Mizoguchi et al., http://www.jcb.org/cgi/content/full/jcb.200806139/DC1

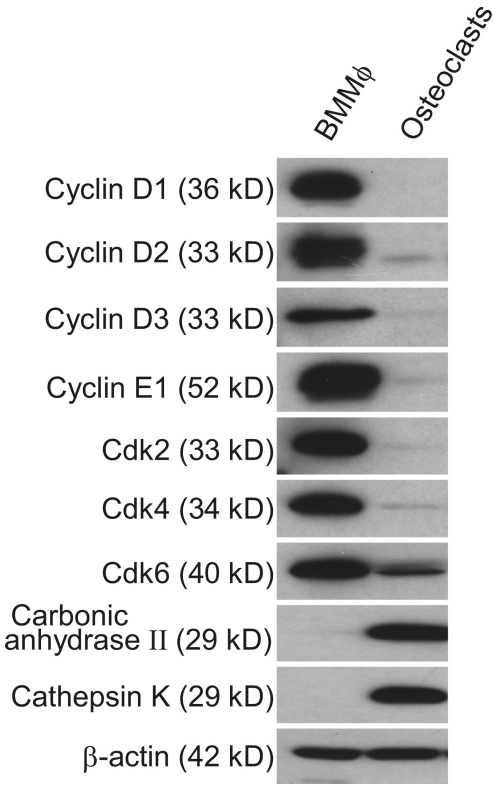

Figure S1. **Expression of cell cycle regulatory molecules in BMMΦ and purified osteoclasts.** Osteoclasts were prepared from cocultures of osteoblasts and bone marrow cells performed on collagen gel-coated dishes treated with $1\alpha,25(OH)_2D_3$ (10^{-8} M; Wako Chemicals USA, Inc.) as described previously (Akatsu, T., T. Tamura, N. Takahashi, N. Udagawa, S. Tanaka, T. Sasaki, A. Yamaguchi, N. Nagata, and T. Suda. 1992. *J. Bone Miner. Res.* 7:1297–1306). Primary osteoblasts were prepared from calvariae of newborn mice as previously described (Akatsu et al., 1992). Osteoclasts were formed within 6 d in the cocultures. All of the cells in the cocultures were recovered from the dishes by treatment with 0.2% collagenase (Wako Chemicals USA, Inc.). The purity of osteoclasts in the crude preparation was ~5%. To purify osteoclasts, the crude osteoclast preparation was plated in culture dishes (60-mm diameter dishes). After cells were cultured for 4 h, osteoblasts were removed by treatment with 0.05% trypsin and EDTA for 5 min (Invitrogen). The purity of osteoclasts was 95% after removal of osteoblasts (Akatsu et al., 1992). Cell lysates were prepared from BMMΦ and purified osteoclasts and were subjected to immunoblot analyses of cyclins (D1, D2, D3, and E1), Cdks (2, 4, and 6), carbonic anhydrase II, and cathepsin K.

