Supplementary Figures and Table

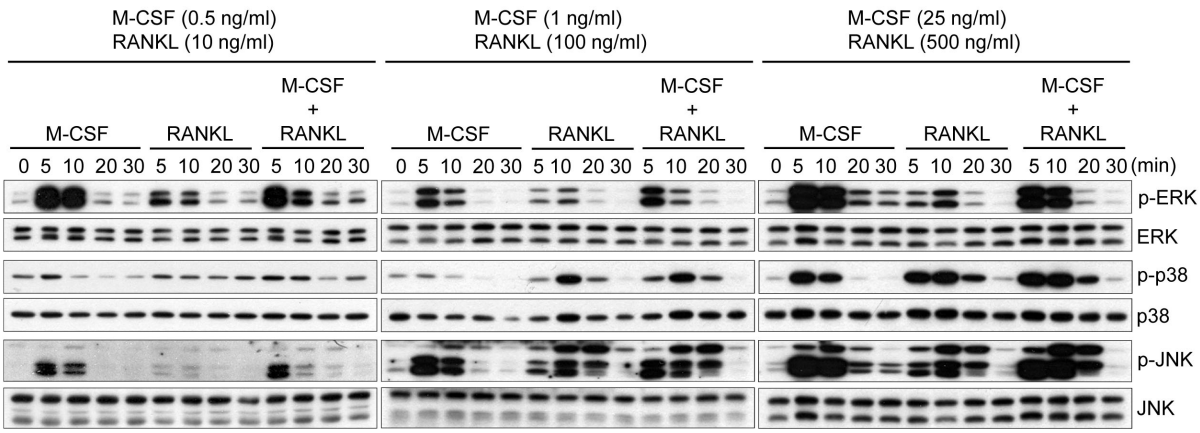

Figure S1. MAPK activation by different concentrations of M-CSF or RANKL. Osteoclast precursors were treated with M-CSF (0.5, 1, and 25 ng/ml) or RANKL (10, 100, and 500 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies to total or phosphorylated forms of ERK, p38, and JNK. Data are representative of three independent experiments.

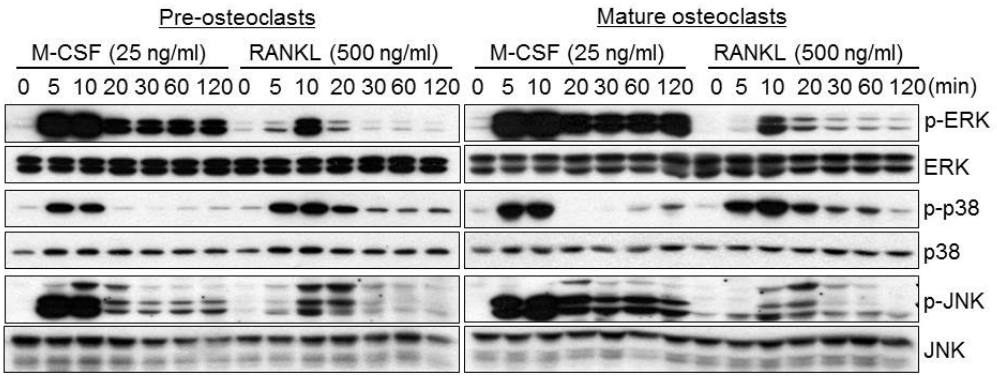

Figure S2. MAPK activation by M-CSF or RANKL at different stages of osteoclast differentiation. Pre-osteoclasts or mature osteoclasts were prepared by treatment of osteoclast precursors with M-CSF (30 ng/mL) and RANKL (100 ng/mL) for 2 or 4 days, respectively, after which the cells were deprived of both ligands for 6 hours before stimulation with M-CSF (25 ng/mL) or RANKL (500 ng/mL) for the indicated times. Cell lysates were then subjected to immunoblot analysis of MAPK activation. The gel images are representative of three independent experiments.

