Supporting Information

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SI Methods

Isolation and Culture of Osteoblasts. Mouse calvarial osteoblasts were isolated from the calvarial bones of 2-day-old C57BL/6 mice by sequential collagenase digestion and grown to confluence as previously described (1). Both mouse and human cells (human osteosarcoma HosTe85) were seeded into 96-well plates at a density of 1×10^4 cells/well and allowed to adhere overnight. Fresh medium containing O-1602 with or without CBD or 40 nM bovine parathyroid hormone (Bachem) as a positive control was added the following day. After 24, 48, and 72 h, cell viability was determined using an AlamarBlue assay; then alkaline phosphatase activity was measured using a colorimetric assay (2). Results were corrected for total cell viability.

To determine effects of O-1602 on mineralization, mouse calvarial osteoblasts were cultured in mineralization medium that contained 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate. Cells were maintained under these conditions for 21 days; medium and test substances were refreshed every 2 to 3 days. Cells were fixed and maintained in ethanol before staining for mineral deposits (2) using 40 mM Alizarin Red S (Sigma) at pH 4.1 or von Kossa reagent.

Primary human osteoblasts were isolated from the femoral heads of patients undergoing total hip replacement surgery using an adaptation of methods previously described (3). Bone fragments were seeded into 75-cm^2 tissue-culture flasks containing D-MEM Glutimax (Gibco) supplemented with 10% FCS and 100 IU/mL penicillin. Confluent monolayers (approximately 5 weeks) were trypsinized, and osteoblasts were seeded onto glass coverslips at a density of 0.5×10^5 cells/well in 24-well plates and left to adhere overnight before being fixed in 4% paraformaldehyde for immunostaining.

GPR55 Expression. Total RNA was isolated from human M-CSF–dependent monocytes and multinucleated osteoclasts (generated from the same blood donor) using TRIzol (Invitrogen) and purified using the RNeasy micro kit (Qiagen). cDNA was prepared using the Omniscript reverse transcription kit (Qiagen) and then was quantified using TaqMan Primers (Applied Biosystems): GPR55 Hs00271662, FAM-labeled and GAPDH NM_002046, VIC-labeled; 2 μL cDNA in 20-μL reaction at 95 °C for 15 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min using an Opticon2 qPCR machine (MJ Research). There was no difference in the GAPDH mRNA levels in monocytes and osteoclasts.

To detect GPR55 protein, osteoclasts and osteoblasts were fixed with 4% paraformaldehyde in PBS and stained using a rabbit antibody to GPR55 (4) and Alexa Fluor goat anti-rabbit 488 antibody (Invitrogen) and then were counterstained with 0.5 μM TO-PRO3 iodide (Invitrogen). Control cells were stained either with normal rabbit IgG (Santa Cruz) or with anti-GPR55 in the presence of 5 μg/mL immunizing protein. Cells were examined on a Zeiss LSM510 Meta confocal microscope, and the images were captured using the LSM software.

Generation of GPR55 Knockout Mice. For targeting vector construction, a 1.98-kb 5′ homology and a 5.11-kb 3′ homology fragment were amplified by Pfu PCR from a bacterial artificial chromosome that contained the mouse GPR55 gene. Both fragments were subcloned and sequenced with 3100 BigDye terminator (v3.1 Matrix Standard, Applied Biosystems). A region containing the complete GPR55 coding sequence in exon 2 was replaced with a loxP-flanked Neo cassette. The targeting vector was linearized and electroporated into R1/E ES cells and selected with G418 (300 mg/mL). ES-cell clones that successfully underwent homologous recombination were identified by PCR screening and then were confirmed by Southern blotting. The targeted ES-cell clones were injected into C57BL/6 blastocysts, which were implanted into pseudo pregnant female mice. Chimeric males were used for breeding with C57BL/6 mice to produce germ line-transmitted N1 offspring. The Neo cassette was deleted by breeding the N1 mice with Rosa26Cre mice having a pure C57BL/6 background. The GPR55-mutant mice were obtained by intercrossing the heterozygous mice. Genotyping of the offspring was performed by PCR to detect wild-type and mutant alleles by using the following primers: 5′-ATGCGGAATTCCTGTTACCCA-3′ (forward), 5′-CACCCTAGGGCCTCAGTTGTA-3′ (reverse) and 5′-GGAAAGCTGAGATACAGACTT-3′ (reverse). For RT-PCR analysis, the cerebrums were dissected from 8-week littermates of GPR55 knockout homozygous, heterozygous, and wild-type mice. Reverse transcription was run with SuperScript First-Strand kit (Invitrogen) followed by PCR using 1 primer located in the upstream exon (5′-GGAAAGGGGACTTCTCTTGG-3′) and 1 primer in the deleted region (5′-CACTCCACCAGAGTGCAGAA-3′). The mouse strain was backcrossed 6 generations toward C57BL/6. Mice that were heterozygous for the deletion were intercrossed to produce homozygous knockout and wild-type littermate control mice.