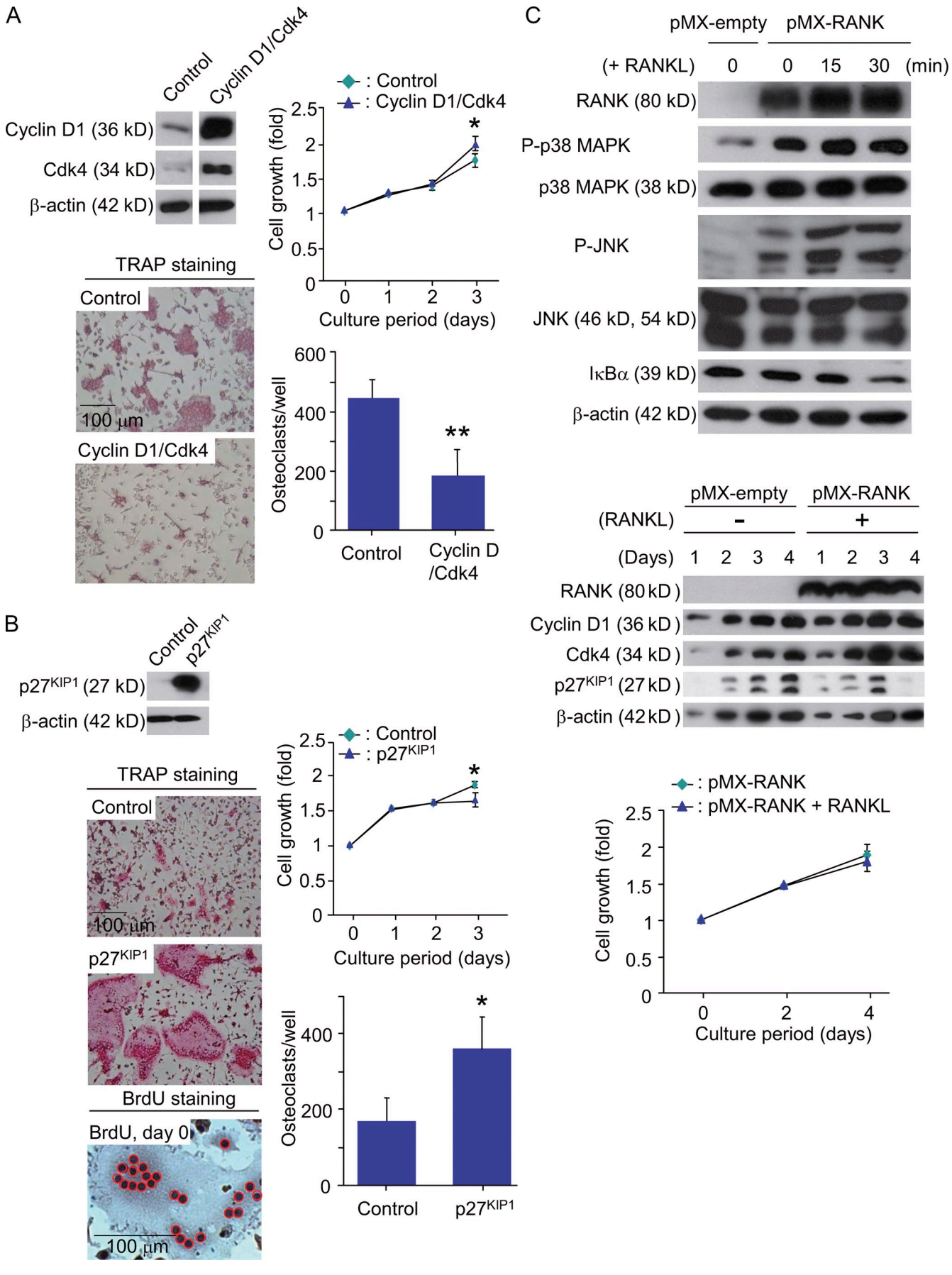
A
Control
Cyclin D1/Cdk4
Cyclin D1 (36 kD)
Cdk4 (34 kD)
β-actin (42 kD)
: Control
: Cyclin D1/Cdk4
Cell growth (fold)
Culture period (days)
TRAP staining
Control
100 μm
Cyclin D1/Cdk4
Osteoclasts/well
Control
Cyclin D /Cdk4
B
Control
p27KIP1
p27KIP1 (27 kD)
β-actin (42 kD)
: Control
: p27KIP1
TRAP staining
Control
100 μm
p27KIP1
BrdU staining
BrdU, day 0
100 μm
Osteoclasts/well
Control
p27KIP1
C
pMX-empty
pMX-RANK
(+ RANKL)
0
0
15
30
(min)
RANK (80 kD)
P-p38 MAPK
p38 MAPK (38 kD)
P-JNK
JNK (46 kD, 54 kD)
IκBα (39 kD)
β-actin (42 kD)
pMX-empty
pMX-RANK
(RANKL)
-
+
(Days)
1 2 3 4 1 2 3 4
RANK (80 kD)
Cyclin D1 (36 kD)
Cdk4 (34 kD)
p27KIP1 (27 kD)
β-actin (42 kD)
: pMX-RANK
: pMX-RANK + RANKL
Cell growth (fold)
Culture period (days)

Figure S2. **Overexpression of cell cycle regulatory molecules in BMMΦ and RANK in osteoblasts.** (A) Coexpression of cyclin D1 and Cdk4 in BMMΦ. BMMΦ were infected with empty pMX retrovirus or coinfected with pMX retrovirus expressing cyclin D1 and Cdk4. BMMΦ infected with empty pMX retrovirus (control) or pMX retrovirus expressing cyclin D1 and Cdk4 were cultured with 10^4 U/ml M-CSF for 24 h. (top left) Cell lysates were prepared and subjected to immunoblotting with anti–cyclin D1, anti-Cdk4, and anti–β-actin antibodies. BMMΦ infected with empty pMX retrovirus or pMX retrovirus expressing cyclin D1 and Cdk4 were cultured with 10^4 U/ml M-CSF. (top right) After the indicated periods, cell growth was measured by the AlamarBlue assay (Invitrogen) and expressed as the increase in fluorescence emission at 590 nm (excitation wavelength, 560 nm) relative to the control at day 0. BMMΦ infected with empty pMX retrovirus or pMX retrovirus expressing cyclin D1 and Cdk4 were cultured with 10^4 U/ml M-CSF and 100 ng/ml RANKL. (bottom left) Cells were fixed and stained for TRAP on day 3. (bottom right) The $TRAP^+$ multinucleated cells containing more than three nuclei were enumerated as osteoclasts. Results are expressed as the mean ± SD for three to six cultures. *, $P < 0.05$; **, $P < 0.01$; significantly different from the culture infected with empty pMX retrovirus (control). (B) Expression of $p27^{KIP1}$ in BMMΦ. BMMΦ were infected with empty pMX retrovirus or pMX retrovirus expressing $p27^{KIP1}$. BMMΦ infected with empty pMX retrovirus (control) or pMX retrovirus expressing $p27^{KIP1}$ were cultured with 10^4 U/ml M-CSF for 24 h. (top left) Cell lysates were prepared and subjected to immunoblotting with anti-$p27^{KIP1}$ and anti–β-actin antibodies. BMMΦ infected with empty pMX retrovirus or pMX