Supporting Information

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SI Materials and Methods

Mice. The generation and characterization of $Shn3^{-/-}$ animals were previously described (1). For this study, mice were backcrossed to a BALB/c background for >10 generations, and age- and sex-matched BALB/c WT mice were used as controls. Conditional Shn3 KO mice were generated at Taconic and maintained on a C57/BL6 background. Prx1-Cre mice on a C57/BL6 background were purchased from the Jackson Laboratory. Conditional NFATc1-deficient mice were as previously described (2). Briefly, $NFATc1^{fl/fl}$ $Shn3^{+/-}$ $MxCre^{+}$ mice were crossed with $NFATc1^{fl/fl}$ $Shn3^{+/-}$ $MxCre^{-}$ mice. Before genotyping at postnatal days 10, 12, and 14, all mice were treated with polyinosinic:polycytidylic acid (poly dI:C) (2). Animals were killed at 10 wk of age, and midshaft femur total BV/TV was analyzed by microcomputed tomography (micro-CT).

Serum Measurements. Serum was collected from WT and $Shn3^{-/-}$ mice and stored at −80 °C. Samples for comparison were always collected at the same time of day. Measurements for Pyd (Quidel), CTX (IDS), calcium (BioAssay), and RANKL (R&D Systems) were performed per the instructions of the manufacturers.

Whole-Mount TRAP Histochemistry. Calvariae from 8-wk-old mice were dissected, soft tissue was removed, and the calvariae were fixed in 100% methanol for 5 min. Bones were thoroughly washed in water, and TRAP staining was performed per the instructions of the manufacturer (Sigma).

Histology and Immunohistochemistry. Femurs were isolated from 8-wk-old mice, decalcified, paraffin-embedded, and sectioned. Sections were dewaxed and blocked in 3% H_2O_2 in PBS for 20 min, and TRAP staining was performed per the manufacturer's protocol. For subsequent RANKL immunohistochemistry, proteinase K treatment was then performed. Sections were blocked in 3% donkey serum with 1% BSA and 0.1% Triton X-100 for 1 h at room temperature and then incubated with RANKL antibody (sc-7628; Santa Cruz Biotechnology) at 4 °C overnight. Detection was performed with donkey anti-goat horseradish peroxidase (HRP), trichostatin A (TSA)-biotin, streptavidin-HRP, and diaminobenzidine (DAB) amplification. Sections were counterstained in hematoxylin and mounted.

Osteoblast/Osteoclast Cocultures. Calvarial osteoblasts were derived from P2–P5 neonatal BALB/c mice as previously described (1) and cultured in α-MEM supplemented only with 10% FCS and antibiotics (penicillin/streptomycin). Cells were allowed to reach confluency, and were then split into 96-well plates at a density of 30,000 cells/mL. Two days later, osteoclast precursors were added in the presence of calciotropic agents. Vitamin D (Sigma) was added at a concentration of 10 nM. PGE2 (Calbiochem) was used at a concentration of 10 μM. PTH (Sigma) was used at 10 nM. Isoproterenol (Sigma) was used at 10 μM. Anti-OPG antibody (R&D Systems) was used at 10 μg/mL. Osteoclast precursors were derived from adult male BALB/c mice. Briefly, long bones were carefully dissected, marrow was flushed, and all cells were plated overnight in α-MEM plus 10% FCS with macrophage-colony stimulating factor (M-CSF) (10 ng/mL; Peprotech). Eighteen hours later, nonadherent cells were collected and mononuclear osteoclast precursor cells were purified using Histopaque 1083 gradient (Sigma) centrifugation. Four to five days later, tissue culture supernatant was harvested from TRAP assay (Sigma) or cells were fixed and stained for microscopy. Secreted TRAP was used to quantify osteoclastogenesis in these experiments because we found this led to a more robust and reproducible quantification than manual counting of osteoclasts.

RNA Isolation and Real-Time Quantitative PCR Assay. RNA was isolated from adult calvariae and tissue culture using TRIzol (Invitrogen) reagent per the instructions of the manufacturer. To analyze gene expression, cDNA was synthesized and quantitative PCR assays for actin, RANKL, Runx2, OPG, osteocalcin (OCN), Col1a1, calcitonin receptor, and cathepsin K were performed as described (1). Primer sequences are available on request.

