Supplementary Information

Interferon regulatory factor 8 regulates bone metabolism by suppressing osteoclastogenesis

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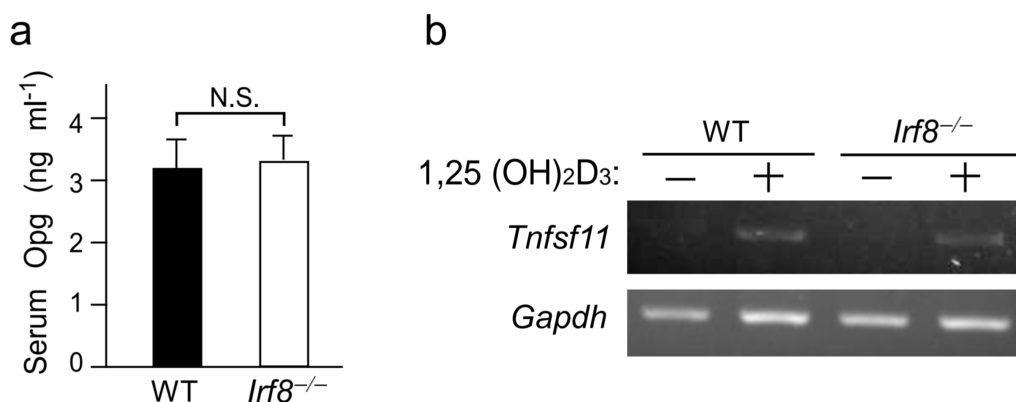
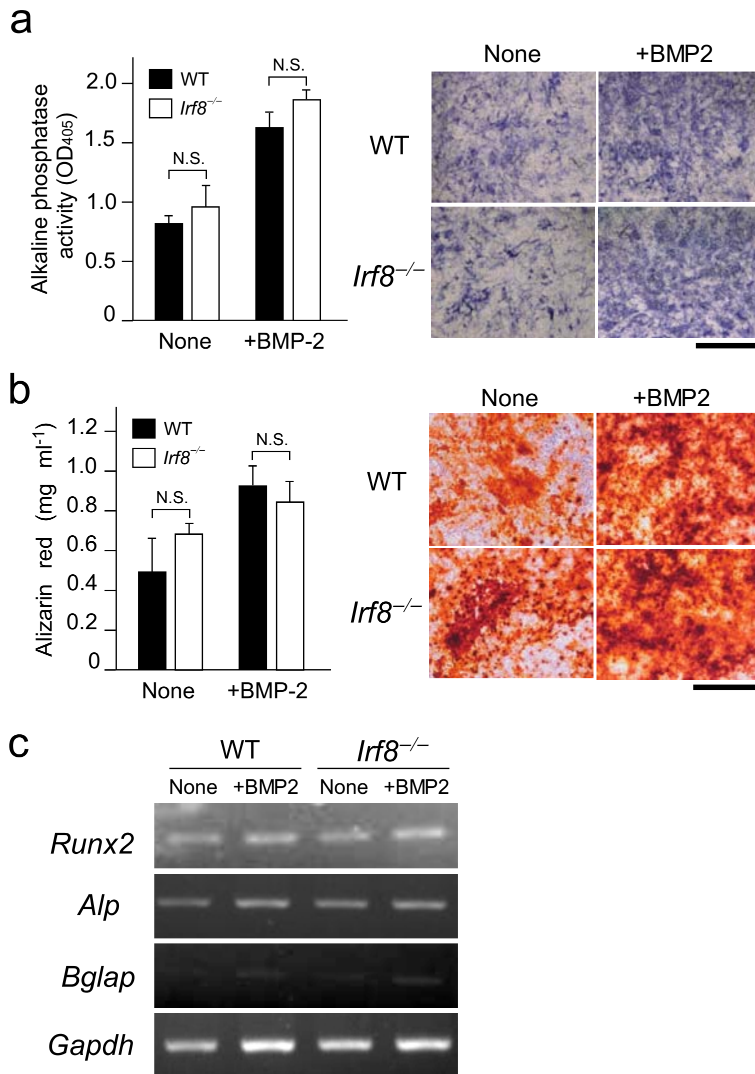
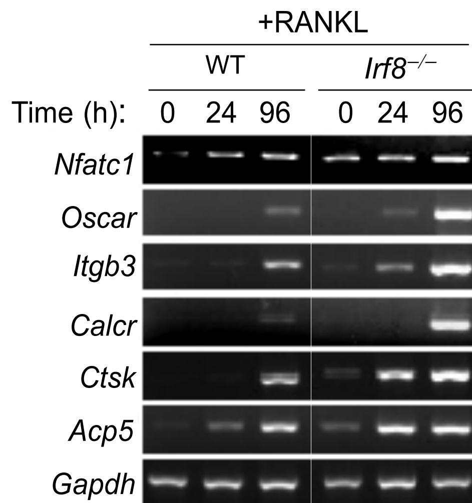


