Activin A receptor type 1-mediated BMP signaling regulates RANKL-induced osteoclastogenesis via canonical SMAD signaling pathway

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Running title: *ACVR1 required for osteoclastogenesis*

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Supporting information

Experimental procedures

Genomic real-time PCR

Genomic quantitative PCR was performed using TaqMan Gene Expression Assays. The following custom designed primer sets to detect *Acvr1* exon 7 (AIKAL5S_F 5′-CTCACTACTCTGGATACGGTTAGCT-3′, AIKAL5S_R 5′-GGGTCCCAAATATCTCTATGTGCAA-3′, AIKAL5S_M FAM 5′-CTATGGACAGTACAATCCG-3′) and *Egfp* (5′-GAGCGCACCATCTTCTTCAAG-3′, 5′-TGTCGCCCTCGAACTTCAC-3′, FAM 5′-ACGACGGCAACTACA-3′) were purchased from Taqman (Applied Biosystems, Foster City, CA). Data were normalized to *Gapdh* by the 2-ΔΔct method.

X-gal staining

Cells were fixed in 4% paraformaldehyde (PFA) for 10 min on ice and then stained with X-gal solution (containing 1 mg/ml X-gal, 5 mM potassium hexacyanoferrate, 5 mM potassium hexacanoferrate, 0.1% sodium deoxycholate, 2% NP-40, 2 mM $MgCl_2$ in 0.1 M sodium phosphate buffer) at 37 °C overnight.

Gene silencing

siRNAs for negative control (scrambled, #4390843), targeting *Smad1* (#4390771, siRNAID s69488), *Smad5* (#4390771, siRNAID s69502) and *Smad4* (#4390771, siRNAID s201660) were from Thermofisher. Transfection procedure was performed according to Lipofectamine RNAiMAX reagent (Invitrogen) protocol. Final siRNA amount was 25pmol for each 6-well dish. After incubation for 24 hours, cells were treated with M-CSF/RANKL to induce osteoclast differentiation.