retrovirus expressing $p27^{KIP1}$ were cultured with 10^4 U/ml M-CSF. (top right) After the indicated periods, cell growth was measured by the AlamarBlue assay. BMMΦ infected with empty pMX retrovirus or pMX retrovirus expressing $p27^{KIP1}$ were cultured with 10^4 U/ml M-CSF and 100 ng/ml RANKL. (bottom left) Cells were fixed and stained for TRAP on day 3. BMMΦ infected with pMX retrovirus expressing $p27^{KIP1}$ were also cultured with 10^4 U/ml M-CSF and 100 ng/ml RANKL in the presence of 10 nM BrdU for 3 d and stained with an antibody against BrdU. (bottom right) The $TRAP^+$ multinucleated cells containing more than three nuclei were enumerated as osteoclasts. Red circles indicate $BrdU^+$ nuclei in RANKL-induced multinucleated cells. Results are expressed as the mean ± SD for three to six cultures. *, $P < 0.01$; significantly different from the culture infected with empty pMX retrovirus (control). (C) Expression of RANK in osteoblasts. Primary osteoblasts were infected with empty pMX retrovirus (pMX-empty) or pMX retrovirus expressing RANK (pMX-RANK). (top) Osteoblasts infected with pMX retrovirus expressing RANK were incubated with or without 100 ng/ml RANKL for the indicated periods. Cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. Anti-RANK antibody (M-20) was obtained from Santa Cruz Biotechnology, Inc., and anti-p38 MAPK, anti–phosphorylated p38 MAPK, anti-JNK, anti–phosphorylated JNK, and anti-IκBα antibodies were obtained from Cell Signaling Technology. Phosphorylated p38 MAPK and JNK in the lysates were detected by reblotting the membrane with the indicated antibodies. Phosphorylation of p38 MAPK and JNK was up-regulated in osteoblasts infected with RANK cDNA and further enhanced by the treatment with RANKL. RANKL induced the degradation of IκBα (activation of NF-κB) in osteoblasts infected with RANK cDNA. (middle) Osteoblasts infected with pMX retrovirus expressing RANK or empty pMX retrovirus were treated with or without 100 ng/ml RANKL for the indicated periods. Cell lysates were prepared and immunoblotted with the indicated antibodies. Neither down-regulation of cyclin D1 and Cdk4 nor up-regulation of $p27^{KIP1}$ was observed in RANK-transfected osteoblasts even in the presence of RANKL. (bottom) Osteoblasts infected with pMX retrovirus expressing RANK were cultured in the presence or absence of 100 ng/ml RANKL. After the indicated periods, cell growth was measured by the AlamarBlue assay. Results are expressed as the mean ± SD for six cultures. Osteoblasts expressing functional RANK proliferated similarly in the presence and absence of RANKL.