1. Crockett JC, et al. (2007) The matricellular protein CYR61 inhibits osteoclastogenesis by a mechanism independent of alphavbeta3 and alphavbeta5. *Endocrinology* 148:5761–5768.
2. Scutt A, Reading L, Scutt N, Still K (2003) Mineralizing fibroblast-colony-forming assays. *Methods in Molecular Medicine* 80:29–39.
3. Beresford JN, Gallagher JA, Poser JW, Russell RG (1984) Production of osteocalcin by human bone cells in vitro. Effects of 1,25(OH)2D3, 24,25(OH)2D3, parathyroid hormone, and glucocorticoids. *Metabolic Bone Disease and Related Research* 5:229–234.
4. Lauckner JE, et al. (2008) GPR55 is a cannabinoid receptor that increases intracellular calcium and inhibits M current. *Proc Natl Acad Sci USA* 105:2699–2704.

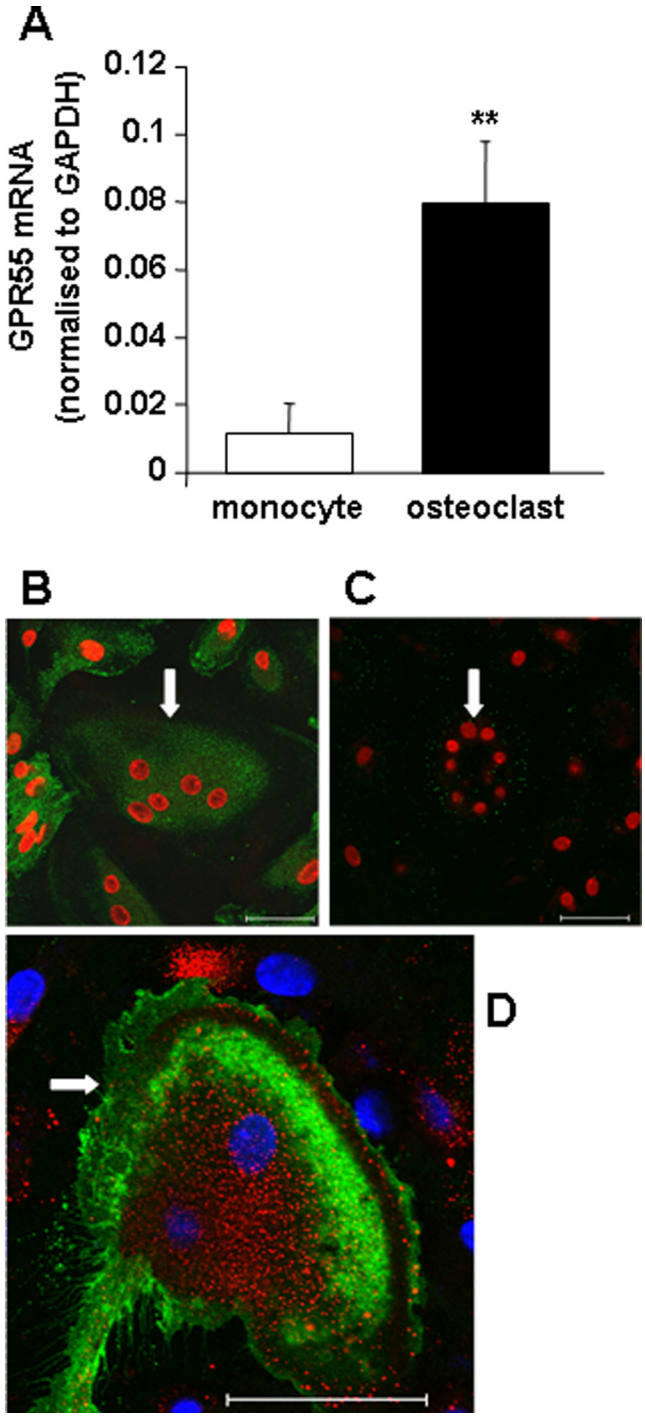

Fig. S1. GPR55 is expressed in human osteoclasts. (*A*) Quantitative real-time RT-PCR was performed using a primer TaqMan probe set specific for GPR55 on RNA isolated from human monocytes and differentiated osteoclasts (after 7 days' treatment with RANKL + M-CSF). GPR55 mRNA levels were normalized to GAPDH. The ratio of GPR55/GAPDH expression is shown; values are mean of 7 donors (3 male and 4 female) ± SEM. Levels were measured in triplicate for each donor. **, $P < 0.01$, Student's *t* test. (*B*) GPR55 expression in human, multinucleated osteoclasts (*arrow*) was confirmed by immunofluorescence staining for GPR55 (*green*), with a nuclear counterstain in red. (*C*) Immunostaining control was normal rabbit IgG. (*D*) Punctate staining of GPR55 (*red*) in the cytosol and adjacent to the plasma membrane in a human osteoclast (*arrow*) co-stained for $\alpha v\beta 3$ (*green*) and nuclei (*blue*). (Scale bars: 50 μm.)