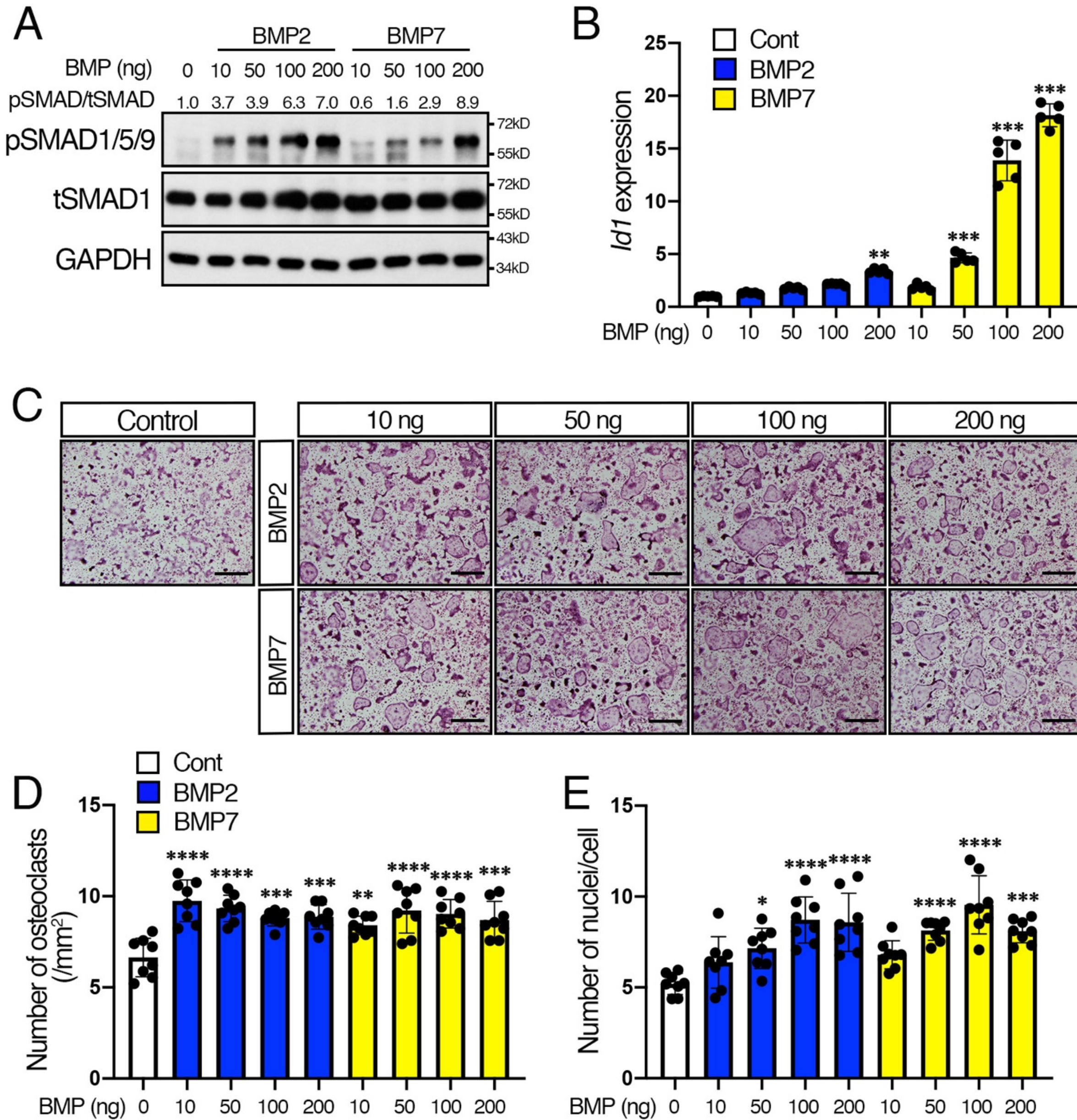

Figure S1. BMP-2 and BMP-7 enhanced RANKL-induced osteoclastogenesis**.** (*A*) Bone marrow mononuclear cells (BMMs) from control mice were cultured in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 2 days. Differentiated osteoclasts were stimulated with BMP-2 and BMP-7 for 30 minutes, and protein lysates were harvested. The activation of SMAD1/5/9 signaling in osteoclasts was examined by western blot. Fold increases of protein levels are relative to unstimulated control cells. Representative images of protein bands are shown, n = 3. (*B*) Differentiated osteoclasts were stimulated with BMP-2 and BMP-7 for 6 hours. Total RNA was extracted from osteoclasts, and the expression levels of *Id1* were assessed by quantitative reverse-transcribed PCR (qRT-PCR). n = 5. (*C*) BMMs were seeded in 48-well plates (1.5 x 10^4 cells/well) in the presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml). Cells were stained with TRAP at day 5. BMP-2 and BMP-7 were added every other day. Scale bars: 500 µm. (*D*) TRAP-positive cells containing 3 or more nuclei were counted as osteoclasts. n = 8. (*E*) The number of nuclei per cell was analyzed. n = 8. The value of each group was compared to the non-

treatment group. Values represent the mean ± SD. Differences were assessed by one-way ANOVA, followed by a Turkey test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