SV40-Transformed Osteoblast Cell Lines. WT and $Shn3^{-/-}$ primary calvarial osteoblasts were transformed with a plasmid encoding the SV40 large T antigen, and various transformed subclones were obtained and characterized. Cells expressing osteoblastic markers (Runx2 and Osterix) were used for further studies. WT cells were infected with control, Shn3 shRNA, and Shn3 overexpression lentiviruses containing a puromycin resistance gene. Infected cells were selected with puromycin, and RNA was harvested and analyzed as above. We used a combination of primary calvarial osteoblasts and SV40-transformed osteoblastic cells in these experiments because of different expression levels of receptors for these agents in various cell types.

Luciferase Reporter Assays. C3HT101/2 cells were transiently transfected as previously described (1). Luciferase reporters were used containing the proximal OPG promoter (3) and the distal RANKL upstream enhancer region with mutated CREB binding sites (4). Forty-eight hours after transfection (Effectene; Qiagen), cells were harvested and firefly and renilla luciferase activities were determined on a luminometer using Promega dual-luciferase reagent.

Coimmunoprecipitation. 293T cells were transfected with Shn3 (1), HA-Runx2 (a gift from Jane Lian, University of Massachusetts, Worcester, MA), and HA-CREB (a gift from Michael Greenberg, Children's Hospital, Boston, MA). Forty-eight hours later, cells were lysed in buffer [200 mM NaCl, 20 mM Tris·HCl (pH 8.0), 0.5% Triton X-100] supplemented with protease inhibitors. Lysates were incubated with anti–HA-coupled agarose (F7; Santa Cruz Biotechnology) overnight at 4 °C. Immune complexes were washed three times in lysis buffer, and precipitated proteins were resolved by SDS/PAGE followed by immunoblotting for Shn3 or HA.

BM Transplantation. WT or $Shn3^{-/-}$ mice on a pure (backcrossed for >10 generations) BALB/c genetic background were lethally irradiated and rescued with 0.5×10^6 BM cells via tail vein injection from either WT or $Shn3^{-/-}$ mice. Ten weeks later, chimeras were killed, splenic B cells were harvested using $CD19^{+}$ selection (Miltenyi), and genomic DNA was analyzed by PCR assay using previously published primer sequences (1). At the same time, femurs were harvested and analyzed by micro computed tomography (μCT).

Th17 Cell Generation and RANKL (CD254) FACS. Spleen and lymph node were harvested from adult WT or $Shn3^{-/-}$ C57B/6 animals. RBCs were lysed, and single-cell suspensions were prepared according to standard methods. $CD4^{-}$ selection was performed using Robosep (Stem Cell) according to the manufacturer's instructions. Naive $CD4^{+}CD62L^{high}CD25^{-}$ cells were purified by cell sorting and incubated at a 1:3 ratio with irradiated spleno-

cytes (2,000 rad) in RPMI in the presence of anti-CD3, TGF-β1 (2 ng/mL; R&D Systems), IL-6 (20 ng/mL; R&D Systems), and anti–IL-4 (10 μg/mL) and anti–IFN-γ (10 μg/mL) antibodies. Two to three days later, cells were collected and transferred into fresh medium containing the aforementioned cytokines and antibodies, except in the absence of anti-CD3, cells were then split at a 1:2 ratio as needed. Two to three days later, cells were collected and restimulated with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 30 min and fluorescence-activated cell sorter staining with isotype control or anti-RANKL (CD254; 510003; Biolegend) antibody.

Low-Calcium Diet. WT or Shn3$^{-/-}$ mice on a BALB/c genetic background were placed on either a control (0.6% Ca, 0.4% P; TD.97191; Harlan Tekland) or low-calcium (0.02% Ca, 0.4% P; TD.02279; Harlan Tekland) diet from the age of 11–13 wk. Animals were then killed, and femurs were harvested and analyzed by μCT. PTH levels were not measured because serum levels of calcium and phosphorus were comparable in animals of both genotypes on both diets.