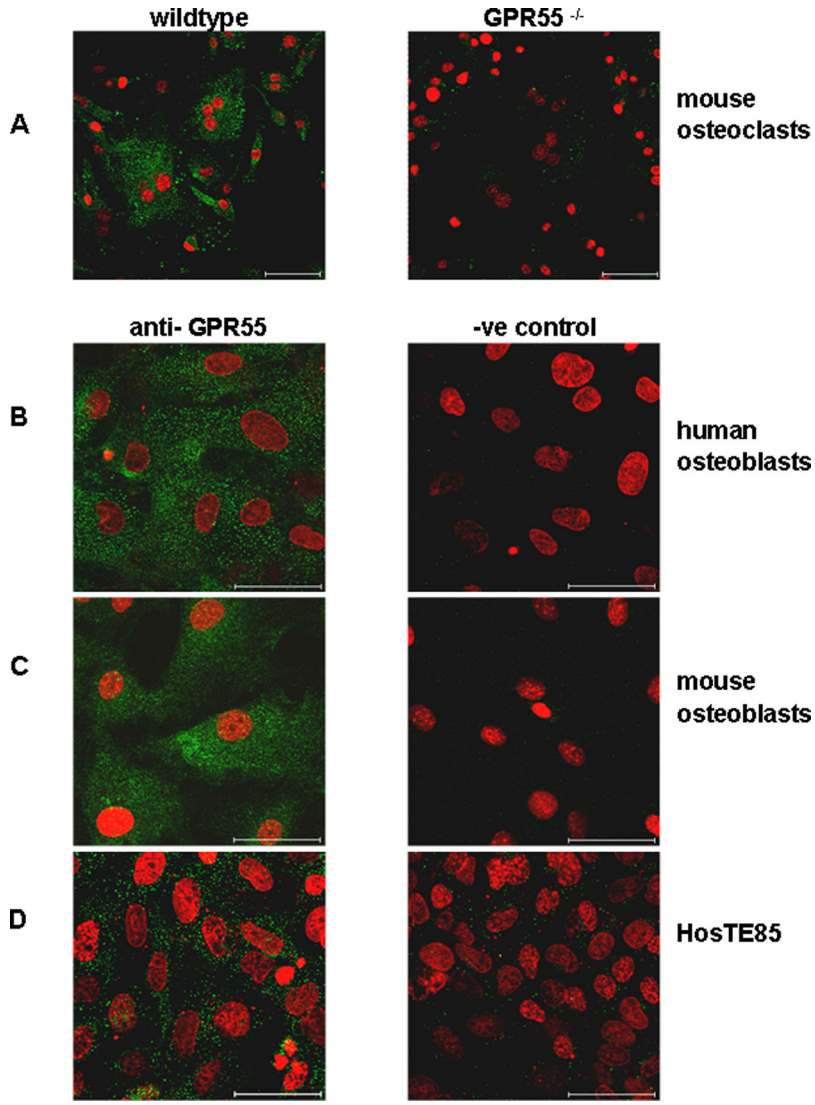

Fig. S2. GPR55 is expressed in osteoclasts and osteoblasts. GPR55 expression was detected by immunofluorescence staining for GPR55 (*green*), with a nuclear counterstain (*red*) and visualized by confocal microscopy. (*A*) Mouse osteoclasts generated from wild-type and GPR55$^{-/-}$ BMMs. (*B–D*) Human primary osteoblasts, mouse calvarial osteoblasts, and HosTe85 osteoblast-like cells. Negative controls were stained with normal rabbit IgG or with anti-GPR55 in the presence of 5 μg/mL immunizing protein. (Scale bar: 50 μm.)