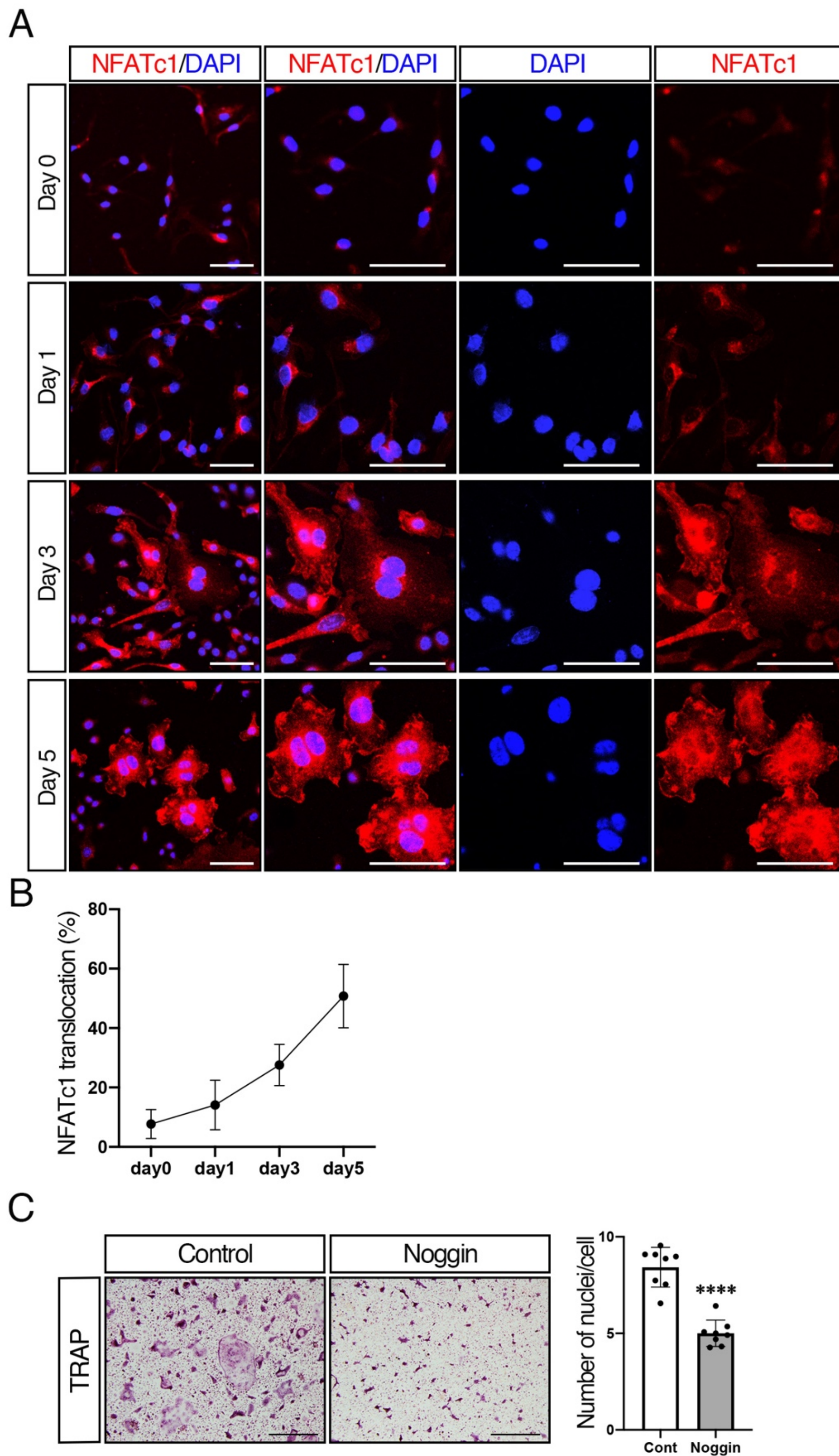

Figure S2. NFATc1 nuclear translocation was increased during osteoclastogenesis. (*A*) BMMs from control mice were incubated with M-CSF at 20 ng/mL (Day 0). M-CSF (20 ng/mL) and RANKL (50 ng/mL) were added every 48 hours up to 5 days. NFATc1 nuclear localization was assessed by immunofluorescence

(red), and nuclei (blue) were stained with DAPI. Low- and high-magnification images were shown. Scale bars: 50 µm. (*B*) NFATc1 nuclear translocation was expressed as a percentage of the total number of osteoclasts. Cells were rated positive for nuclear localization of NFATc1 if the fluorescence intensity of the nuclei exceeded that of the cytoplasm. n = 4. (*C*) BMMs from control mice were cultured in 48-well plates (1.5 x 10^4 cells/well) with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for 5 days, and TRAP staining was conducted. Cells were treated with Noggin (250 ng/mL) every other day. The number of nuclei per cell was analyzed. n = 8, Scale bars: 500 µm. Values represent the mean ± SD. Differences were assessed by Student's *t*-test. ****p<0.0001.