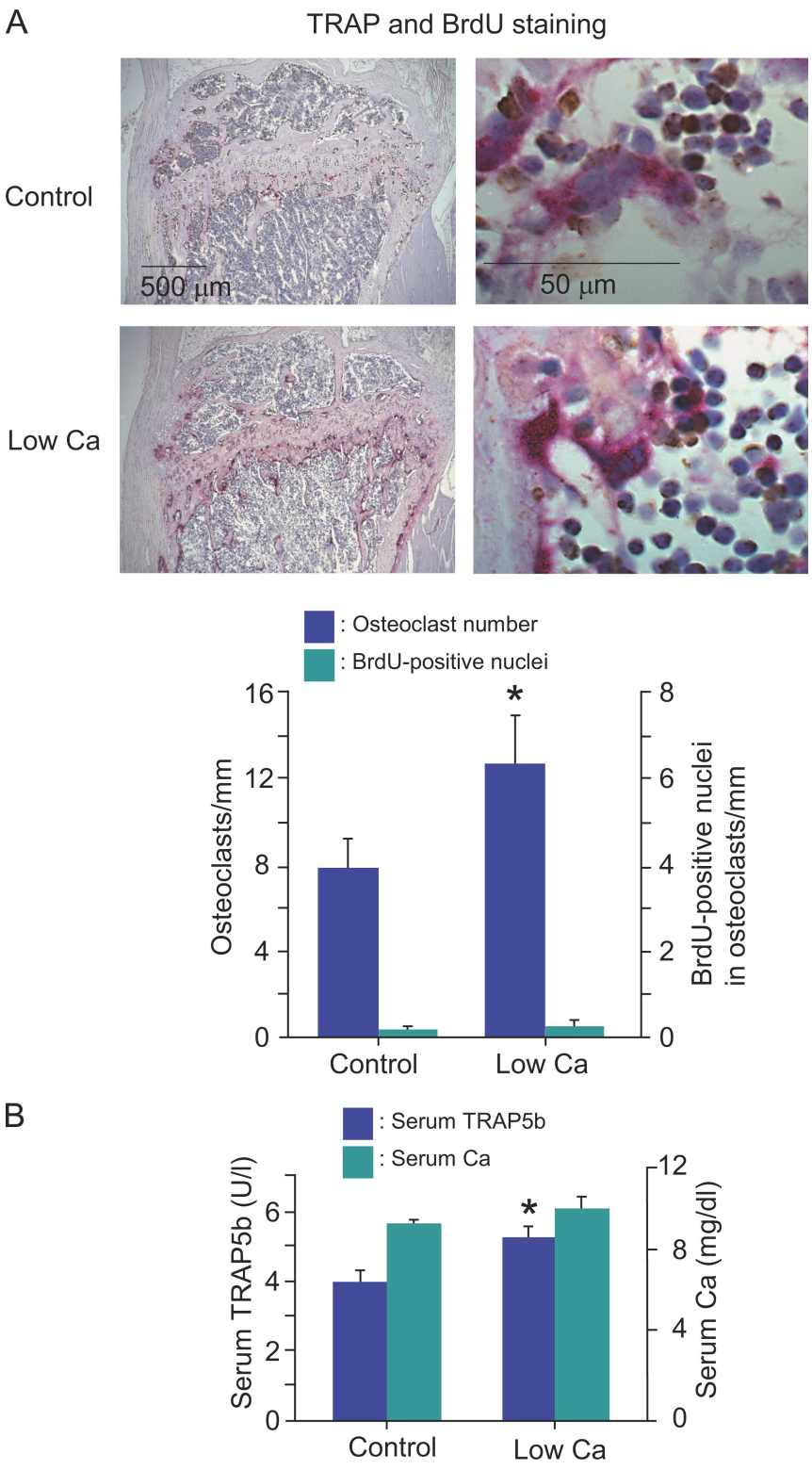

Figure S3. **Effects of a low calcium diet on the incorporation of BrdU into nuclei of osteoclasts.** (A) Detection of TRAP$^+$ osteoclasts and BrdU$^+$ nuclei. 7-wk-old mice were fed a control diet (calcium content, 10 mg/g) or a low calcium diet (calcium content, 0.3 mg/g) for 3 d together with 1 mg/ml BrdU in drinking water. Mice were killed, and blood and tibiae were collected. (top) Sections of tibiae were double stained for TRAP and BrdU. (bottom) BrdU$^+$ and BrdU$^-$ nuclei in osteoclasts were counted, and percentages of BrdU$^+$ nuclei in osteoclasts were determined. *, $P < 0.05$; significantly different from the mice fed on a control diet. (B) Serum calcium and TRAP5b activity. Serum concentrations of calcium and TRAP5b activity were measured in mice fed on control and calcium-deficient diets. The low calcium diet was provided by T. Shinki (Nihon Pharmaceutical University, Saitama, Japan). Results are expressed as the mean ± SD for three animals. *, $P < 0.01$; significantly different from the mice fed on a control diet.

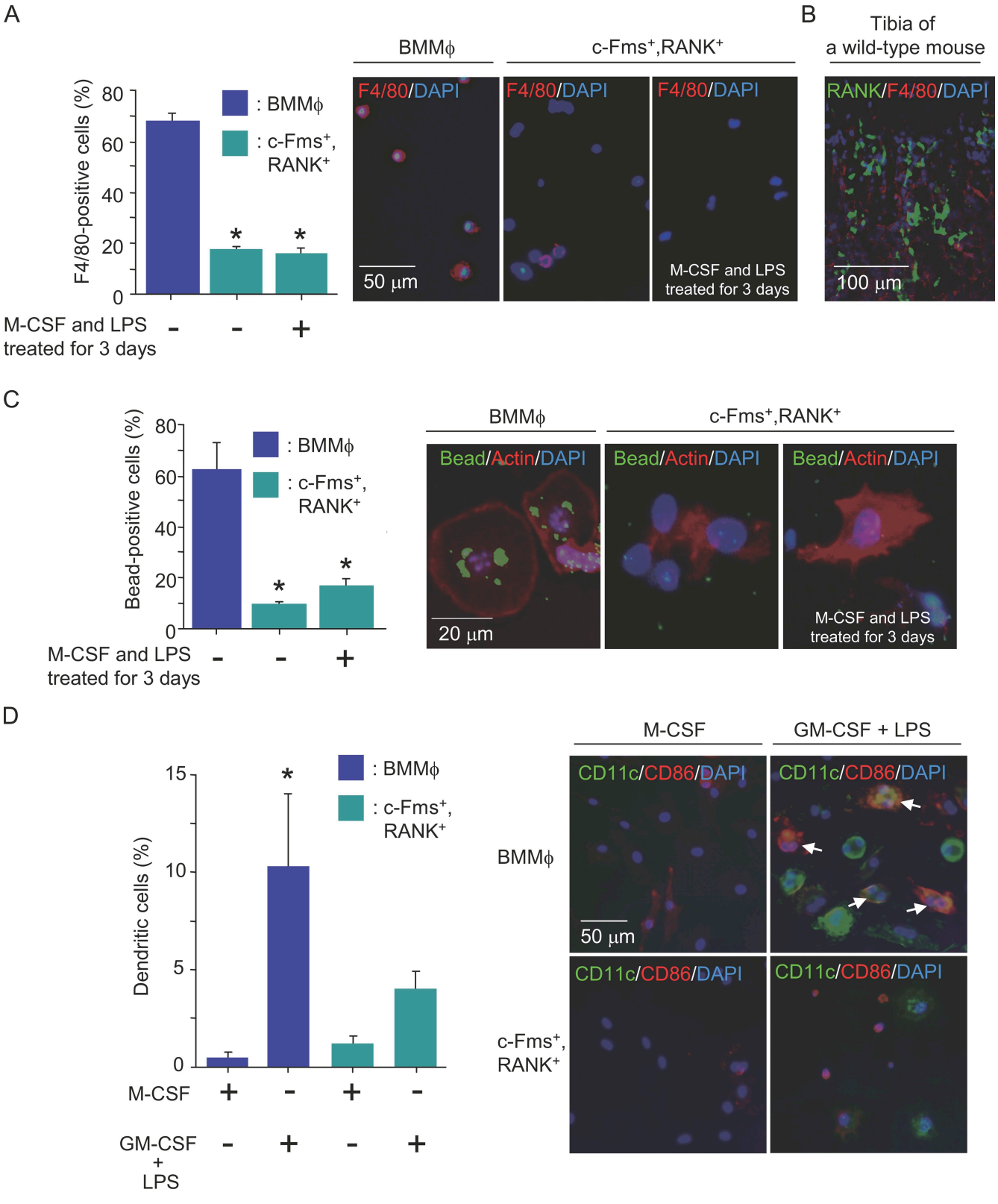
A
B
Tibia of
a wild-type mouse
BMMϕ
c-Fms+,RANK+
F4/80-positive cells (%)
80
60
40
20
0
: BMMϕ
: c-Fms+,
RANK+
*
*
M-CSF and LPS
treated for 3 days
- - +
F4/80/DAPI
F4/80/DAPI
F4/80/DAPI
50 μm
M-CSF and LPS