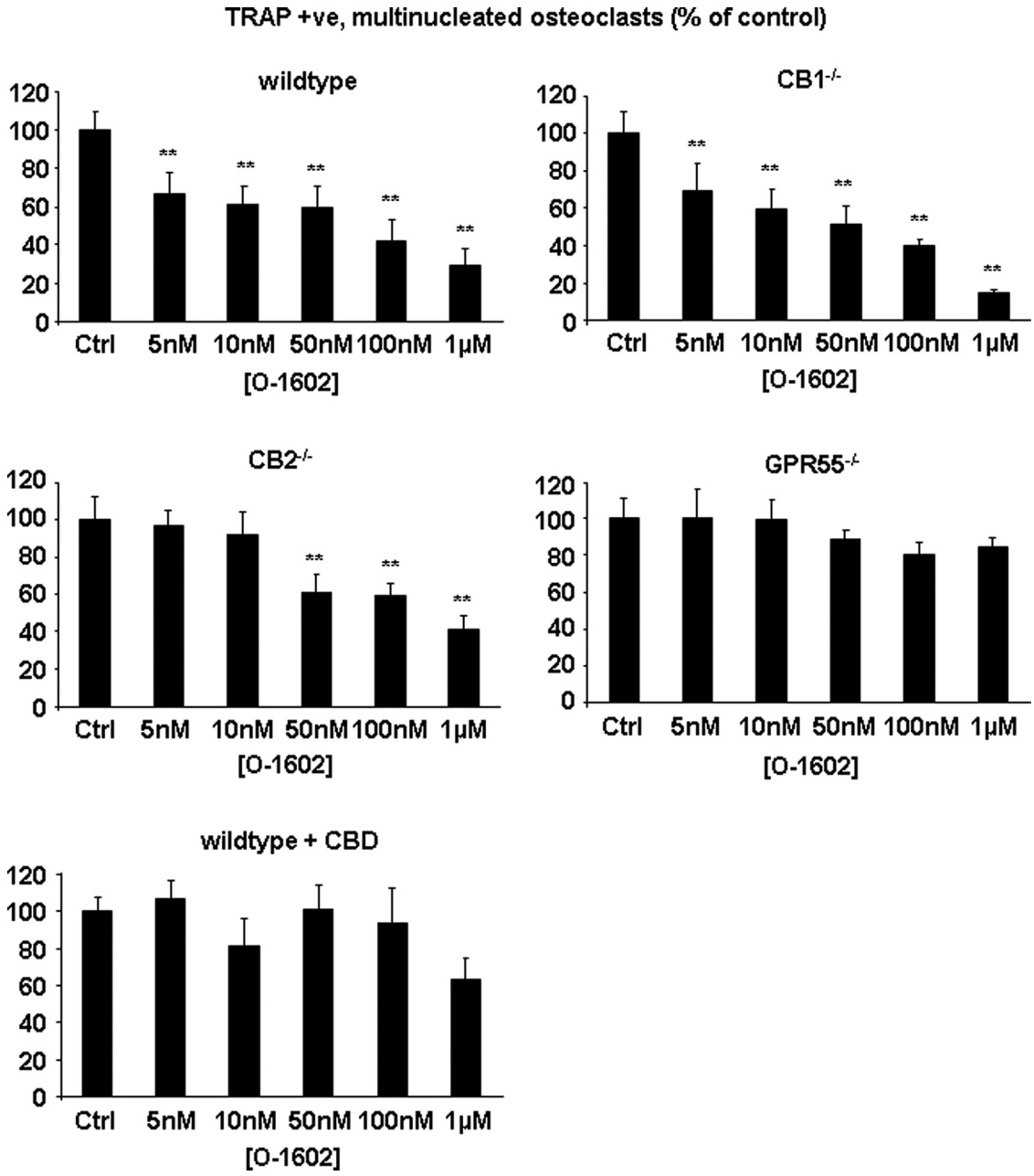

Fig. S3. O-1602 inhibits osteoclast formation from wild-type, CB1$^{-/-}$, and CB2$^{-/-}$, but not GPR55$^{-/-}$, macrophages. M-CSF–dependent BMMs from wild-type, CB1$^{-/-}$, CB2$^{-/-}$, or GPR55$^{-/-}$ mice were cultured in 96-well plates with M-CSF plus RANKL in the presence of 1 nM to 1 μM O-1602 ($\pm$ 500 nM CBD with wild-type cells) for 5 days and then were fixed and stained for TRAP. The number of TRAP-positive multinucleated cells was counted and expressed as a percentage of control. Data are mean $\pm$ SEM; $n = 4$ or 5 experiments, 5 replicates for each. ANOVA with Bonferroni post-test. **, $P < 0.01$ compared with control cultures. The data on GPR55$^{-/-}$ cells from Fig. 1*B* are shown for comparison.