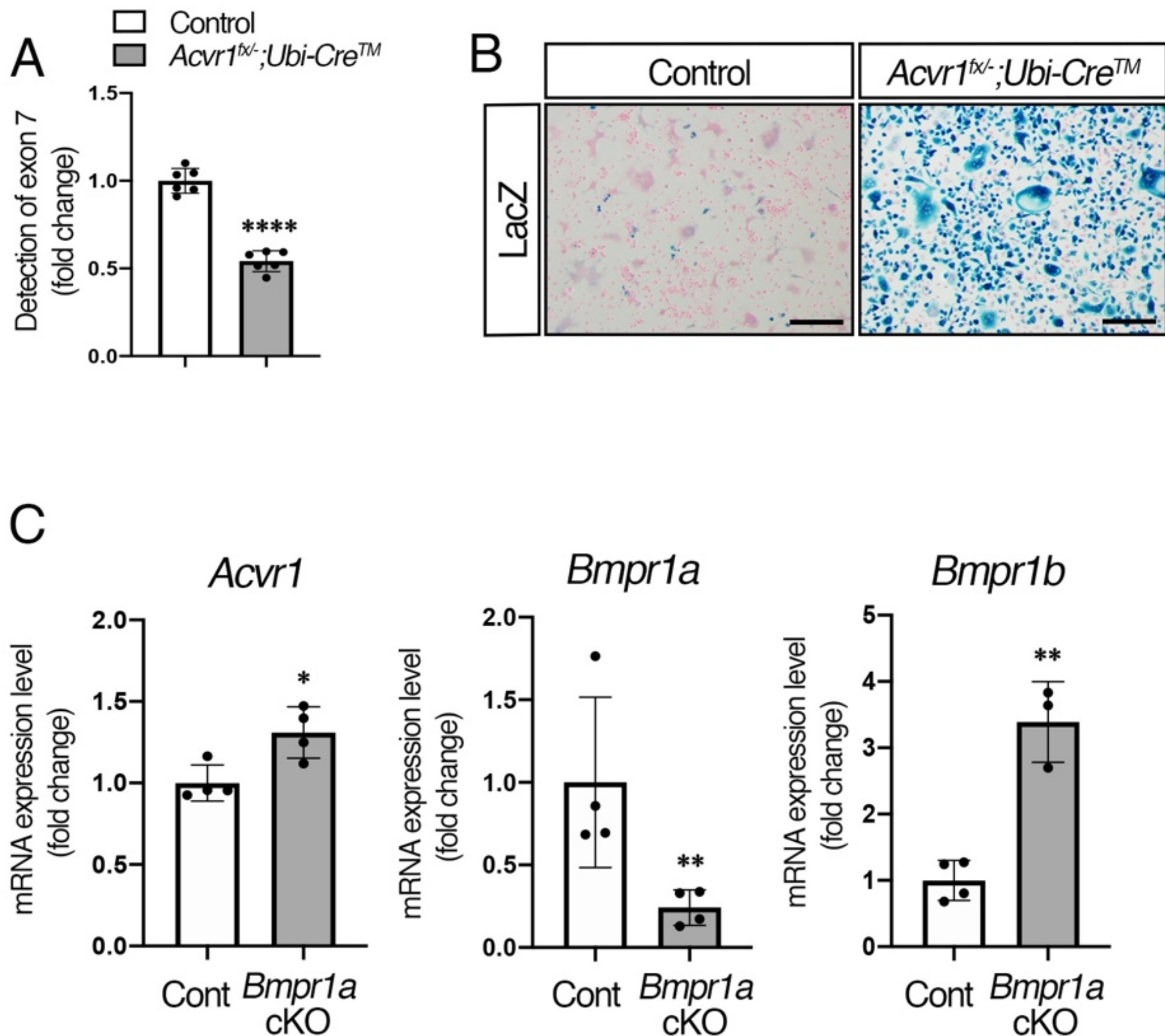

Figure S3. *Acvr1*-floxed alleles were deleted in *Acvr1* cKO osteoclasts. (*A*) BMMs from control and *Acvr1* cKO (*Acvr1*$^{fx/-}$*;Ubi-Cre*^{TM}) mice were incubated with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for 5 days. Cre activity was induced by 4-hydroxytamoxifen (100 ng/mL) in culture. Deletion of *Acvr1* exon 7 was measured by genomic qPCR. n = 6. (*B*) Differentiated osteoclasts were stained with X-gal at day 5. n = 4, Scale bars: 50 µm. (*C*) BMMs from control (*Bmpr1a*$^{fx/+}$*;Ubi-Cre*^{TM}) and *Bmpr1a* cKO (*Bmpr1a*$^{fx/-}$*;Ubi-Cre*^{TM}) mice were incubated with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for 5 days. Cre activity was induced by 4-hydroxytamoxifen (100 ng/mL) in culture. Comparison of expression levels of *Acvr1*, *Bmpr1a,* and *Bmpr1b* between control and *Bmpr1a* cKO osteoclasts were assessed by qRT-PCR. n = 4. Values represent the mean ± SD. Differences were assessed by Student's *t*-test. **p*<0.05, ***p*<0.01, *****p*<0.0001.

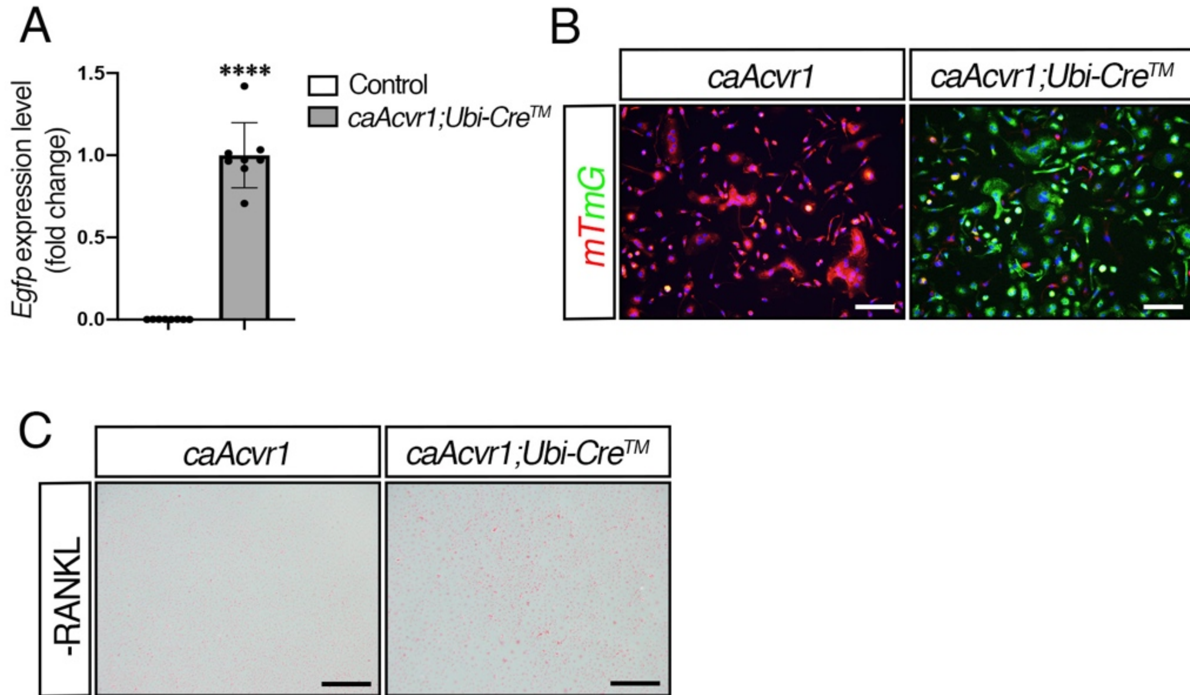

Figure S4. *caAcvr1*-mutant cells did not differentiate into osteoclasts without RANKL. (*A*) Genomic DNA was isolated from control and *caAcvr1*-mutant (*caAcvr1;Ubi-Cre^{TM}*) osteoclasts. Expression levels of *Egfp* were quantified by quantitative real-time PCR as a surrogate of *caAcvr1*. n = 8. (B) BMMs from control (*caAcvr1*) and *caAcvr1*-mutant (*caAcvr1;Ubi-Cre^{TM}*) mice were seeded in 48-well plates (1.5 x 10^4 cells/well) in the presence of M-CSF (20 ng/mL) and RANKL (50 ng/mL), and Cre activity was induced by 4-hydroxytamoxifen (100 ng/ml) at the same time. Cre activity in osteoclasts was monitored by mTmG expression at day 5 of RANKL treatment. n = 4, Scale bars: 200 µm. (*C*) BMMs from control (*caAcvr1*) and *caAcvr1*-mutant (*caAcvr1;Ubi-Cre^{TM}*) were cultured without RANKL for 5 days, and Cre activity was induced by 4-hydroxytamoxifen (100 ng/mL) at the same time. Cells were stained with TRAP and Nuclear Fast Red. n = 4, Scale bars: 500 µm. Values represent the mean ± SD. Differences were assessed by Student's *t*-test. ****p<0.0001.