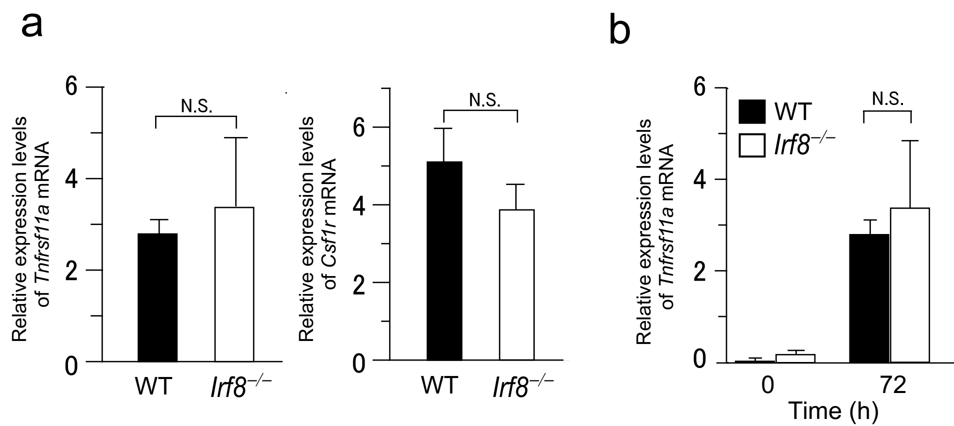
Supplementary Figure 1 Serum Opg protein levels and *Rankl* mRNA expression levels in osteoblasts from wild-type and *Irf8*$^{-/-}$ mice. (**a**) Mouse sera were collected and Opg protein levels determined using an immunoassay kit (R&D Systems) (n = 6 for each group). N.S., not significant. (**b**) Mouse calvarial osteoblasts derived from wild-type and *Irf8*$^{-/-}$ mice were stimulated with 10 nM 1,25$(OH)_2D_3$ for 24 h. The expression of *Tnfsf11* mRNA encoding RANKL protein was examined using RT-PCR.