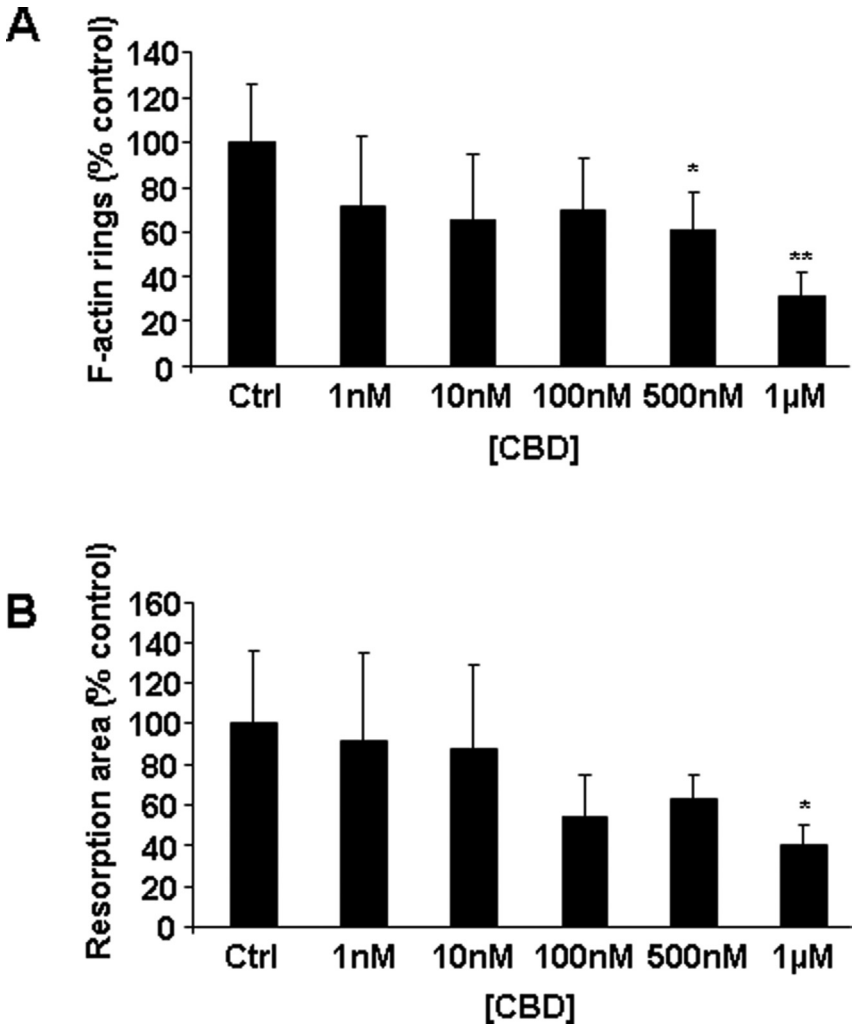

Fig. S4. CBD inhibits human osteoclast polarization and resorption. M-CSF–dependent human monocytes were cultured on dentine in the presence of RANKL for 7 days and then were treated with vehicle (0.1% DMSO) or with 1 nM to 1 μM CBD for a further 5 days. (*A*) The number of F-actin rings expressed as percentage of control cultures ± SEM ($n = 5$ experiments with 5 replicates for each). ANOVA with Bonferroni post-test. *, $P < 0.05$; **, $P < 0.01$. (*B*) Resorption area quantified using reflective light microscopy and expressed as percentage of control ± SEM ($n = 4$ experiments with 5 replicates for each). ANOVA with Bonferroni post-test. *, $P < 0.05$ compared with control cultures.