treated for 3 days
RANK/F4/80/DAPI
100 μm
C
Bead-positive cells (%)
80
60
40
20
0
: BMMϕ
: c-Fms+,
RANK+
*
*
M-CSF and LPS
treated for 3 days
- - +
BMMϕ
c-Fms+,RANK+
Bead/Actin/DAPI
Bead/Actin/DAPI
Bead/Actin/DAPI
20 μm
M-CSF and LPS
treated for 3 days
D
Dendritic cells (%)
15
10
5
0
*
: BMMϕ
: c-Fms+,
RANK+
M-CSF + - + -
GM-CSF
+
LPS - + - +
M-CSF
GM-CSF + LPS
BMMϕ
c-Fms+,
RANK+
CD11c/CD86/DAPI
CD11c/CD86/DAPI
50 μm
CD11c/CD86/DAPI
CD11c/CD86/DAPI

Figure S4. **Characterization of c-Fms$^+$/RANK$^+$ cells isolated from bone marrow cells.** BMMΦ were obtained by culturing mouse bone marrow cells with 10^4 U/ml M-CSF. c-Fms$^+$/RANK$^+$ cells were isolated from bone marrow by magnetic cell sorting. In some experiments, c-Fms$^+$/RANK$^+$ cells were cultured with 10^4 U/ml M-CSF and 1 µg/ml LPS (Sigma-Aldrich) for 3 d. (A) Immunostaining of F4/80 in BMMΦ and c-Fms$^+$/RANK$^+$ cells. Cells were incubated with anti-F4/80 antibody (A3-1; BMA Biomedicals) followed by Histofine Simple Stain Mouse MAX-PO (rat; Nichirei Biosciences, Inc.). (right) HRP was visualized with a Tyramide Signal Amplification kit (PerkinElmer) for cyanine 3 (red). Cells were also stained with DAPI (blue). (left) Percentages of F4/80-positive cells were determined. *, $P < 0.01$; significantly different from BMMΦ. (B) Localization of RANK$^+$ and F4/80$^+$ cells in