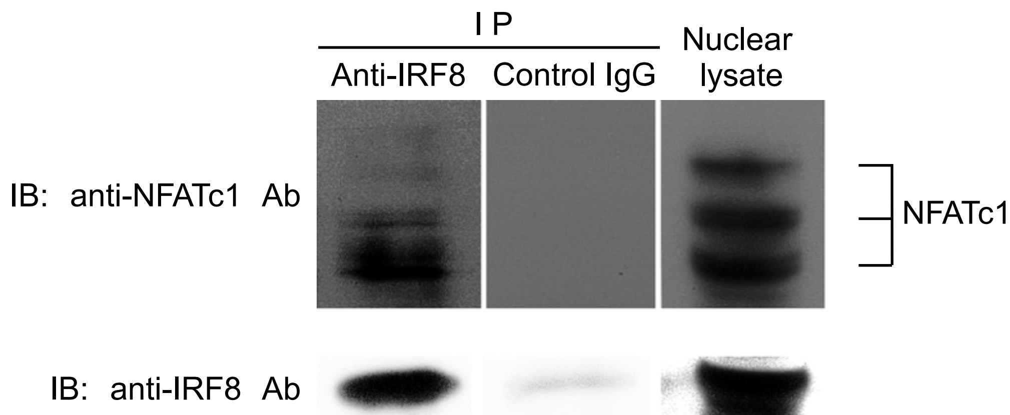
Supplementary Figure 2 Differentiation of osteoblasts derived from wild-type and *Irf8*$^{-/-}$ mice. Primary calvarial osteoblasts prepared from wild-type and *Irf8*$^{-/-}$ mice were cultured in the presence or absence of 100 ng/ml of BMP2 for 3 (**a**,**c**) or 7 (**b**) days, after which the following experiments were performed. (**a**) Alkaline phosphatase (ALP) activities in the cell lysates were determined (left). The cells were also stained for ALP, with ALP–positive cells appearing in blue (right). Scale bar, 50 µm. (**b**) The mineralized matrix was stained with alizarin red (right). Concentrations of dissolved alizarin red in the media were determined (left). Scale bar, 50 µm. (**c**) mRNA expression levels of osteoblast-related genes, including *Runx2* (encoding Runx2), *Alp* (encoding alkaline phosphatase), and *Bglap* (encoding osteocalcin), were analyzed by RT–PCR. N.S., not significant. Data are shown as the mean + s.d. (n = 3).



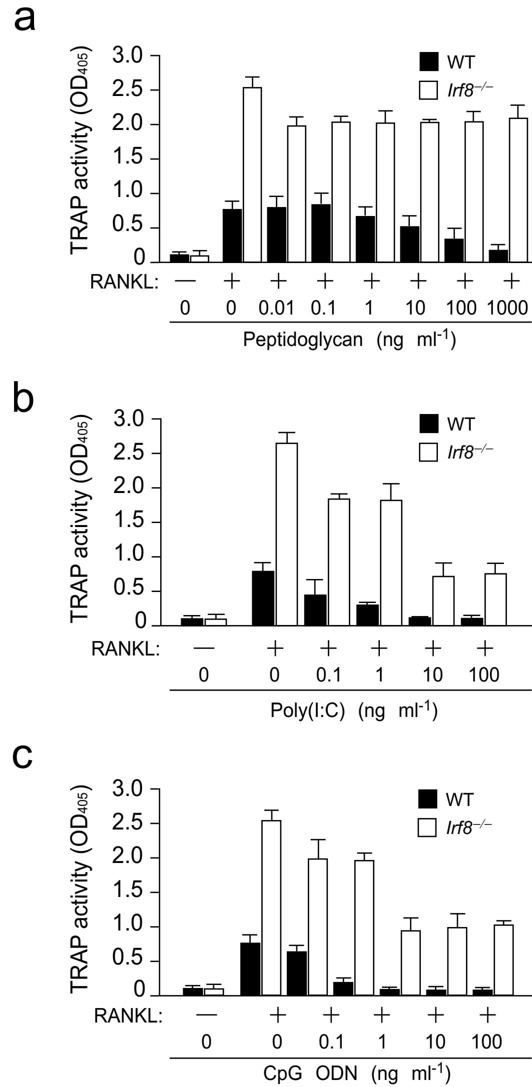
Supplementary Figure 3 Expressions of osteoclast-related genes during osteoclastogenesis BMMs prepared from wild-type and *Irf8*$^{-/-}$ mice were stimulated with 150 ng/ml of RANKL in the presence of 50 ng/ml of M-CSF for the indicated periods. Subsequently, mRNA expression levels of osteoclast-related genes were analyzed by RT-PCR.