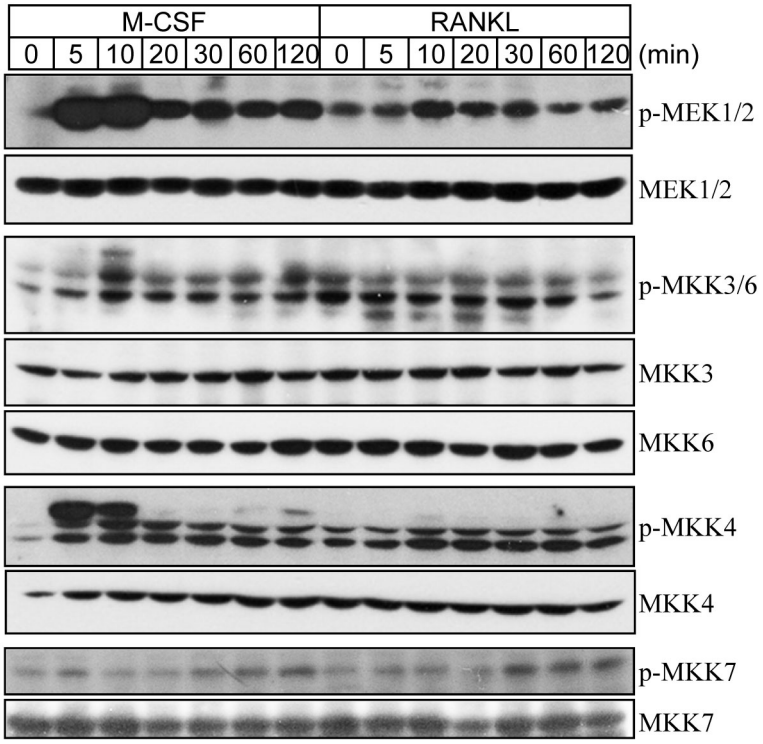

Figure S3. MAPK upstream activators MAPK kinases (MKKs) activation by M-CSF or RANKL. Osteoclast precursors were treated with M-CSF (25 ng/ml) or RANKL (500 ng/ml) for the indicated times, after which cell lysates were subjected to immunoblot analysis with antibodies specific for total or phosphorylated forms of MKKs, including MEK1/2, MKK3/6, MKK4, and MKK7. The gel images are representative of three independent experiments.