In Vivo Transient Muscle Paralysis Model. Under isoflurane anesthesia, each mouse was weighed on day 0 and given a single i.m. injection of botulinum toxin A (2 U per 100 g, 20-μL final volume of Botox; Allergan) or a saline injection of equivalent volume in the right calf muscle group. All mice received an s.c. injection of saline (800 μL) for each of the next 4 d to ensure sufficient hydration. At 24 h postinjection, all mice were visually examined for calf paralysis by assessing whether the mice could extend their toes or demonstrate ankle plantar flexion during transient tail suspension. Mice were group-housed with free ambulation and received food and water ad libitum per procedures approved by the University of Washington Institutional Animal Care and Use Committee.

Micro-CT Imaging and Analysis. In vivo imaging was conducted with a high-resolution micro-CT scanner (10.5-μm voxel resolution; Scanco vivaCT 40). While anesthetized with isoflurane, each mouse underwent imaging of the right proximal tibia metaphysis (i.e., trabecular bone assessment) and right tibia middiaphysis (i.e., cortical bone assessment) on day 0. A subset of Shn3 KO mice (n = 4) also underwent baseline imaging of the left (contralateral) tibia metaphysis and tibia diaphysis. Botox (or saline) intervention occurred following imaging on day 0 while the mice were still under anesthesia. Final imaging was repeated for each mouse on day 21.

Standard image analysis procedures were used to determine trabecular bone and cortical bone parameters (5). User-defined contour lines were drawn every 15 slices, and interim contour lines were morphed along the entire analysis region using algorithms developed by Scanco. For the proximal tibia metaphysis, we determined trabecular fraction (BV/TV), trabecular number (mm), trabecular thickness (mm), and trabecular spacing (mm) within a 0.8-mm region initiating at the distal edge of the metaphyseal growth plate for each mouse at day 0 vs. day 21. Tibia middiaphysis cortical bone morphology was assessed for a 1.0-mm region centered 1.2 mm proximal to the tibia-fibula junction. Cortical bone outcome measures included periosteal volume (mm^3), cortical volume (mm^3), endocortical volume (mm^3), and cortical thickness (mm^3).

Biomechanical Testing. Biomechanical testing of WT (Shn3 f/f) and Shn3 conditional KO (Shn3 f/f × Prx1-Cre) femurs was performed using a BioDent Bio-Indenter according to the instructions of the manufacturer to obtain parameters that included indentation distance increase (μm), mean energy dissipated (μJ), and unloading stiffness (N/μm). Femurs from 4 male mice per genotype were tested.

1. Jones DC, et al. (2006) Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. *Science* 312:1223–1227.
2. Aliprantis AO, et al. (2008) NFATc1 in mice represses osteoprotegerin during osteoclastogenesis and dissociates systemic osteopenia from inflammation in cherubism. *J Clin Invest* 118:3775–3789.
3. Kieslinger M, et al. (2005) EBF2 regulates osteoblast-dependent differentiation of osteoclasts. *Dev Cell* 9:757–767.
4. Fu Q, Manolagas SC, O'Brien CA (2006) Parathyroid hormone controls receptor activator of NF-kappaB ligand gene expression via a distant transcriptional enhancer. *Mol Cell Biol* 26:6453–6468.
5. Warner SE, et al. (2006) Botox induced muscle paralysis rapidly degrades bone. *Bone* 38:257–264.