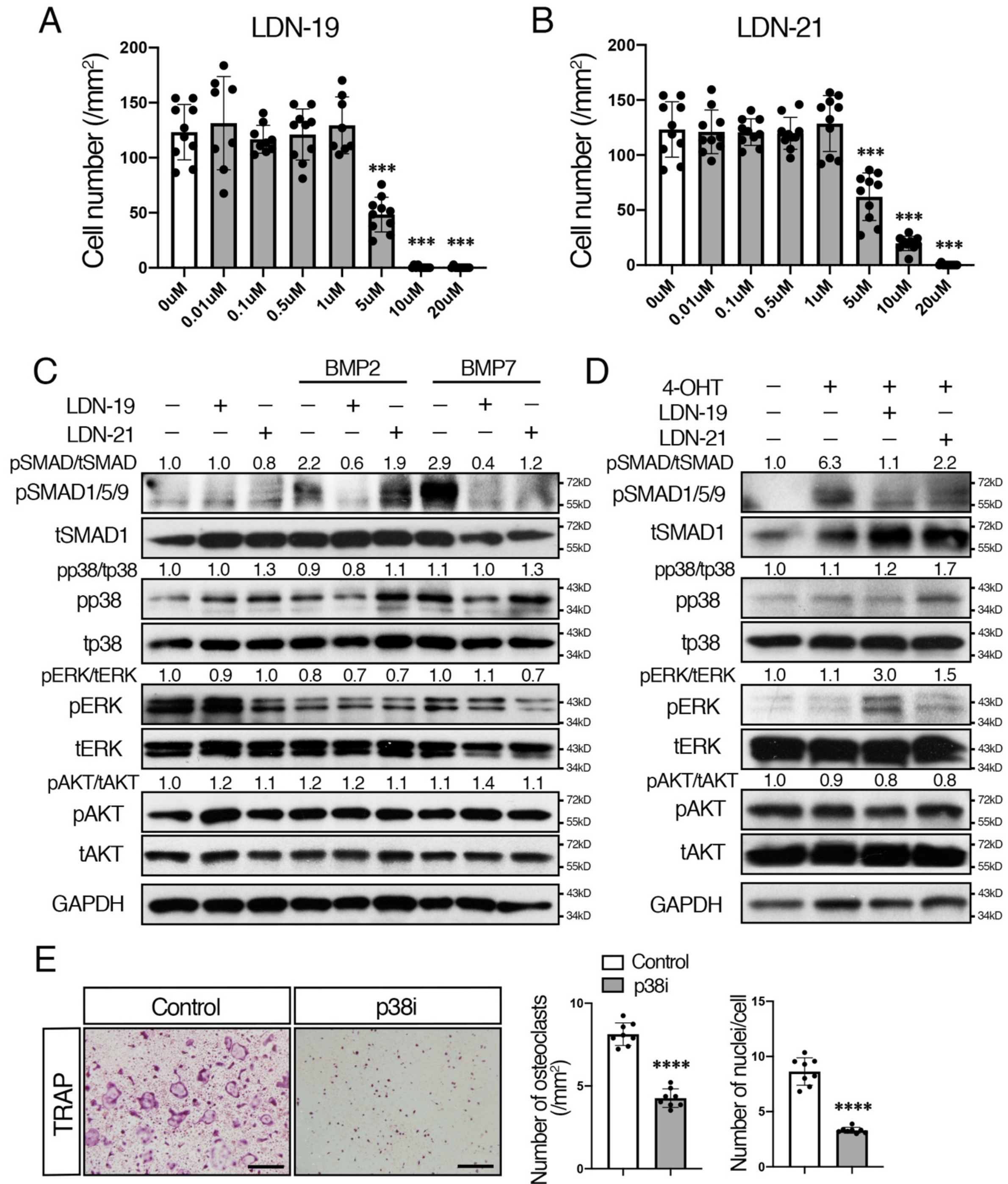

Figure S5. LDN-19 and LDN-21 decreased pSMAD1/5/9 levels but did not alter non-SMAD signaling pathways. (*A*, *B*) BMMs from control mice were treated with LDN-19 (LDN-193189) (A) and LDN-21 (LDN-212854) (B) for 24 hours. Cells were stained with DAPI and cell number was quantified. The value of each group was compared to the non-treatment group. n = 10. (*C*) BMMs from control mice were pre-treated with LDN-19 (1μM) and LDN-21 (1μM) for 2 hours, and cells were stimulated with BMP-2 (100 ng/mL) or BMP-7 (100 ng/mL) for 30 minutes. Control cells were treated with DMSO at a final concentration of 0.1%. Total and phosphorylated forms of SMAD1/5/9, p38, ERK, and AKT were measured by western blot. n = 3. (*D*) BMMs from control (*caAcvr1*) and *caAcvr1*-mutant (*caAcvr1;Ubi-*

Cre^{TM}) mice were incubated with LDN-19 and LDN-21 for 2 days, and Cre activity was induced by 4-hydroxytamoxifen (4-OHT, 100 ng/mL) at the same time. Fold increases of protein levels are relative to unstimulated control cells. Representative images of protein bands are shown, n = 3. (*E*) BMMs from control mice were seeded in 48-well plates (1.5 x 10^4 cells/well) with M-CSF (20 ng/mL) and RANKL (100 ng/mL) for 5 days and TRAP staining was conducted. Cells were treated with p38 MAPK inhibitor (SB203580, 1μM). Control cells were treated with DMSO at a final concentration of 0.1%. n = 8, Scale bars: 500 μm. Values represent the mean ± SD. Differences were assessed by one-way ANOVA, followed by a Turkey test. ***p<0.001.