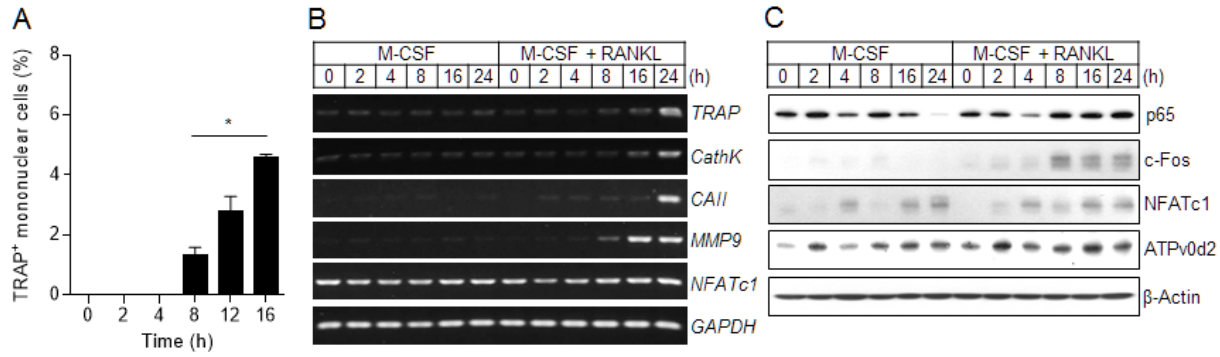

Figure S4. Changes in expression of osteoclastogenic markers during osteoclast differentiation. A, $TRAP^+$ mononuclear cell formation. Osteoclast precursors were cultured in the presence of M-CSF (30 ng/mL) and RANKL (100 ng/mL) for the indicated times. The proportion of $TRAP^+$ mononuclear cells was determined. The quantitative data between groups were analyzed by one-way ANOVA comparison. $*P < 0.01$. B and C, Osteoclastogenic gene expression. Osteoclast precursors were treated with M-CSF (30 ng/mL) or M-CSF (30 ng/mL) plus RANKL (100 ng/mL). Expression levels of osteoclastogenic genes were evaluated by semi-quantitative RT-PCR (B) and immunoblot analysis (C). *GAPDH* (B) and β-Actin (C) were examined as loading controls.

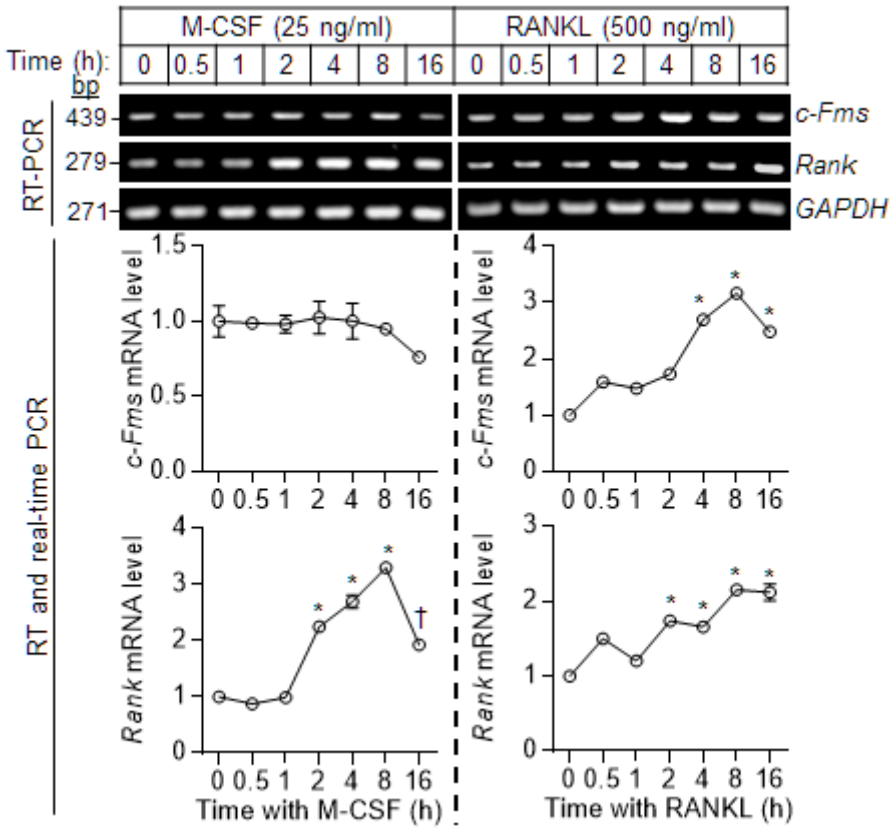

Figure S5. Effects of M-CSF and RANKL on *c-Fms* and *RANK* mRNA abundance in osteoclast precursors. Osteoclast precursors were cultured with M-CSF (25 ng/mL) or RANKL (500 ng/mL) for the indicated times, after which the abundance of *c-Fms* and *RANK* mRNAs was evaluated by semiquantitative (upper panels) or quantitative (lower panels) RT-PCR analysis. *GAPDH* mRNA was examined as a loading control. The gel images for semiquantitative data are representative of three independent experiments, and quantitative data normalized by the amount of *GAPDH* mRNA are expressed relative to the corresponding value for time 0 and are means ± SD (n = 3) for three independent experiments. *$P < 0.01$. †$P < 0.05$. (Student *t* test).