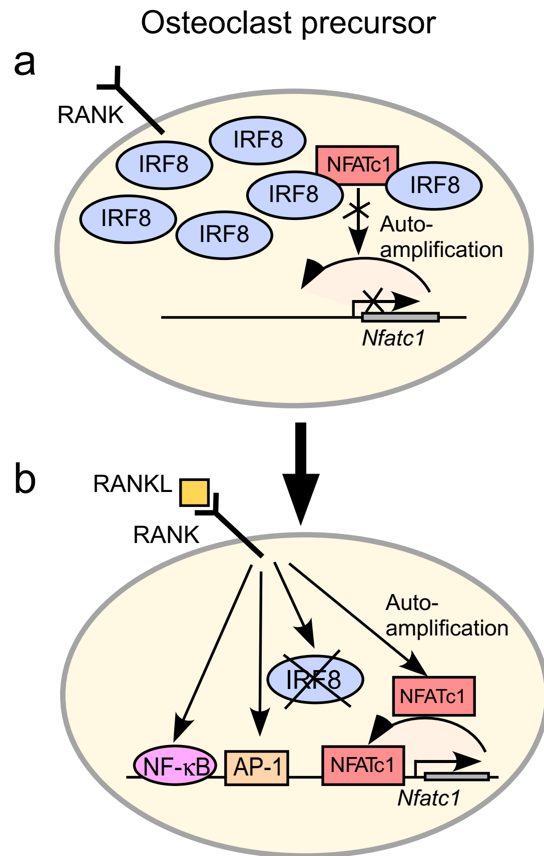
Supplementary Figure 4 Expression levels of *Tnfrsf11a* and *Cfs1r* mRNAs in BMMs (**a**) Bone marrow cells obtained from wild-type and *Irf8*$^{-/-}$ mice were stimulated with 20 ng/ml of M-CSF for 3 days. Subsequently, mRNA expression levels of *Tnfrsf11a* (encoding RANK protein) and *Cfs1r* (encoding c-Fms protein) were analyzed by real-time quantitative PCR. (**b**) *Tnfrsf11a* mRNA expression levels before and after stimulation with RANKL (150 ng/ml) were analyzed by real-time quantitative PCR. Data are shown as the mean + s.d. (n = 3). N.S., not significant.



Supplementary Figure 5 Interaction between endogenous NFATc1 and IRF8 proteins. Immunoprecipitation (IP) using IRF8 or control irrelevant antibodies (Abs) was performed from nuclear extracts of human THP1 monocytic cells that had been stimulated with ionomycin (100 ng/ml) to induce NFATc1 nuclear translocation. Each immunoprecipitate was analyzed by immunoblotting with specific antibodies to NFATc1and IRF8.



Supplementary Figure 6 Effects of TLR ligands on osteoclast formation. BMMs prepared from wild-type and *Irf8*$^{-/-}$ mice were stimulated with various doses of peptidoglycan (**a**), poly(I:C) (**b**), and CpG oligodeoxynucleotides (CpG ODN) (**c**) in the presence or absence of RANKL (150 ng/ml) with M-CSF (50 ng/ml) for 5 days. TRAP activity in each cell lysate was measured. Data are shown as the mean + s.d. ($n = 3$).



Supplementary Figure 7 Model for inhibition of osteoclastogenesis by IRF8. (**a**) Abundant IRF8 was found expressed in osteoclast precursors, in which there was also moderate basal NFATc1 expression. IRF8 bound to NFATc1 to suppress its activity and induced NFATc1 target genes. (**b**) RANKL stimulation induced NF-κB and AP-1 activation, and reduced IRF8 levels. Cooperation between positive signals delivered by NF-κB and AP-1, and release of NFATc1 from inhibition by IRF8, which enables auto-amplification of NFATc1 expression, resulted in subsequent robust induction of NFATc1 expression. Increased levels of NFATc1 promoted osteoclastogenesis by inducing osteoclast-related genes, such as *Acp5* (encoding TRAP protein), *Oscar*, and *Ctsk* (encoding cathepsin K protein).