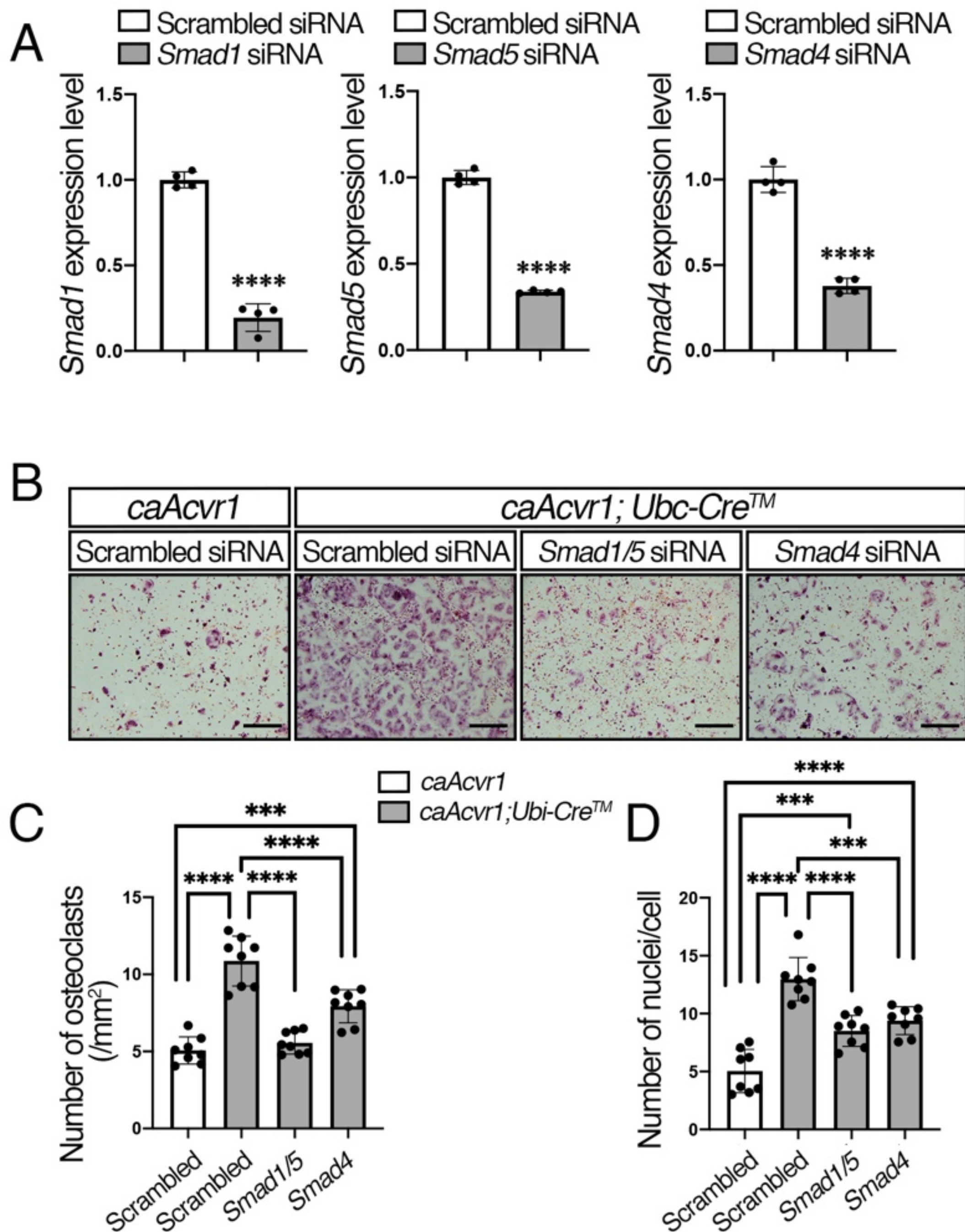

Figure S6. *Smad1/5* and *Smad4* gene silencing reduced osteoclast differentiation of *caAcvr1*-mutant cells. (*A*) BMMs from control mice were transfected with siRNAs against *Smad1*, *Smad5* and *Smad4* for 24 hours. Total RNAs were extracted and gene expression levels were measured by qRT-PCR. n = 4. (*B*) BMMs from control (*caAcvr1*) and *caAcvr1*-mutant (*caAcvr1;Ubi-Cre^{TM}*) mice were transfected with siRNAs against *Smad1*, *Smad5* and *Smad4,* and seeded in 48-well plates (1.5 x 10^4 cells/well) with M-CSF (20 ng/mL), RANKL (100 ng/mL) and 4-hydroxytamoxifen (100 ng/mL) for 5 days and TRAP staining was conducted. Scale bars: 500 µm. (*C*) TRAP-positive cells containing 3 or more nuclei were counted as osteoclasts. n = 8. (*D*) The number of nuclei per cell was analyzed. n = 8. Values represent the

mean ± SD. Differences were assessed by one-way ANOVA, followed by a Turkey test, *p<0.05, ***p<0.001, ****p<0.0001.