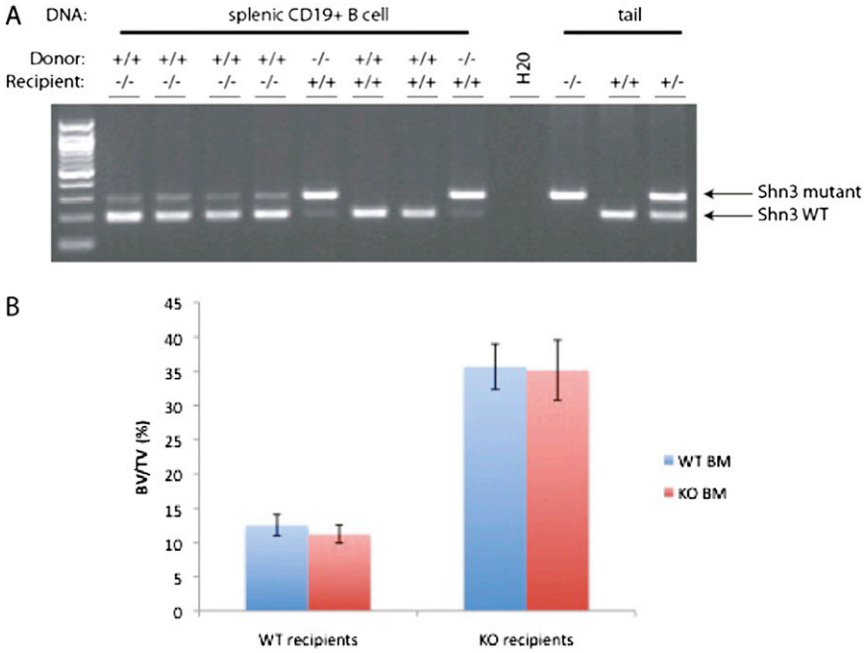

Fig. S1. Reciprocal BM transplantation. (*A*) Genomic DNA was purified from CD19^{+} B cells from the indicated radiation chimeras, and a PCR assay was performed to assess the degree of hematopoietic chimerism qualitatively. (*B*) Distal femoral BV/TV was determined from the indicated radiation chimeras. Error bars represent SD of $n = 2$ WT recipients and $n = 4$ KO recipients per experimental group.

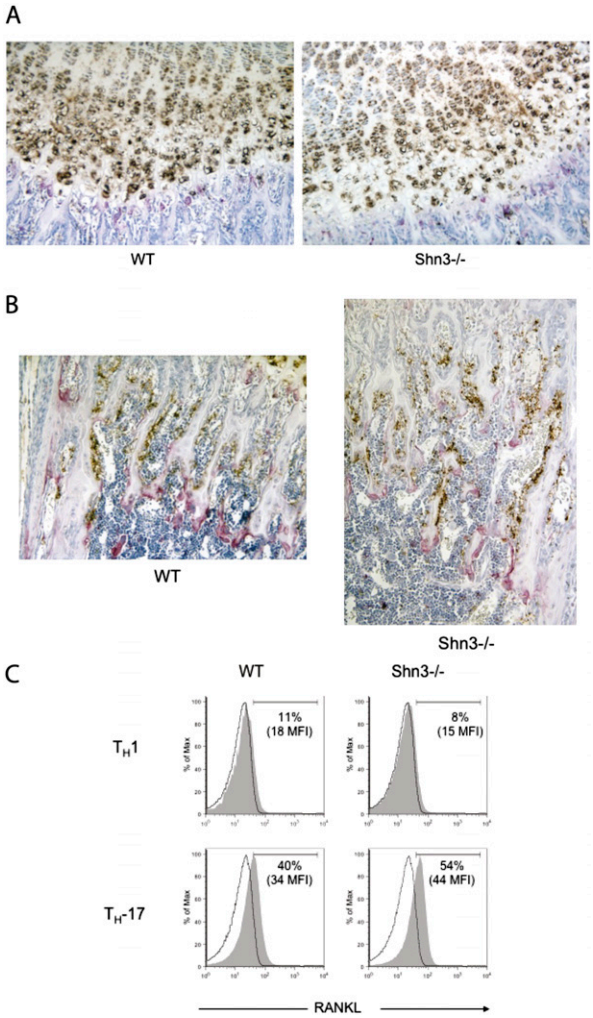

Fig. S2. RANKL expression in hypertrophic chondrocytes and Th17 cells. (*A*) p10 sections from WT or Shn3$^{-/-}$ animals were processed for RANKL immunohistochemistry (brown) and TRAP histochemistry (red) simultaneously. Comparable RANKL expression was observed in hypertrophic chondrocytes from WT and Shn3$^{-/-}$ sections. (*B*) Representative sections from similar animals shown at the level of the metaphysis. Again, comparable RANKL expression in metaphyseal bone-lining cells and TRAP activity were observed. (*C*) Th1 (T_H1) and Th17 (T_H17) cells were generated from WT and Shn3$^{-/-}$ mice, and surface RANKL expression was determined by fluorescence-activated cell sorting. No significant differences were observed comparing WT and Shn3$^{-/-}$ Th17 cells. MFI. (Magnification: 10× in *A* and *B*.)