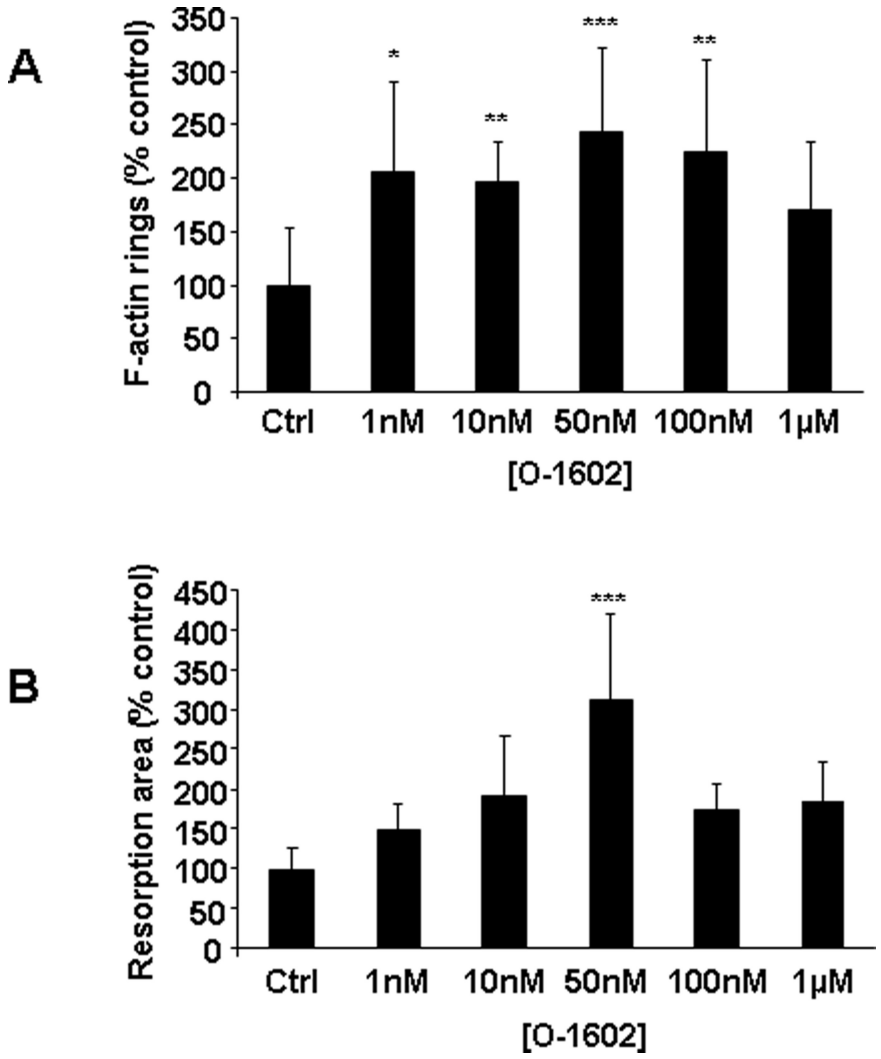

Fig. S5. O-1602 stimulates mouse osteoclast polarization and resorption. Mouse osteoclast-like cells, generated from a co-culture of osteoblasts and bone marrow cells on a collagen matrix, were removed by collagenase digestion, and the resulting cells were cultured on dentine discs for 48 h in the presence of vehicle (0.1% DMSO) or 1 nM to 1 μM O-1602. (*A*) The number of F-actin rings expressed as a percentage of control ± SEM. (*B*) Resorption area was quantified using reflected light microscopy and expressed as percentage of control ± SEM ($n = 3$ experiments with 3–5 replicates for each). ANOVA with Bonferroni post-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control cultures.

