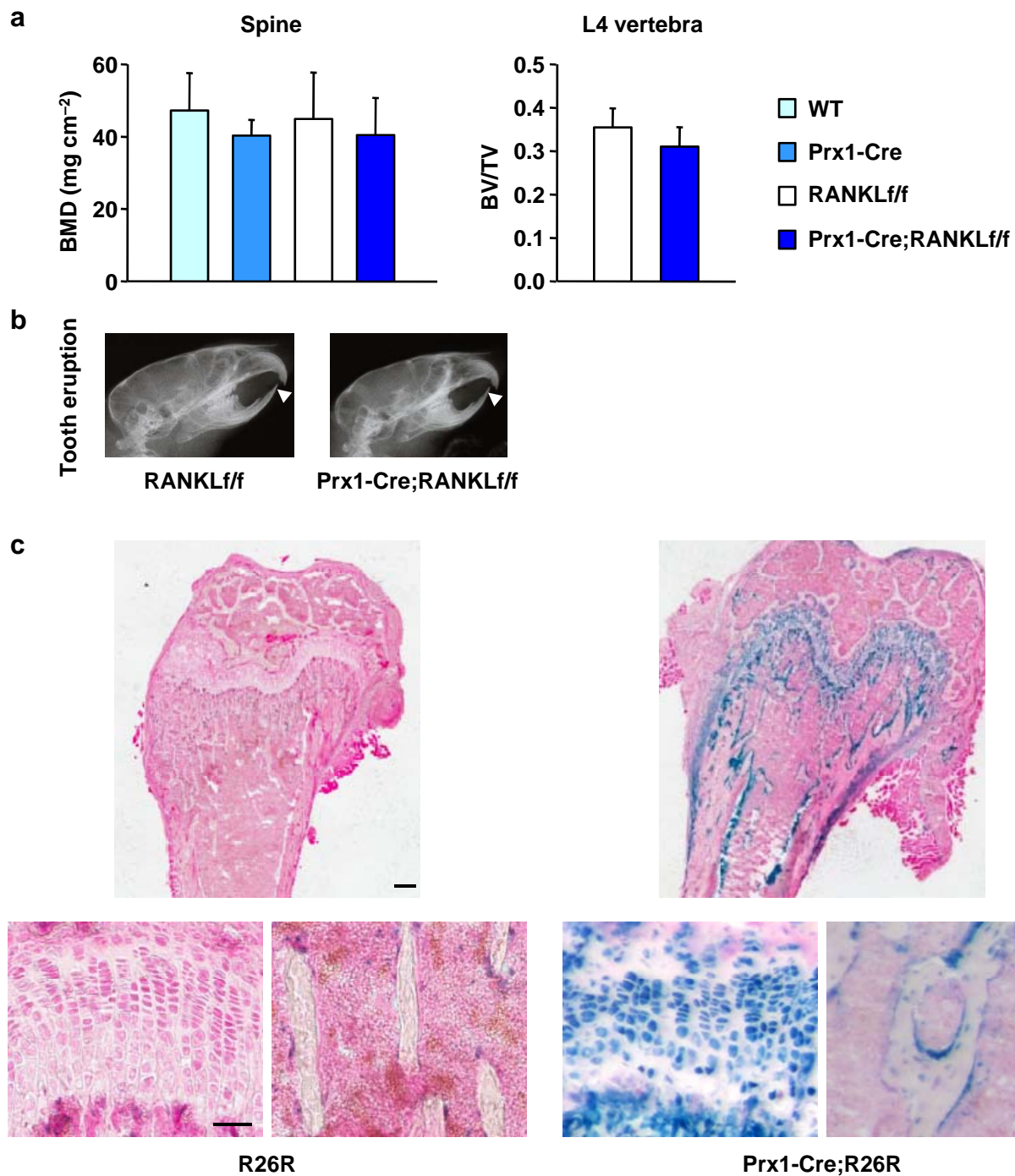
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## Supplementary Figures

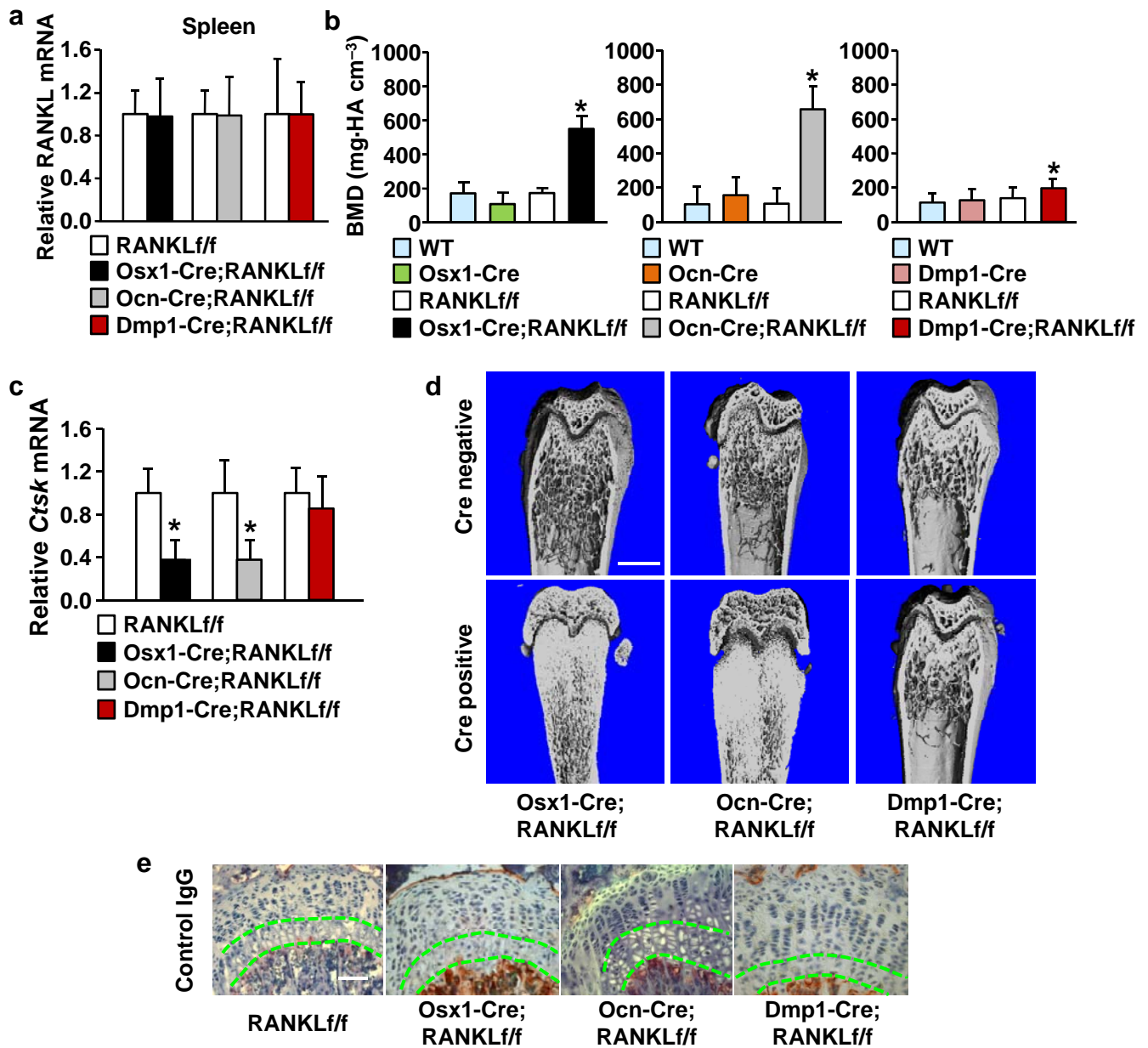




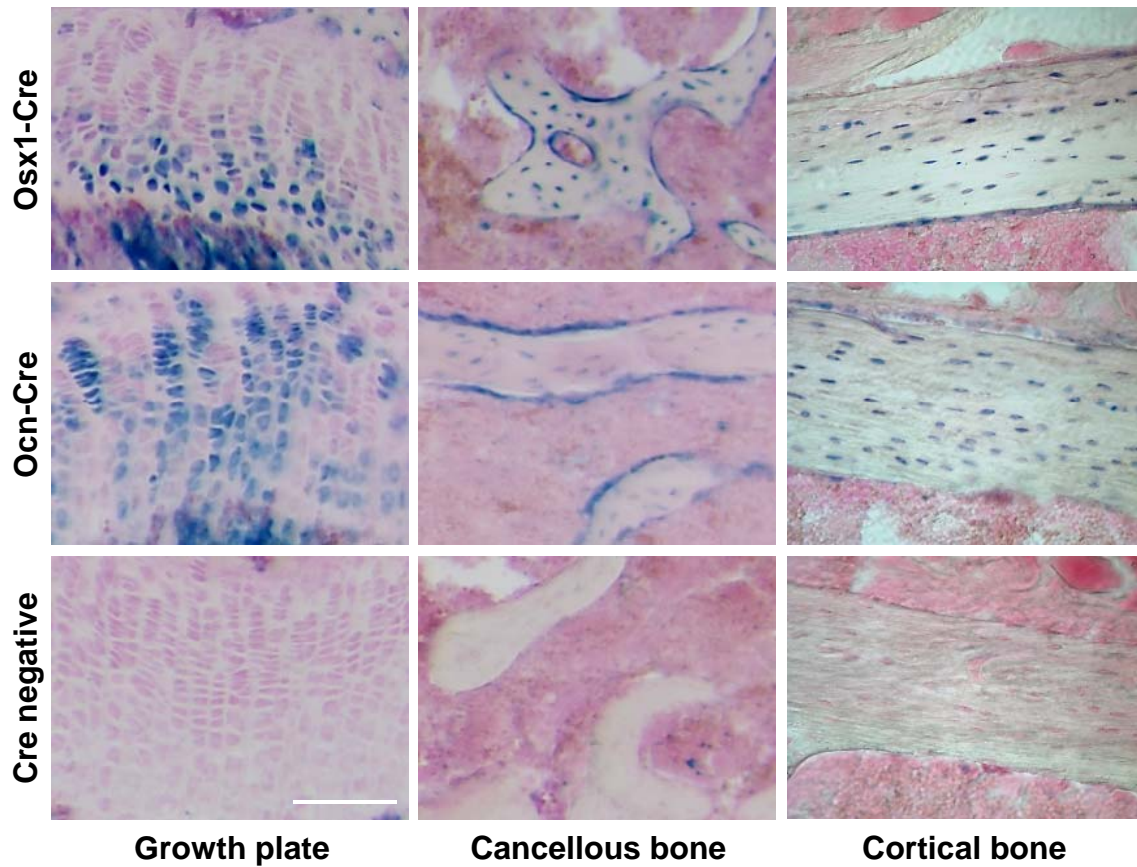
**Figure S1. Targeting strategy for the conditional RANKL allele.**



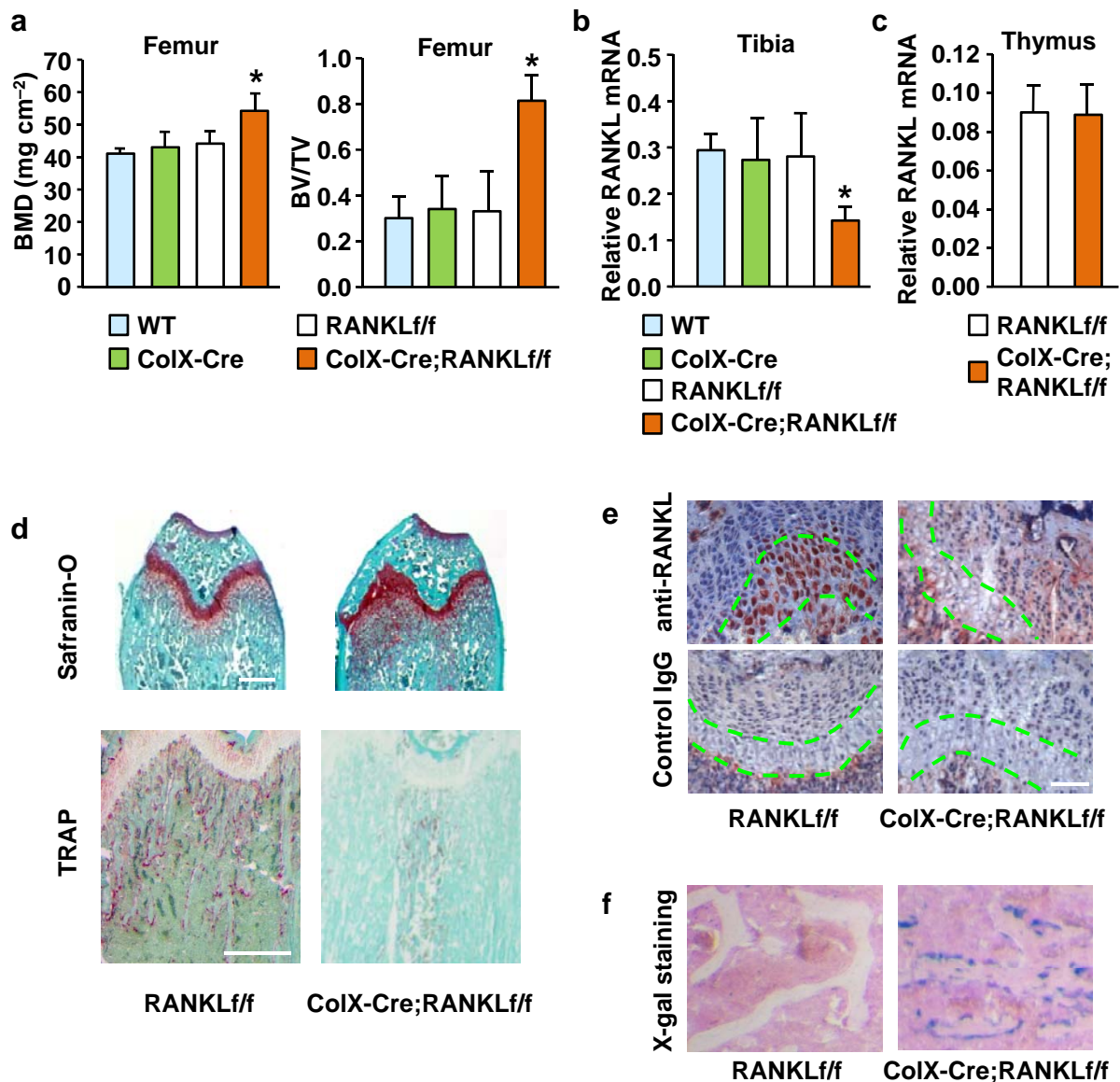
**Figure S2. Prx1-Cre transgene activity.** (a) Left, spinal BMD measured by dual-energy X-ray absorptiometry (DEXA) of WT ( $n = 7$ ), Prx1-Cre ( $n = 7$ ), RANKLf/f ( $n = 8$ ), and Prx1-Cre;RANKLf/f ( $n = 10$ ) littermates. Right, cancellous bone volume measured by  $\mu$ CT in L4 vertebra of RANKLf/f ( $n = 10$ ) and Prx1-Cre;RANKLf/f ( $n = 10$ ) littermates. (b) X-ray images from 5-week-old RANKLf/f and Prx1-Cre;RANKLf/f littermates. Arrowheads in the X-rays indicate position of normally erupted incisors. (c) X-gal stained histological frozen sections of the distal femur from 5-week-old R26R control and Prx1-Cre;R26R mice. Upper panel shows a low magnification image of the distal femur, scale bar, 200  $\mu$ m. Lower panel shows a higher magnification image of the growth plate and cancellous bone. Scale bar, 50  $\mu$ m.