tibiae. Tibiae were recovered from 7-wk-old wild-type mice. Sections of tibiae were prepared and subjected to double staining for RANK (green) and F4/80 (red). Specimens were also stained with DAPI (blue). Note that cells double positive for RANK and F4/80 (yellow cells) are rarely observed in the specimen. (C) Phagocytic activity of BMMΦ and c-Fms$^+$/RANK$^+$ cells. The phagocytic activity of cells was determined as previously described (Li, X., N. Udagawa, M. Takami, N. Sato, Y. Kobayashi, and N. Takahashi. 2003. *Endocrinology*. 144:4999–5005). Cells were prepared on cell desks (13.5 mm in diameter; Sumitomo Bakelite) in 24-well plates. Cells were maintained in serum-free medium in the presence of 10^4 U/ml M-CSF for 4 h and incubated for 40 min with latex beads (Polysciences, Inc.). (right) Cells were fixed, treated with 0.2% Triton X-100 in PBS for 10 min, and stained with rhodamine-conjugated phalloidin (red; Invitrogen) and DAPI (blue). Latex beads appeared as green dots with FITC-filtered light. (left) Cells incorporating >30 beads were counted as bead-positive cells, and percentages of bead-positive cells were determined. *, $P < 0.01$; significantly different from BMMΦ. (D) The ability of BMMΦ and c-Fms$^+$/RANK$^+$ cells to differentiate into dendritic cells. Dendritic cell differentiation was induced as previously reported (Mochizuki, A., M. Takami, T. Kawawa, R. Suzumoto, T. Sasaki, A. Shiba, H. Tsukasaki, B. Zhao, R. Yasuhara, T. Suzawa, Y. Miyamoto, Y. Choi, and R. Kamijo. 