SUPPLEMENTARY METHODS

***In vitro* osteoblast differentiation and matrix calcification assay.** Primary osteoblast progenitor cells were isolated from the calvarial bone of newborn (1–3 d) mice and cultured in α−MEM with 10% FBS containing 50 mg/ml ascorbic acid, 10 mM β−glycerophosphate and 10 nM dexamethasone. ALP staining and activity assay were performed after 3 d culture as described[1]. Alizarin red staining and matrix calcification assay were performed after 7 d culture. The cells were fixed with 95% methanol and stained with 1% Alizarin red for 5 min, washed with PBS, and observed under a microscope. Matrix calcification was quantified by Alizarin Red S binding assay. Brifely, the cell cultures were first stained with 1% Alizarin red for 5 min, and then the plates were washed four times with PBS. 10% (wt/vol) cetylpyridinium chloride was added to each well for 10 min to release the remaining calcium−bound Alizarin Red S. The solution was collected, diluted at a ratio of 1:10 and read at OD_{570}.

Retroviral gene transduction. Retrovirus packaging was performed by transfecting the retroviral vectors into BOSC23 cells using FuGENE6 (Roche), as reported previously[2]. Bone marrow cells or macrophages derived from the bone marrow or spleen were infected with the recombinant retroviruses in the presence of 50 ng/ml M−CSF and 10 μg/ml polybrene for 8 h, and the media was changed for the various types of stimulation.

RT−PCR, real−time quantitative PCR and Northern blot assays. The primers for mouse *Irf8* were as follows: 5'−GATCGAACAGATCGACAGCA−3' (sense) and 5'−TGGGCTCCTCTTGGTCATAC−3' (antisense). The other primers and probes used for *Nfatc1*− and *Acp5*−specific Northern blotting have been described previously[3,4]. DNA−free RNA was obtained with the RNeasy MiniKit (Qiagen, Valencia, CA) with DNase treatment, and 1μg of total RNA was reverse−transcribed with random hexamers and MMLV−RT. Real−time quantitative PCR was done in triplicate with the iCycler iQ thermal cycler and detection system (BioRad) and the PCR Core Reagents kit (Applied Biosystems, Foster City, CA) with 500 nM primers. Amounts of mRNAs were normalized relative to glyceraldehyde−3−phosphate dehydrogenase (*Gapdh*) mRNA. When RT was omitted, threshold cycle number increased by at least ten, signifying lack

of genomic DNA contamination or nonspecific amplification; the generation of only the correct size amplification products was confirmed with agarose gel electrophoresis. Oligonucleotide primers used were as follows: human *Irf8*, 5'–TGCGCTCCAAACTCATTCTCGTG–3' (sense) and 5'–GTCTGGCGGCGGCTCCTC–3' (antisense); mouse *Tnfrsf11a* (*Rank*), 5'–TTCGGCGTTTACTACAGGA–3' (sense) and 5'–CATTGACCCAATTCCACAAA–3' (antisense); mouse *Csf1r* (*c–fms*), 5'–TCCACCGGGACGTAGCA–3' (sense) and 5'–CCAGTCCAAAGTCCCCAATCT–3' (antisense); mouse *Gapdh*, 5'–GTGAAGGTCGGAGTCAAC–3' (sense) and 5'–TGGAATTTGCCATGGGTG–3' (antisense).

Luciferase reporter assay. HEK293 cells were plated in 24–well plates at a density of 2×10^5 cells/well the night before transfection. A total of 8.05 µg of plasmid DNA, including 0.5 µg of luciferase reporter (p3×*Nfatc1–Luc* or *pApc5–Luc*), 0.05 µg of phRL–*TK* control reporter (Promega), 1.5 µg of pcDNA3–*Nfatc1* expression vector, and various amounts of pcDNA3–*Irf8* expression vector (1.5, 3, and 6 µg), were cotransfected into HEK293 cells using Lipofectamine 2000 (Invitrogen). The amount of transfected DNA was held constant at 8.05 µg by the addition of empty vector DNA (pcDNA3) when necessary. After 36 h, luciferase activity was determined using a dual–luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Preparation of GST fusion proteins, immunoprecipitation and immunoblot analyses. *Irf8* was subcloned into the GST fusion vector pGEX–6P–2 (Amersham Biosciences). Gst–Irf8 fusion proteins were expressed in *E. coli* BL21 cells and purified using GSTrap 4B columns (GE Healthcare) according to the manufacturer's instructions. GST and Gst–Irf8 proteins were used for immunoprecipitation and EMSA assays. Cell extracts from HEK293 cells obtained 48 h after transfection with the pFH–*Nfatc1* vector were harvested using lysis buffer [20 mM Tris (pH8.0), 5 mM $MgCl_2$, 0.1 M KCl, 10% glycerol, 0.1% Tween 20, 0.1% NP–40, complete proteinase inhibitor cocktail (Roche), and phosphatase inhibitor cocktail I and II (Sigma)]. The cell extracts (200 µg) were incubated with either GST (4 µg) or Gst–Irf8 (4 µg) for 4 h at 4°C in the lysis