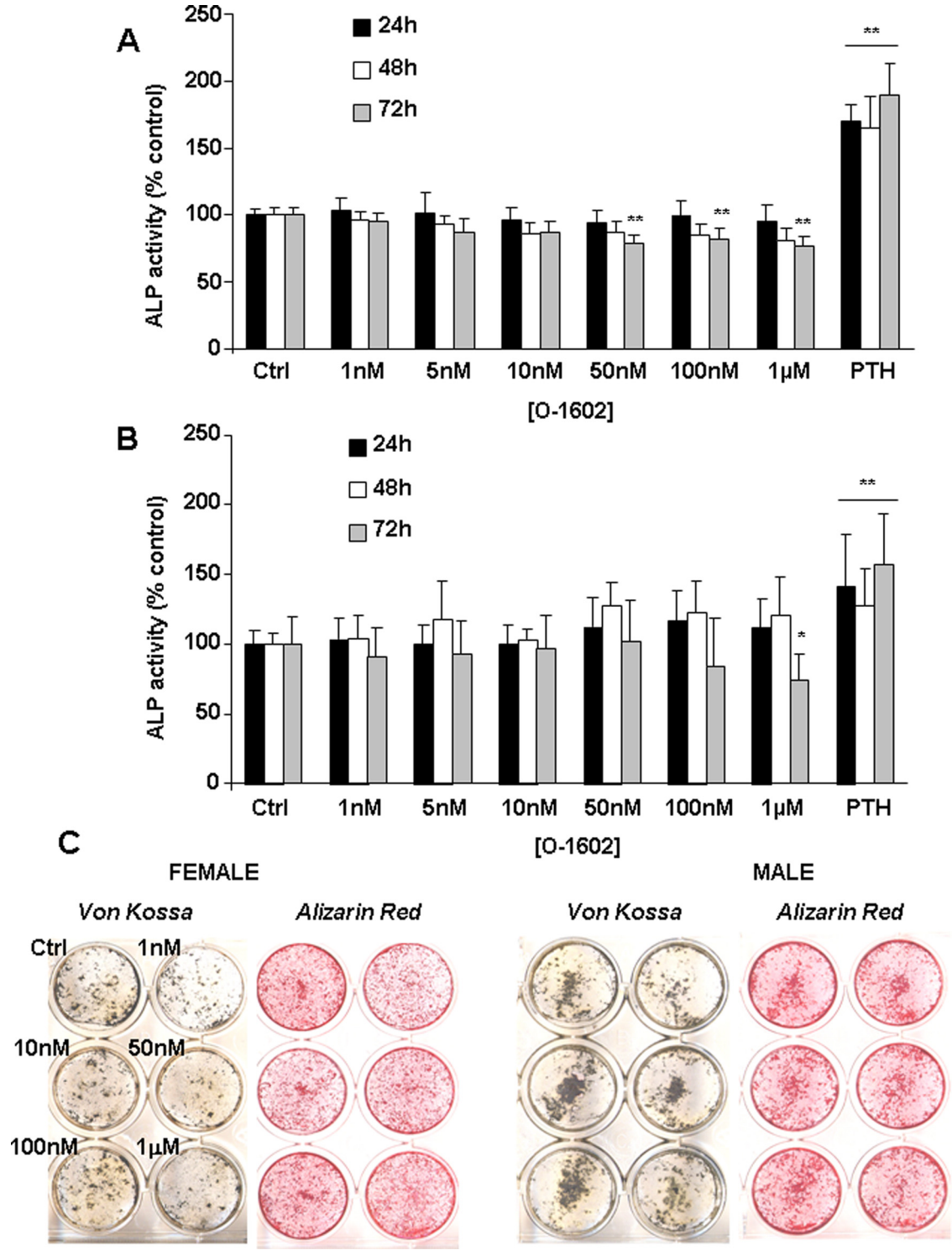

Fig. S6. Effect of O-1602 on osteoblastic alkaline phosphatase activity and mineralization. (*A*) Mouse calvarial osteoblasts and (*B*) human HosTe85 osteoblast-like cells were treated with O-1602 for 24, 48, or 72 h and were assayed for alkaline phosphatase activity using a colorimetric assay. Data were corrected for cell viability and presented as mean ± SEM; $n = 7$–10 experiments (mouse); $n = 4$ experiments (human). ANOVA with Bonferroni post-test; *, $P < 0.05$; **, $P < 0.01$, compared with control cultures at the same time point. (*C*) Representative images of confluent mouse calvarial osteoblasts that had been cultured in mineralization medium with vehicle or 1 nM to 1 μM O-1602 for 3 weeks, fixed, and stained using Alizarin Red or von Kossa reagent.

Table S1. Histomorphometric analysis of sections of femora from 12-week-old male and female wild-type or GPR55$^{-/-}$ mice

	Male		Female	
Section	Wild-Type	GPR55$^{-/-}$	Wild-Type	GPR55$^{-/-}$
BV/TV (%)	14.3 ± 1.741	20.4 ± 1.5*	8.8 ± 1.6	9.6 ± 1.3