2006. *J. Immunol.* 177:4360–4368). Cells were cultured for 3 d in RPMI 1640 medium (Invitrogen) supplemented with 5% FBS in the presence of 10 ng/ml granulocyte M-CSF (GM-CSF; R&D Systems) and further treated with 10 ng/ml granulocyte M-CSF and 1 µg/ml LPS (Sigma-Aldrich) for 24 h. Cells were fixed, incubated with FITC-conjugated anti-CD11c antibody (HL3; BD), and treated with a HRP-conjugated secondary anti-FITC antibody. (right) HRP-conjugated antibodies were visualized with a Tyramide Signal Amplification kit for FITC (green). Cells were incubated for 1 h with the peroxidase-blocking reagent (Dako) to quench the peroxidase and were incubated with anti-CD86 antibody (GL-1; BD) followed by Histofine Simple Stain Mouse MAX-PO (rat). HRP was visualized with a Tyramide Signal Amplification kit for cyanine 3 (red). Cells were also stained with DAPI (blue). Arrows indicate CD11c and CD86 double-positive (CD11c$^+$/CD86$^+$) cells (mature dendritic cells, yellow cells). (left) Percentages of CD11c$^+$/CD86$^+$ cells were determined. *, $P < 0.01$; significantly different from the culture treated with M-CSF alone. Results are expressed as the mean ± SD for three cultures.