Figure 2B

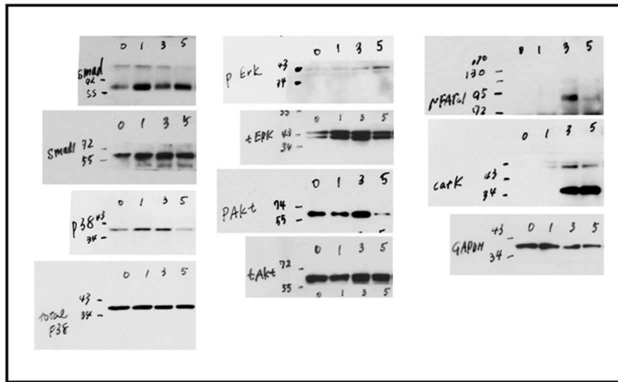

Figure 2C

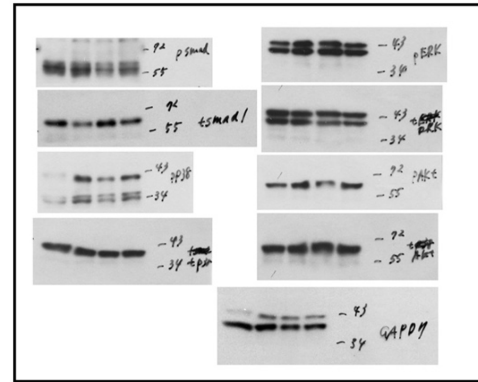

Figure 4A

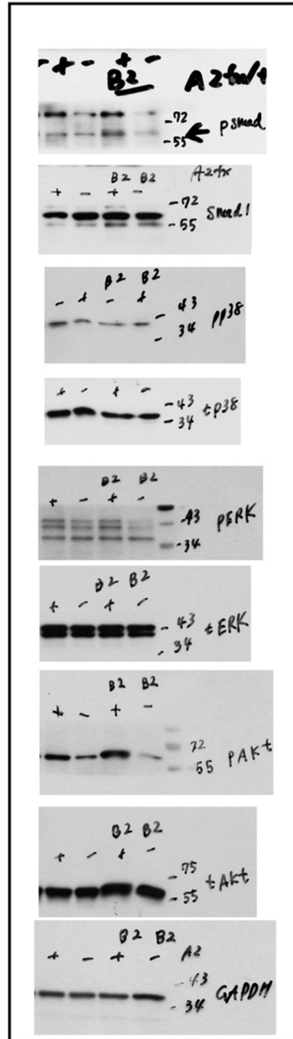

Figure 4B

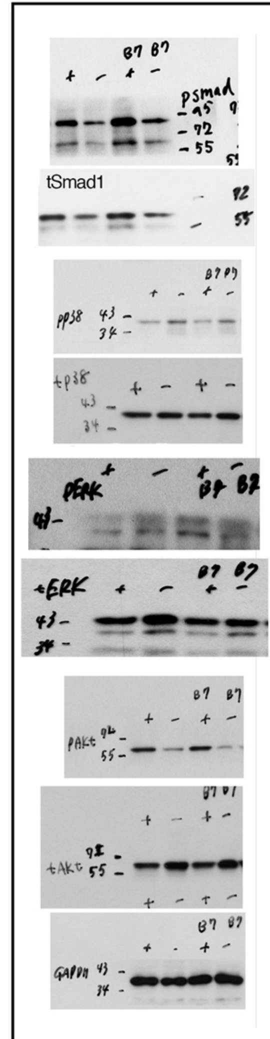

Figure 4C

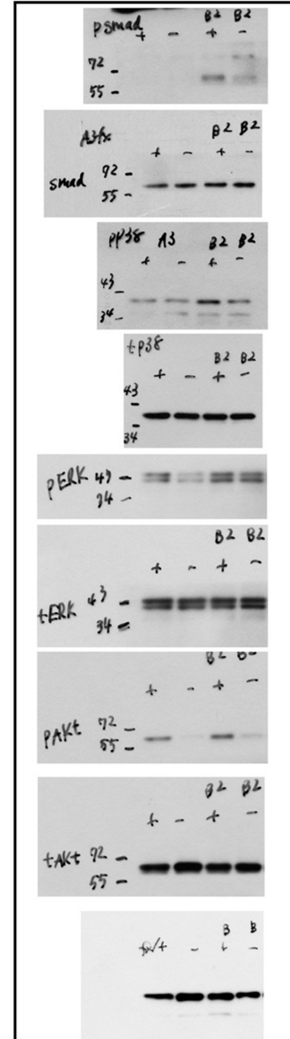

Figure 4D

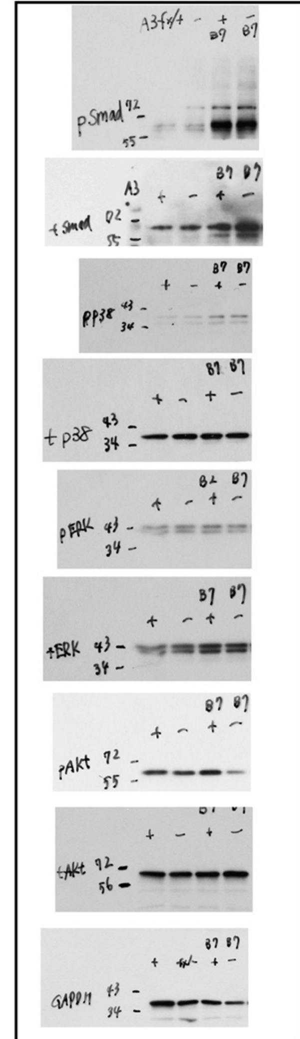

Figure 5H

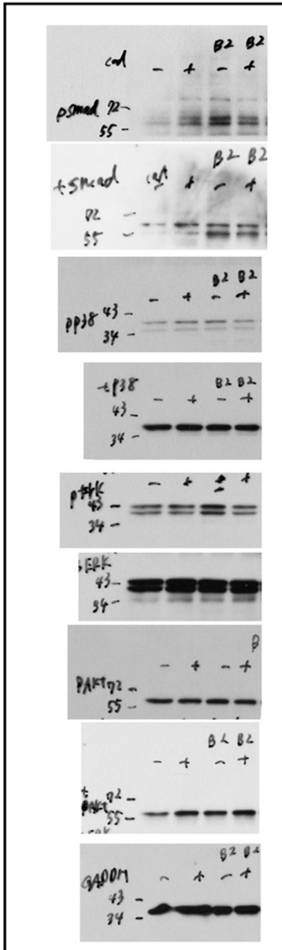

Figure 5I

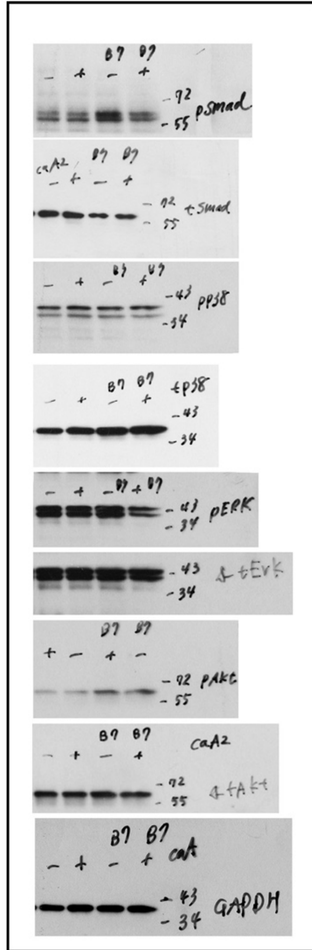

Figure 7A