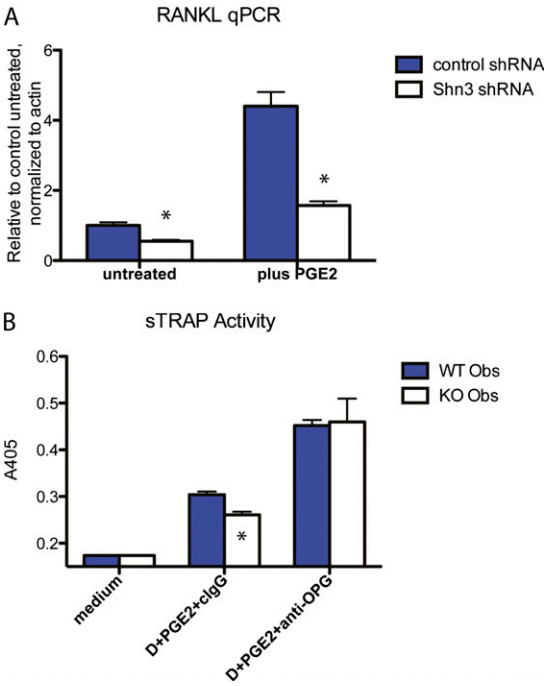

Fig. S3. (*A*) RANKL mRNA levels were determined by quantitative PCR (qPCR) assay in SV40-transformed osteoblast cell lines transduced with control shRNA-expressing lentivirus or lentivirus expressing shRNA targeting Shn3. Cells were harvested following treatment with PGE2 (10 μM) for 3 h. Data are expressed as mRNA levels relative to control untreated sample, and all samples were normalized to actin. Error bars represent SD of experimental triplicates. Data shown are representative of three independent experiments. $*P < 0.05$. (*B*) Cocultures were performed using calvarial osteoblasts from WT or Shn3$^{-/-}$ (KO) neonates, with WT BM responder cells, and in the presence of the indicated calciotropic agents and neutralizing anti-OPG antibody. Secreted TRAP activity was determined at day 5. Obs. Error bars represent experimental triplicates. Data shown are representative of three independent experiments. $*P < 0.05$.