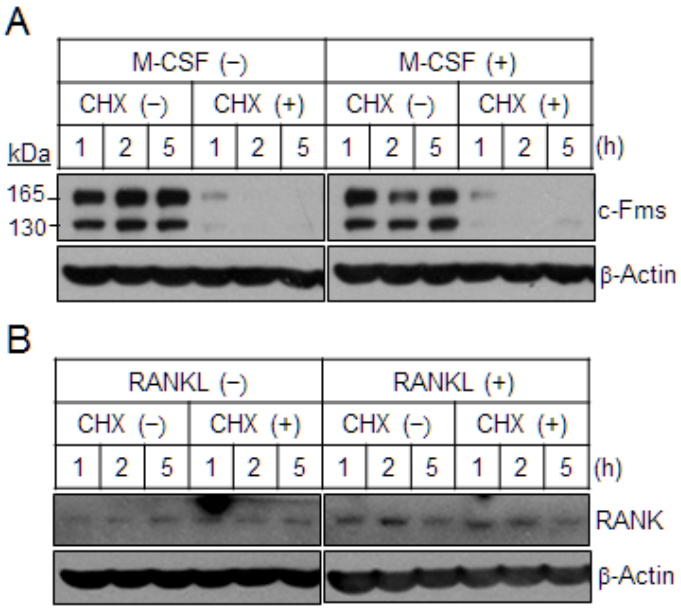

Figure S6. The stability of c-Fms and RANK. A and B, Osteoclast precursors were incubated for the indicated times in the absence or presence of cycloheximide (CHX) at 5 μg/mL and of either M-CSF at 25 ng/mL (A) or RANKL at 500 ng/mL (B), after which cell lysates were subjected to immunoblot analysis with antibodies to c-Fms (A) or to RANK (B). β-Actin was examined as a loading control. The gel images are representative of three independent experiments.

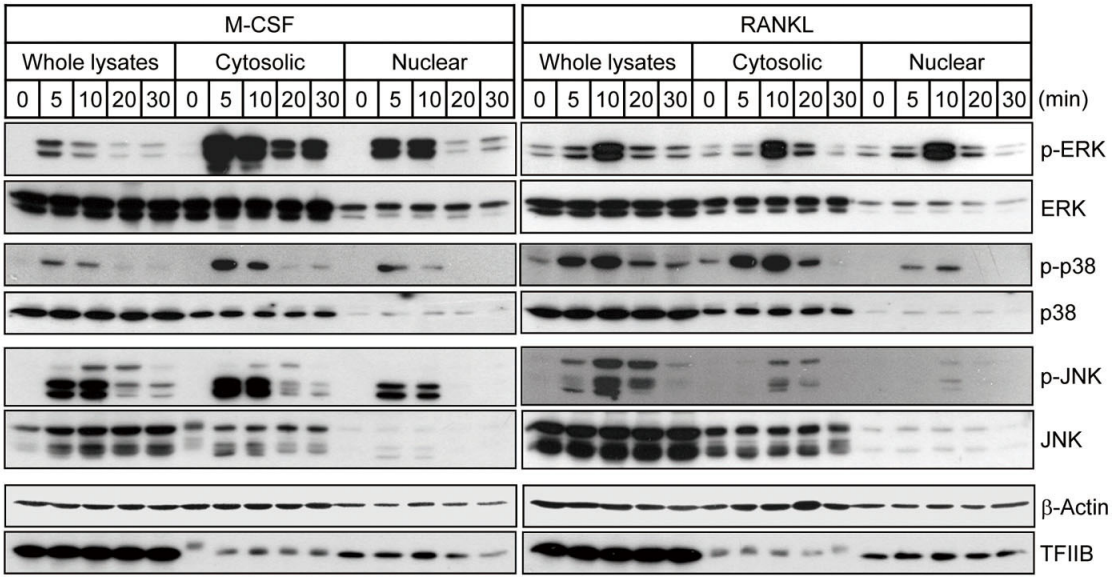

Figure S7. Nuclear translocation of activated MAPKs in osteoclast precursors. Osteoclast precursors were cultured with M-CSF (25 ng/mL) or RANKL (500 ng/mL) for the indicated times, after which whole cell lysates or cytosolic or nuclear fractions prepared therefrom were subjected to immunoblot analysis of MAPK activation. β-Actin and TFIIB were examined as loading controls. The gel images are representative of three independent determinations.