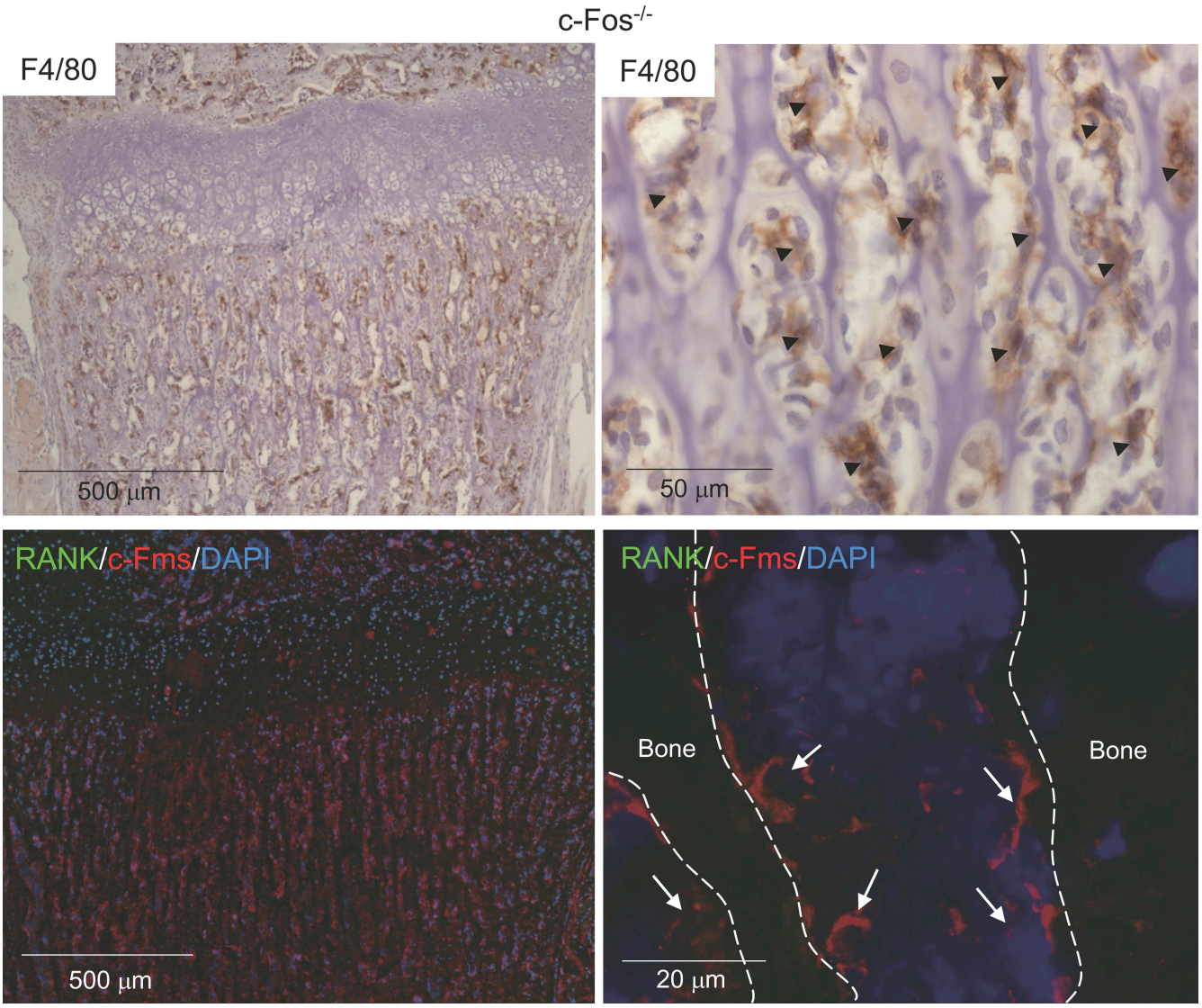

Figure S5. **Analysis of QuOPs in c-Fos$^{-/-}$ mice.** c-Fos$^{-/-}$ mice (C57BL/6) were obtained from The Jackson Laboratory. Tibiae were recovered from 6-wk-old c-Fos$^{-/-}$ mice. (top) Sections of tibiae were prepared and subjected to staining for F4/80. (top right) A high power view of F4/80 staining is shown. Arrowheads indicate F4/80-positive cells (brown cells). (bottom) Sections of tibiae were subjected to double staining of RANK (green) and c-Fms (red). Sections were also stained with DAPI (blue). (bottom right) A high power view of RANK and c-Fms double staining is shown. Arrows indicate c-Fms–positive cells (red cells). No RANK-positive cells (green) are observed in the specimen.