TbTh (mm)	37.4 ± 3.7	46.5 ± 3.3	34.1 ± 1.4	37.3 ± 2.6
TbN (/mm)	3.80 ± 0.24	4.44 ± 0.29	2.55 ± 0.38	2.52 ± 0.17
TbSp (mm)	228 ± 17	184 ± 12*	382 ± 56	373 ± 30
OTh (mm)	2.47 ± 0.29	2.44 ± 0.16	2.48 ± 0.17	2.95 ± 0.16
ObS/BS (%)	13.0 ± 3.4	18.1 ± 1.9	28.4 ± 3.2	31.5 ± 1.8
NOb/BPm (/mm)	8.9 ± 2.6	12.2 ± 1.3	18.3 ± 2.0	21.6 ± 1.5
OcS/BS (%)	4.7 ± 0.9	14.2 ± 2.4*	15.2 ± 2.0	11.9 ± 0.9*
NOc/BPm (/mm)	1.21 ± 0.20	3.66 ± 0.64*	3.89 ± 0.39	2.94 ± 0.18*
OcL (μm)	38.5 ± 1.4	38.9 ± 1.1	38.9 ± 2.0	40.4 ± 1.4
CtgV/BV (%)	4.36 ± 1.56	9.22 ± 1.69*	0.70 ± 0.35	2.79 ± 0.59*

All values are mean ± SEM from 4 wild-type and 8 GPR55$^{-/-}$ animals. *, $P < 0.05$. BV/TV, bone volume/tissue volume; CtgV/BV, cartilage volume; NOb/BPm, osteoblast number/bone perimeter; NOc/BPm, osteoclast number/bone perimeter; ObS/BS, osteoblast surface as % of bone surface; OcL, osteoclast length; OcS/BS, osteoclast surface as % of bone surface; OTh osteoid thickness; TbN, trabecular number; TbSp, trabecular separation; TbTh, trabecular thickness.