buffer, and then were incubated with anti−FLAG antibodies (Sigma, 3 μg/sample) at 4°C for 1 h. The targets were subsequently immunoprecipitated with protein−G beads (Upstate) at 4°C for 1 h, and subjected to SDS−PAGE and immunoblot analysis as previously described[5]. Cytoplasmic and nuclear fractions were extracted using NE−PER Nuclear and Cytoplasmic Extraction Reagents (Pierce), and protein concentrations were determined using a BCA Protein Assay Reagent Kit (Pierce). Cell extracts of 10 μg were loaded into each lane. The antibodies used in the present experiment were as follows: anti−mouse NFATc1 (7A6, Santa Cruz Biotechnology, 1/200 dilution), anti−mouse IRF8 (C−19, Santa Cruz Biotechnology, 1/200 dilution), anti−mouse TBP (TATA binding protein) (Abcam, 1/200 dilution), anti−mouse p38 (Cell Signaling, 1/200 dilution), anti−mouse HRP−conjugated secondary antibodies (Amersham Biosciences, 1/5000 dilution), and anti−goat HRP−conjugated secondary antibodies (Amersham Biosciences, 1/10000 dilution).

EMSA. Nuclear fractions of HEK293 cells obtained 36 h after transfection with pcDNA3−*Nfatc1* and/or pcDNA3−*Irf8* were extracted using NE−PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). Double−stranded oligonucleotides were used as probes. The sequences corresponding to the distal NFATc1 binding site of the human *IL2* promoter were as follows: 5'−GGAGGAAAAACTGTTTCATACAGAAGGC−3' (sense) and 5'−GCCTTCTGTATGAAACAGTTTTTCCTCC−3' (antisense). Binding reactions were performed at room temperature for 30 min by incubating [γ−^{32}P] ATP−labeled probes (> 4×10^4 cpm) with 4 μg of nuclear extracts prepared from HEK293 transfected with pcDNA3−*Nfatc1* and/or pcDNA3−*Irf8* without or with various doses of GST or GST−Irf8 in a final volume of 20 μl of binding buffer [75 mM Tris (pH 7.5), 375 mM NaCl, 7.5 mM EDTA, 7.5 mM DTT, 37.6% glycerol, 1.5% NP−40, and 5 mg/ml bovine serum albumin]. In cold competition experiments, a 25−fold excess of unlabelled oligonucleotides were then incubated with the protein mixture for 10 min at room temperature prior to the addition of the labeled probes. For supershift assays, the protein–probe mixtures were incubated with 2 μg of anti−NFATc1 antibodies (7A6, Santa Cruz Biotechnology) for an additional 20 min at room temperature. After incubation, 20 μl of each mixture was electrophoresed at 150 volts on nondenaturing 4% polyacrylamide gels (4% polyacrylamide, 0.025% glycerol, 0.1% APS, 0.1% TEMED, and 0.25× TBE) using 0.25× TBE electrophoresis buffer at room

temperature. Gels were dried and exposed to an image plate, which was then scanned and analyzed using ImageQuant software (Amersham Biosciences).

SUPPLEMENTARY REFERENCES

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