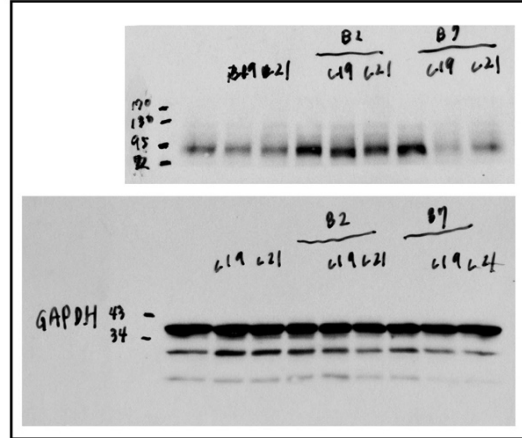

Figure 7B

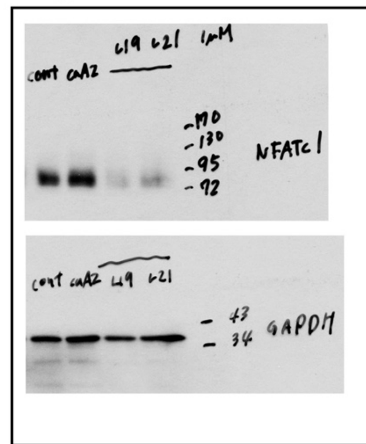

Figure 8D

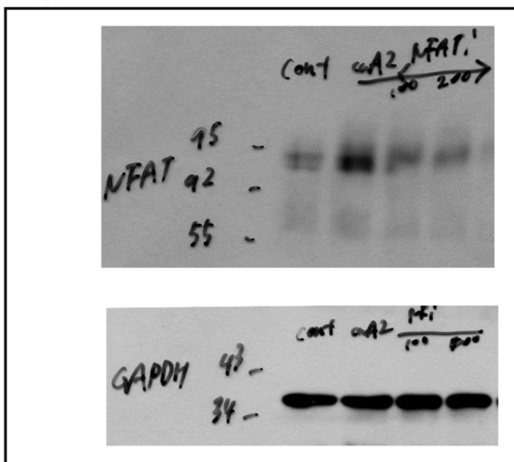

Figure S7. The uncropped films of western blot results shown in Fig. 2, Fig. 4, Fig. 5, Fig. 7 and Fig. 8. For all the experiments detections of SMAD1/5/9 and AKT, and ERK and p38 were performed on different membranes, due to the similar molecular weight. The samples were derived from the same experiment.