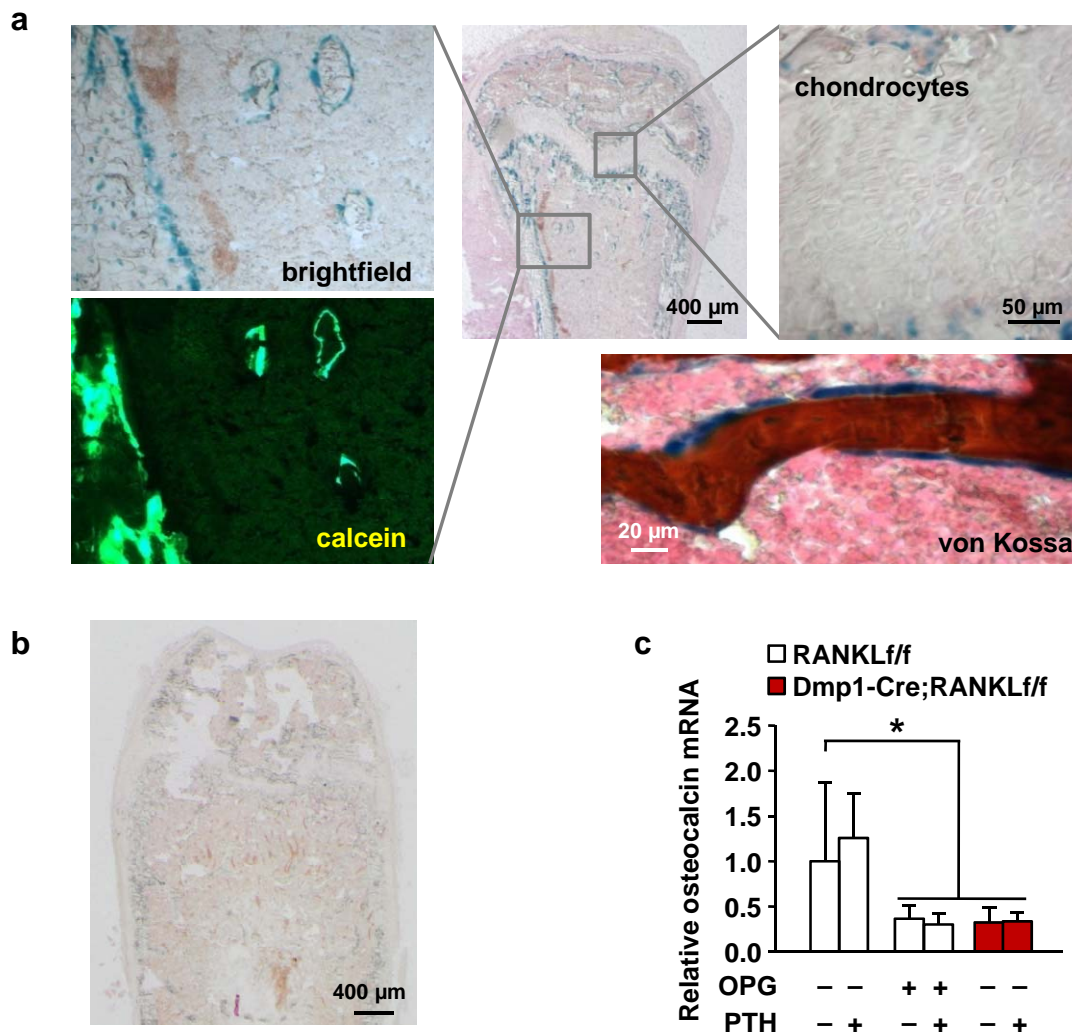
**Figure S3. Deletion of RANKL in *Osx1-Cre* and *Ocn-Cre* expressing cells causes osteopetrosis.** (a) RANKL mRNA levels in spleen of *Osx1-Cre;RANKL<sup>f/f</sup>* ( $n = 8$ ), *Ocn-Cre;RANKL<sup>f/f</sup>* ( $n = 6$ ), *Dmp1-Cre;RANKL<sup>f/f</sup>* ( $n = 9$ ), and their respective *RANKL<sup>f/f</sup>* littermates ( $n = 4$  to 11). (b) Femoral BMD measured by  $\mu$ CT of *Osx1-Cre;RANKL<sup>f/f</sup>* ( $n = 8$ ), *Ocn-Cre;RANKL<sup>f/f</sup>* ( $n = 6$ ), *Dmp1-Cre;RANKL<sup>f/f</sup>* mice ( $n = 9$ ) and their WT ( $n = 9$  to 11), Cre transgenic ( $n = 8$  to 12), and *RANKL<sup>f/f</sup>* ( $n = 4$  to 11) littermates. \* $P < 0.05$  versus WT, Cre transgenic, and *RANKL<sup>f/f</sup>* mice, using 2-way ANOVA. (c) Cathepsin K (*Ctsk*) expression measured in RNA from tibia of the same mice used in panel a. \* $P < 0.05$  versus *RANKL<sup>f/f</sup>* littermates, using Student's t-test. (d) Representative  $\mu$ CT images of the distal femur from 5-week-old *Osx1-Cre;RANKL<sup>f/f</sup>*, *Ocn-Cre;RANKL<sup>f/f</sup>*, *Dmp1-Cre;RANKL<sup>f/f</sup>*, and their respective *RANKL<sup>f/f</sup>* (Cre negative) littermates. Scale bar, 1 mm. (e) Goat IgG stained control histological sections of the distal femur from *Osx1-Cre;RANKL<sup>f/f</sup>*, *Ocn-Cre;RANKL<sup>f/f</sup>*, *Dmp1-Cre;RANKL<sup>f/f</sup>*, and a representative *RANKL<sup>f/f</sup>* littermate, as controls for anti-RANKL IHC. Please note that the brown staining of the primary spongiosa, shown here and in figure 2d, is non-specific since it is present in sections incubated with the control IgG. Scale bar, 100  $\mu$ m. All values in Figure S2 were determined in 5-week-old mice (both sexes were included) and are means  $\pm$  s.d.



**Figure S4. Osx1-Cre and Ocn-Cre transgenes are active in both hypertrophic chondrocytes and osteoblast lineage cells.** X-gal stained frozen histological sections of the growth plate (left panel), cancellous bone (middle panel), and cortical bone (right panel) of the femur from 5-week-old Osx1-Cre;R26R, Ocn-Cre;R26R, and Cre negative R26R mice. Scale bar, 100  $\mu$ m.



**Figure S5. Deletion of RANKL in ColX-Cre expressing cells causes osteopetrosis.** (a) Femoral BMD (left) measured by DEXA and cancellous bone volume in the distal femur measured by  $\mu$ CT (right) of WT ( $n = 5$ ), ColX-Cre ( $n = 6$ ), RANKL $f/f$  ( $n = 7$ ), and ColX-Cre;RANKL $f/f$  ( $n = 7$ ) littermates.  $*P < 0.05$  versus WT, ColX-Cre, and RANKL $f/f$ , using 2-way ANOVA. (b) RANKL mRNA levels in tibia of 5-week-old WT ( $n = 5$ ), ColX-Cre ( $n = 6$ ), RANKL $f/f$  ( $n = 7$ ), and ColX-Cre;RANKL $f/f$  ( $n = 7$ ) littermates.  $*P < 0.05$  versus WT, ColX-Cre, and RANKL $f/f$ , using 2-way ANOVA. (c) RANKL mRNA levels in thymus of ColX-Cre;RANKL $f/f$  ( $n = 7$ ) and RANKL $f/f$  ( $n = 7$ ) littermates. (d) Safranin-O-stained and TRAP-stained histological sections of the distal femur from 5-week-old ColX-Cre;RANKL $f/f$  and RANKL $f/f$  littermates. Scale bars, 0.5 mm. (e) Anti-RANKL immunohistochemistry of the distal femur from ColX-Cre;RANKL $f/f$  and RANKL $f/f$  littermates. The region of the growth plate containing hypertrophic chondrocytes in the images is outlined by green dashed lines and RANKL protein is stained reddish brown. Non-immune IgG controls are presented in lower panel. Scale bar, 100  $\mu$ m. (f) X-gal stained histological sections of the cancellous bone from ColX-Cre;RANKL $f/f$  and RANKL $f/f$  littermates. All values in Figure S5 are means  $\pm$  s.d. and both sexes were included in each group.



**Figure S6. The *Dmp1-Cre* transgene is active in both osteoblasts and osteocytes.** (a) All the images are from 2 consecutive frozen sections from a non-decalcified femur of a 5-week-old *Dmp1-Cre;R26R* mouse injected with calcein 3 days before bones were collected. The top 3 panels and the bottom right panel are images of X-gal stained sections. The image at the lower left is an epifluorescent image of the same field as the panel above it. The image at the bottom right is a section stained with X-gal and von Kossa. (b) Low power image of X-gal stained femoral section of R26R littermate of mouse in a. (c) Osteocalcin mRNA levels in tibial cortical bone of 6-month-old *Dmp1-Cre;RANKLf/f* and *RANKLf/f* littermates, treated with vehicle or OPG for 19 days and then injected with vehicle or PTH(1-34) 1 hr before bones were collected ( $n = 6$  to 8 per group). \* $P < 0.05$  versus *RANKLf/f* mice pretreated with vehicle and then acutely injected with vehicle using 2-way ANOVA. All values in Figure S6 are means  $\pm$  s.d. and both sexes were included in each group.