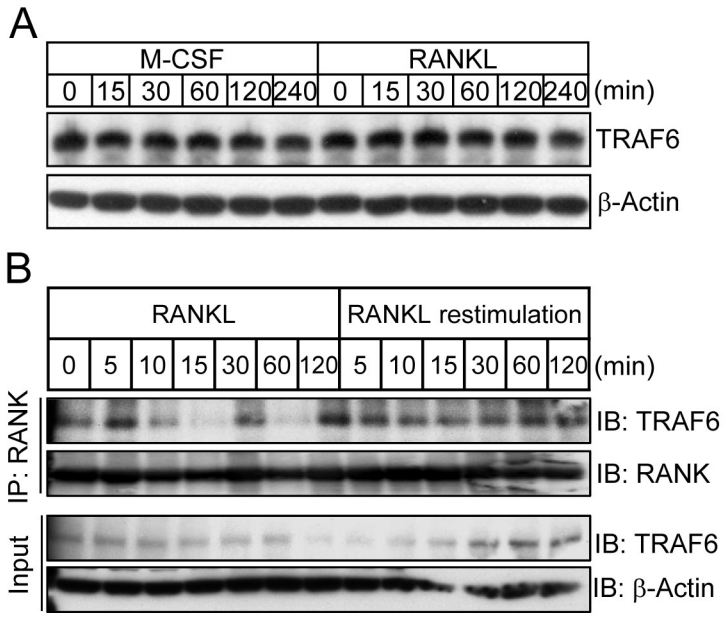

Figure S8. Role of TRAF6 in MAPK restimulation by RANKL. (A) Osteoclast precursors were treated with M-CSF (25 ng/ml) or RANKL (500 ng/ml) for the indicated times. Whole lysates were analyzed by immunoblotting with antibody against TRAF6. (B) Osteoclast precursors were treated with RANKL (500 ng/ml) for 2 h (left panel) and then restimulated with RANKL for the indicated times (right panel). Cell lysates were immunoprecipitated with anti-RANK antibody, and the resulting immunoprecipitates were subjected to immunoblot analysis with anti-TRAF6 antibody to analyze RANK/TRAF6 molecular complex. IP: immunoprecipitate; IB: immunoblotting.

Table S1. Sequences of PCR primers used in this study.

Semiquantitative RT-PCR		
Gene	Sense (5′→3′)	Antisense (5′→3′)
c-Fms	ACTCCGAGGGAGACTCCAGCTAC	CACATCACTCTGAACTGTGTAGACG
RANK	GGTTCACTGCTCCTAATCCAGC	GGTCTGGCTGACATACACCACG
TRAP	ACTTCCCCAGCCCTTACTACC	TCAGCACATAGCCCACACCG
CathK	CTTGTGGACTGTGTGACT	AACACTGCATGGTTCACA
CAII	CTCTCAGGACAATGCAGTGC	ATCCAGGTCACACATTCCAGC
MMP9	CCTGTGTGTTCCCGTTCATCT	CGCTGGAATGATCTAAGCCCA
NFATc1	TGCTCCTCCTCCTGCTGCTC	CGTCTTCCACCTCCACGTCG
GAPDH	CAAGGCTGTGGGCAAGGTCA	AGGTGGAAGAGTGGGAGTTGCTG
Quantitative RT-PCR		
Gene	Sense (5′→3′)	Antisense (5′→3′)
c-Fms	CAGAGCCCCCACAGATAAAA	GTCCACAGCGTTGAGACT
RANK	TTCCCAGTGAAGCAGCAGCCA	GAGATGAACGTGGAGTTACTGTTTC
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGT