

Supplementary information

METHODS:

Osteoclast differentiation. Bone marrow cells were obtained from 6-8 week old mice. To prepare BMMs, cells (1×10^7) were cultured with α -MEM/10%FBS with M-CSF (150 ng/ml) using Corning 100mm Not TC-Treated Culture Dish (Corning Incorporated Life Science, Acton, MA). For retrovirus infection, after the first 2 days of culture, BMMs were incubated with retrovirus stock with M-CSF (150 ng/ml) and polybrene (SIGMA; 5 μ g/ml) for 6 hours and cultured overnight. To select transduced BMMs, BMMs were detached with Trypsin/EDTA and cultured with M-CSF and puromycin (SIGMA; 2 μ g/ml) for 2 days. Transduced BMMs were cultured in 96-well plates (2×10^4 /well) with M-CSF (30 ng/ml) and stimuli for 4-5 days. We used α CD40 (1 μ g/ml) as a stimulus for CD40 mutants and anti-FLAG antibody (10 μ g/ml) plus anti-mouse IgG antibody (5 μ g/ml) for TRANCE-R mutants. Cells were stained for F-Actin with rhodamine phalloidin (Molecular Probes, Eugene, OR) followed by TRAP staining. TRAP(+) cells containing more than 3 nuclei were counted as TRAP(+) MNCs. Statistical analysis was performed using the Student's *t*-test. For bone resorption assays, transduced BMMs were cultured on bone slices for 6-7 days and removed by ultrasonication in 1M NH_4OH . To visualize resorption pits, bone slices were stained with 0.5% toluidine blue.

Immunoprecipitation and western blot analysis. BMMs were lysed with TNE buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 2 mM Na_3VO_4 , 10 mM NaF, 2 mM PMSF, 0.1% deoxycholate, 0.1 U/ml of aprotinin). Equivalent amounts of cell lysates were subjected to immunoprecipitation or western blotting, and quantitated with the NIH imaging program (NIH Image 1.62). The fold induction of phospho-p38 was determined by normalizing

to the density of total p38.

Generation of CD40 mutants. To generate CD40-2T6 and CD40-3T6, the TRAF6 binding site (residues 212-250) of CD40 was inserted behind the original TRAF6 binding site as follows. The XbaI site was first generated behind the original TRAF6 binding site by PCR. Then the TRAF6 binding site of CD40 was cloned by PCR, digested with NheI and XbaI, and inserted into XbaI site generated behind the original TRAF6 binding site. Primer pairs for making XbaI site were as follows:

5'-CCGGTCATAACACCGCTGCTTCTAGACCAGTGCAGGAGACACTGC-3',

5'-GCAGTGTCTCCTGCACTGGTCTAGAAGCAGCGGTGTTATGACCGG-3'

Primer pairs for cloning were the following:

5'-CCTAGCTAGCTTTCTCTATATCAAAAAGGTGGTCAAG-3'

5'-CTAGTCTAGAAGCAGCGGTGTTATGACCGGGATAATC-3'

FIGURES:

Supplementary Figure 1. (A) Surface expression of TRANCE-R mutants. BMMs were transduced with pMX-puro empty-vector (EV), vector carrying TRANCE-R wild-type (TRWT) or mutants. After puromycin selection, BMMs were stained for TRANCE-R with anti-Flag Ab. Each color represents a different mutant as indicated. (B) Osteoclastogenesis using TRANCE-R mutants. Transduced BMMs were stimulated by α Flag Ab, and were stained for TRAP (top) and F-actin (middle) after 4 days. Transduced BMMs were also cultured on the bone slices for 6 days, and bone slices were stained with 0.5% toluidine blue (bottom). Bars = 100 μ m. (C) The effect of TRANCE-R mutants on TRANCE-induced osteoclastogenesis. Transduced BMMs

were cultured with TRANCE (0, 15, 50, 150 ng/ml) and stained for TRAP after 4 days. Statistical analysis was performed using the Student's t-test versus EV at TRANCE 150 ng/ml, #p < 0.05, *p < 0.01, **p < 0.001. (D) TRAF6 expression in transduced BMMs. Total cell lysates from transduced BMMs were subjected to western blotting.

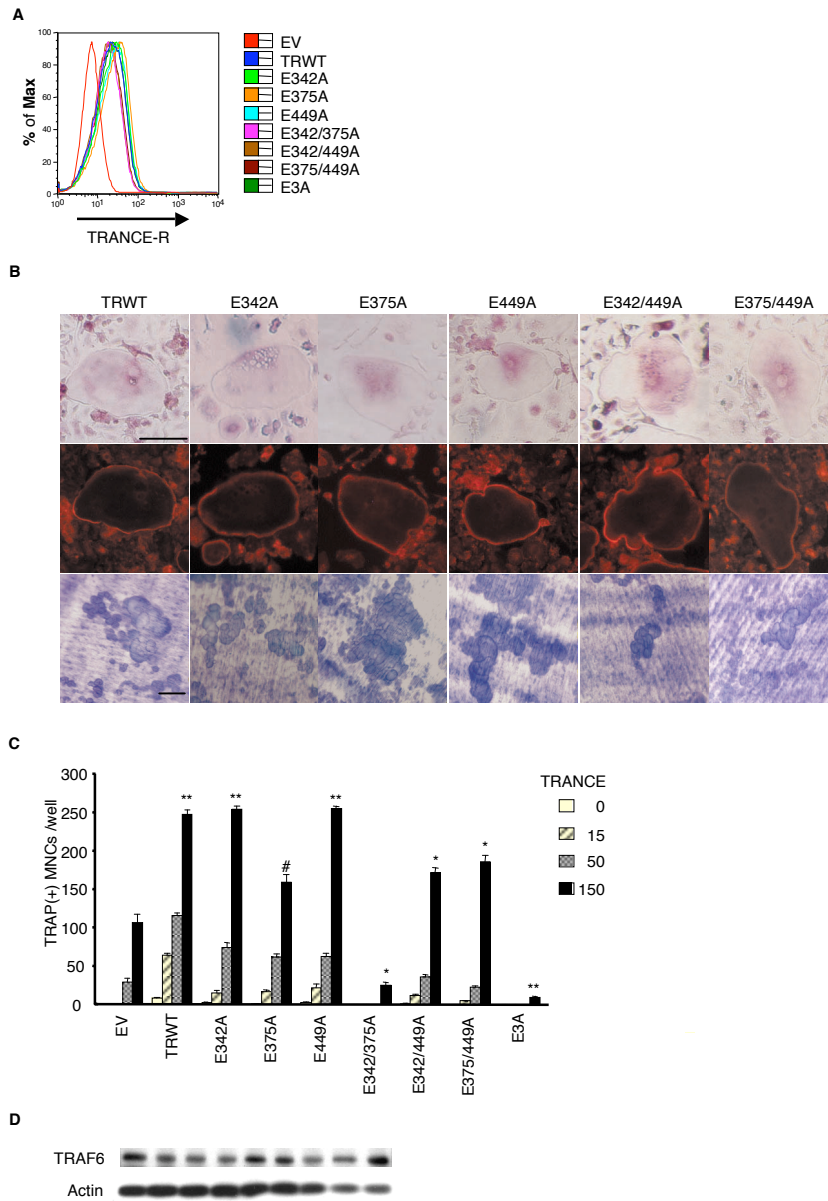
Supplementary Figure 2. Stimulation of endogenous CD40 failed to induce osteoclastogenesis. BMMs were cultured in the presence of CD40L (0, 15, 50, 150 or 500 ng/ml) and/or TRANCE (0, 15, 50, 150 or 500 ng/ml) for 4 days, and stained for TRAP. Statistical analysis was performed using the Student's t-test versus CD40L absent condition, n.s. for not significant.

Supplementary Figure 3. Enhanced CD40 signaling has the potential to induce osteoclastogenesis. (A) BMMs were transduced with pMX-puro empty-vector (EV) or carrying CD40 wild-type (WT), selected with puromycin, and cultured in the absence or presence of TGF- β (1 ng/ml). Transduced BMMs were stimulated with α CD40 (1 μ g/ml) or CD40L (500 ng/ml) for 4 days, and then were stained for TRAP. Statistical analysis was performed using the Student's t-test versus EV, #p < 0.05, **p < 0.001. (B) Transduced BMMs were stained for CD40 and analyzed by FACS. Shown are empty-vector (EV) transduced BMMs (red), CD40-WT-transduced BMMs (WT/M. blue), and CD40-WT-transduced BMMs prepared in the presence of TGF- β (WT/Mt. green). (C) Total cell lysates were collected from transduced BMMs, and were subjected to immunoprecipitation or western blotting.

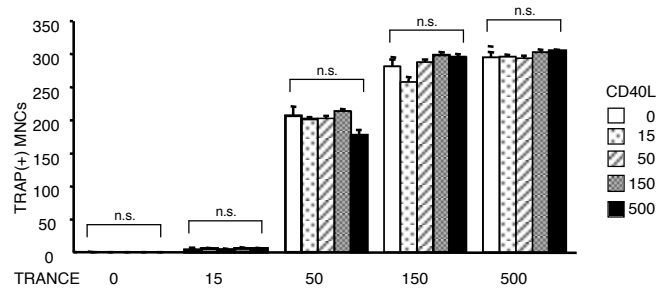
Supplementary Figure 4. CD40 expression in transduced BMMs. BMMs were transduced with pMX-puro empty-vector (EV), CD40 wild-type (WT) or CD40 mutant with two (2T6) or

three (3T6) TRAF6 binding sites. Transduced BMMs were stained for CD40 after puromycin selection and analyzed by FACS. Shown are EV-transduced BMMs (red), WT-transduced BMMs (blue), 2T6-transduced BMMs (green), and 3T6-transduced BMMs (orange).

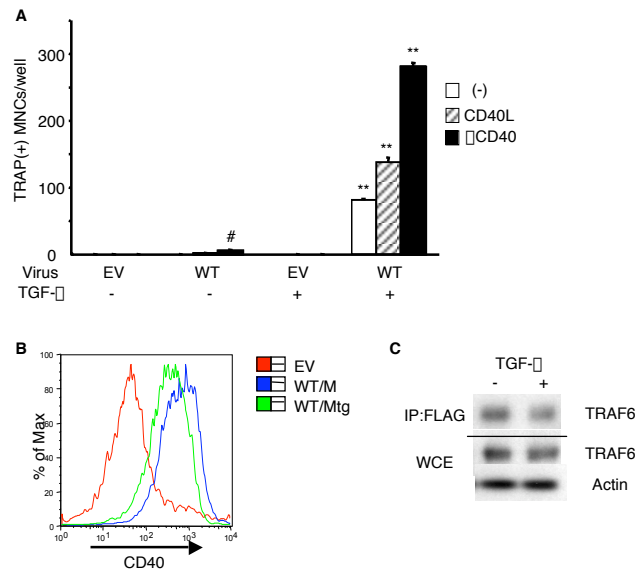
Kadono - Supplementary figure 1



Kadono - Supplementary figure 2



Kadono - Supplementary figure 3



Kadono - Supplementary figure 4