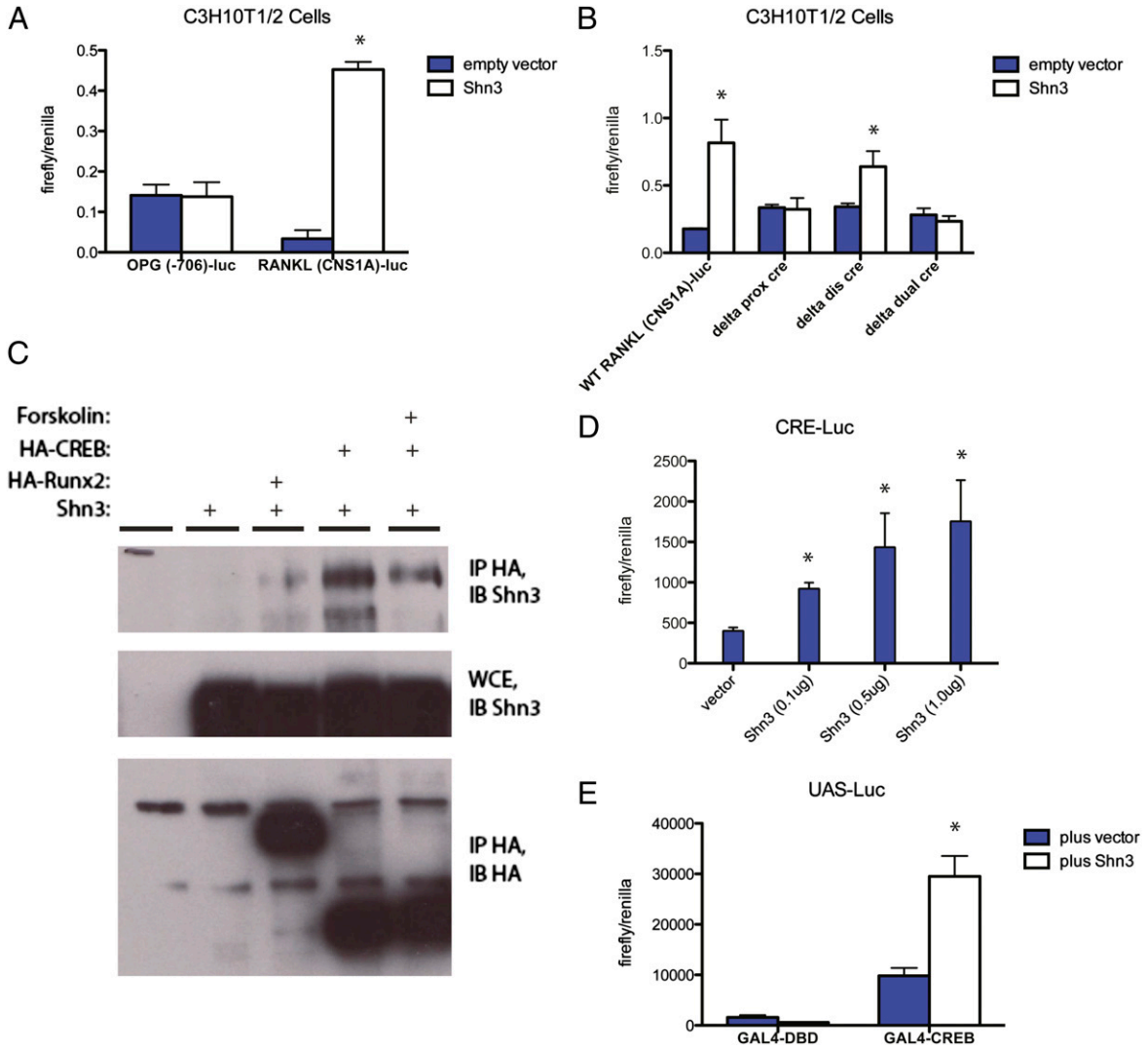

Fig. S4. Shn3 can regulate RANKL gene expression through an upstream regulatory region and CREB. (*A*) C3H10T1/2 cells were transiently transfected with a luciferase reporter element containing either the proximal OPG promoter or the RANKL upstream regulatory element CNS1A with either empty vector or Shn3. Firefly and renilla luciferase activities were determined after 48 h. Error bars represent the firefly/renilla ratio from triplicate experimental wells. (*B*) C3H10T1/2 cells were transfected as in *A* with the indicated CNS1A-luciferase reporter constructs. (*C*) 293T cells were transfected with the indicated combinations of Shn3 and HA-tagged versions of either CREB or Runx2. IB, immunoblot; IP, immunoprecipitation; WCE, whole cell extract. C3H10T1/2 cells were transfected with the indicated combinations of CRE-Luc reporter, empty vector, and Shn3 (*D*) and UAS-Luc reporter, Gal4-DBD, Gal4-CREB, empty vector, and Shn3 (*E*). Firefly and renilla luciferase activities were determined after 48 h. Error bars represent the firefly/renilla ratio from triplicate experimental wells. $*P < 0.05$ compared with empty vector. Each experiment was performed three independent times with similar results.

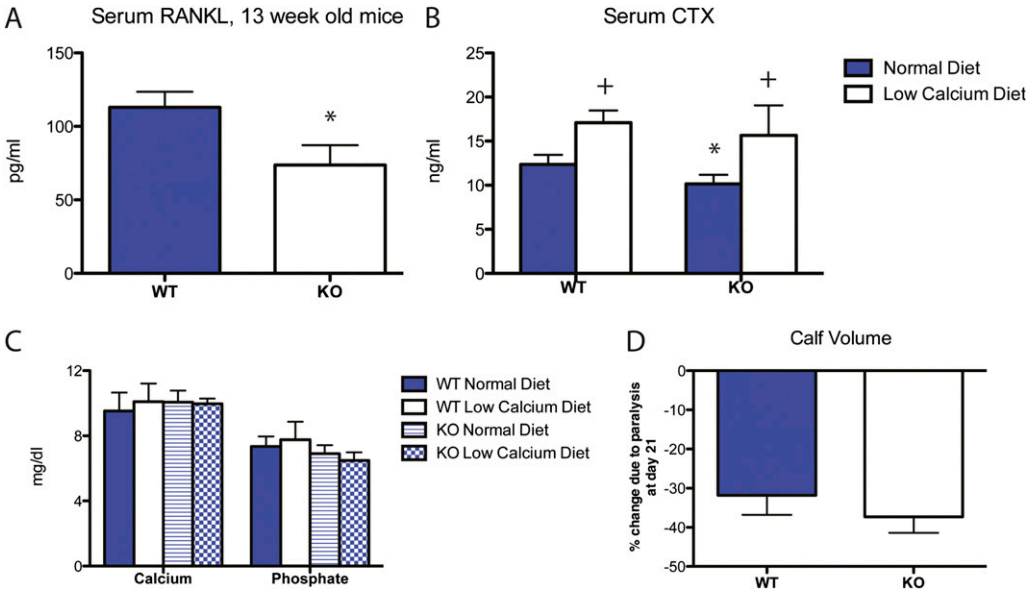

Fig. S5. (*A*) Serum RANKL levels were determined from 13-wk-old WT and Shn3-deficient (KO) mice ($n = 5$ mice per group). *$P < 0.03$. (*B*) Serum CTX levels were determined from WT and Shn3$^{-/-}$ (KO) animals after 2 wk of the indicated diet ($n = 4$ mice per experimental group). *$P < 0.02$ comparing WT normal diet with KO normal diet. $^{+}P < 0.05$ comparing normal diet with low-calcium diet within each genotype. (*C*) Serum calcium and phosphate levels were determined from WT and KO animals fed the indicated diet for 2 wk. No significant differences were observed ($n = 4$ mice per experimental group). (*D*) Volumetric analysis of calf volume atrophy in WT ($n = 6$) or Shn3$^{-/-}$ (KO, $n = 8$) mice following local Botox injection.

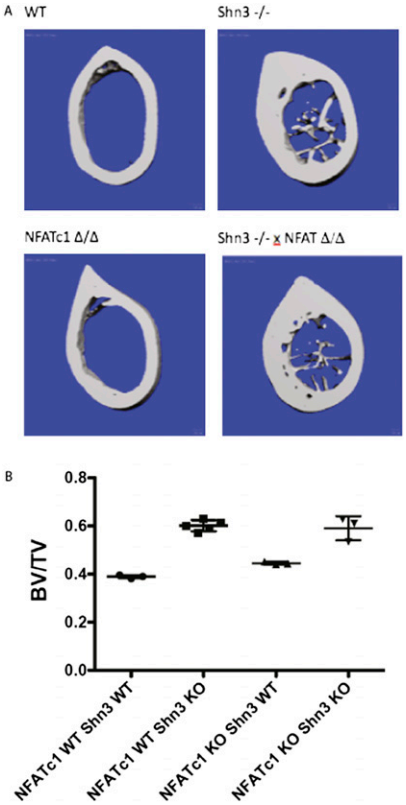

Fig. S6. Effect of inducible deletion of osteoclasts on the diaphyseal phenotype of Shn3-deficient mice. (*A*) Representative midshaft micro-CT images from mice of the indicated genotype. (*B*) Midshaft BV/TV of 10-wk-old Shn3 mice of the indicated genotype with or without osteoclasts (based on NFATc1 genotype). *P* values for two-tailed unpaired Student *t* tests: NFATc1 WT Shn3 WT vs. NFATc1 WT Shn3 KO, $P < 0.0001$; NFATc1 WT Shn3 WT vs. NFATc1 KO Shn3 WT, $P = 0.0005$; NFATc1 WT Shn3 KO vs. NFATc1 KO Shn3 KO, $P = 0.6981$ ($n = 4$ mice per genotype).

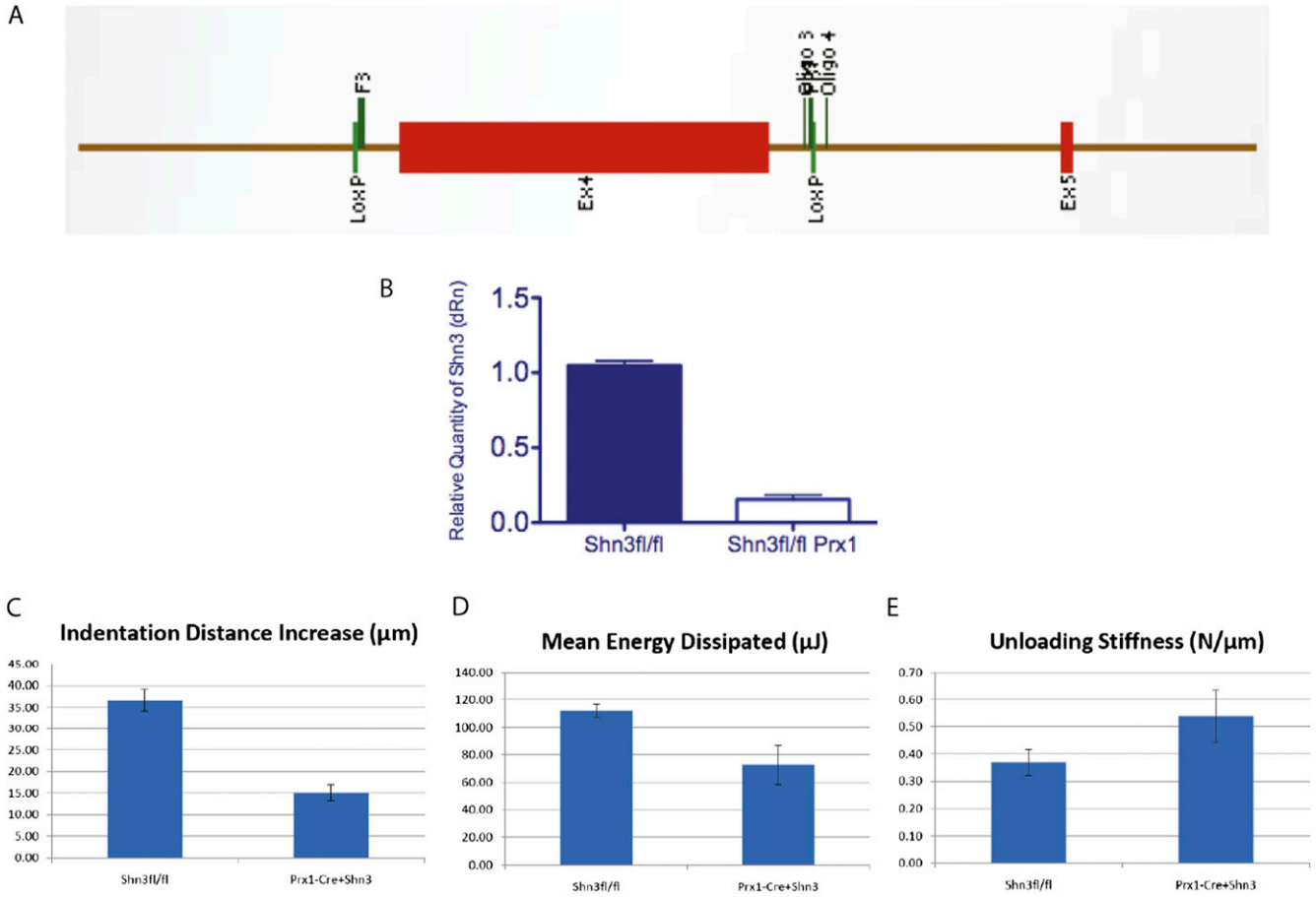

Fig. S7. (*A*) Schema for targeted allele. LoxP sites flank exon 4, which contains the majority of the coding region for the Shn3 protein. (*B*) Quantitative PCR data showing reduced Shn3 expression in Prx1-Cre transgene-positive mice. (*C–E*) Biomechanical parameters tested from Shn3 f/f transgene-negative (*Left*) and transgene-positive (*Right*) mice (n = 4 were tested per group). For all parameters shown, $P < 0.05$ comparing transgene-negative and transgene-positive groups.