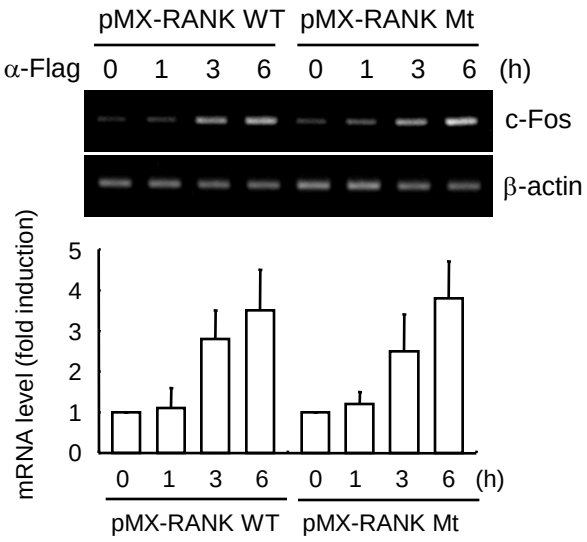
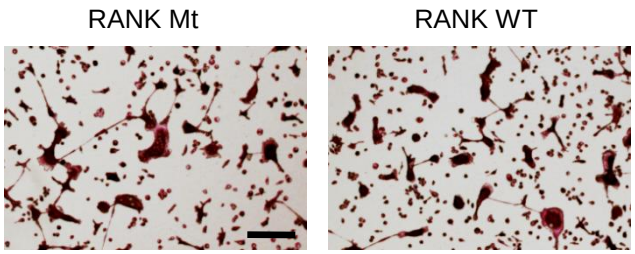
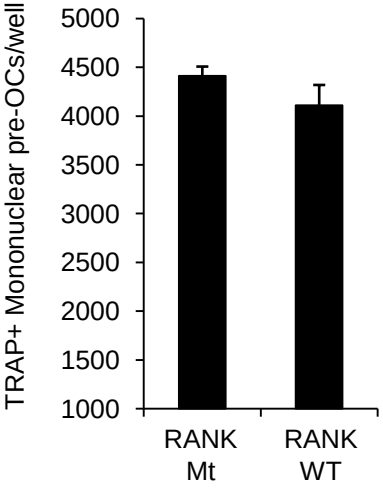
Supplemental Figure 1



Supplemental Figure 2

a





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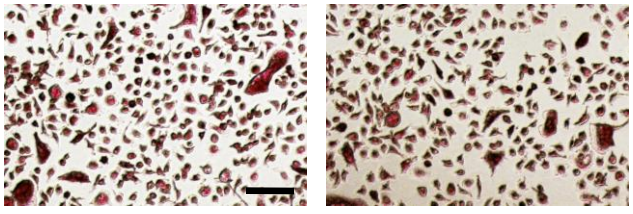

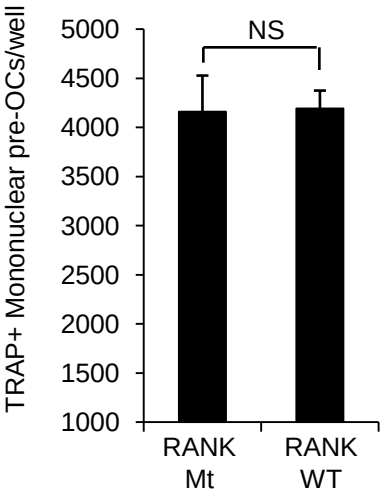

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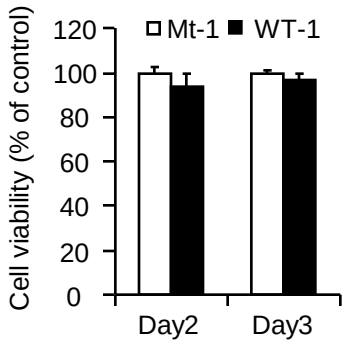

Supplemental Figure 4

a

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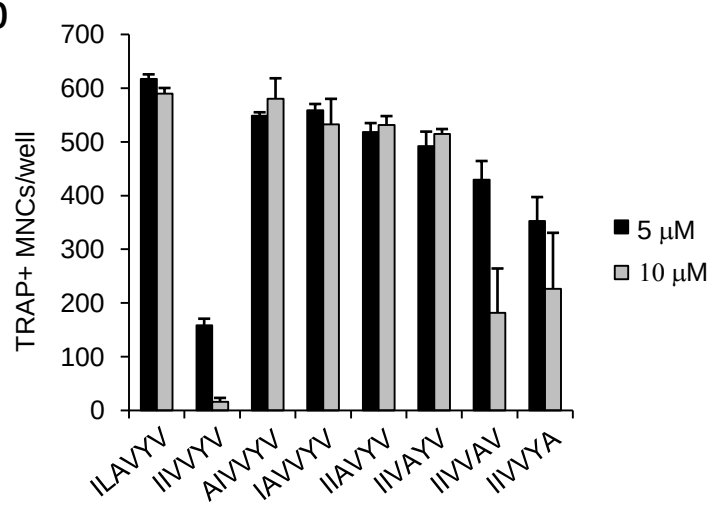
b



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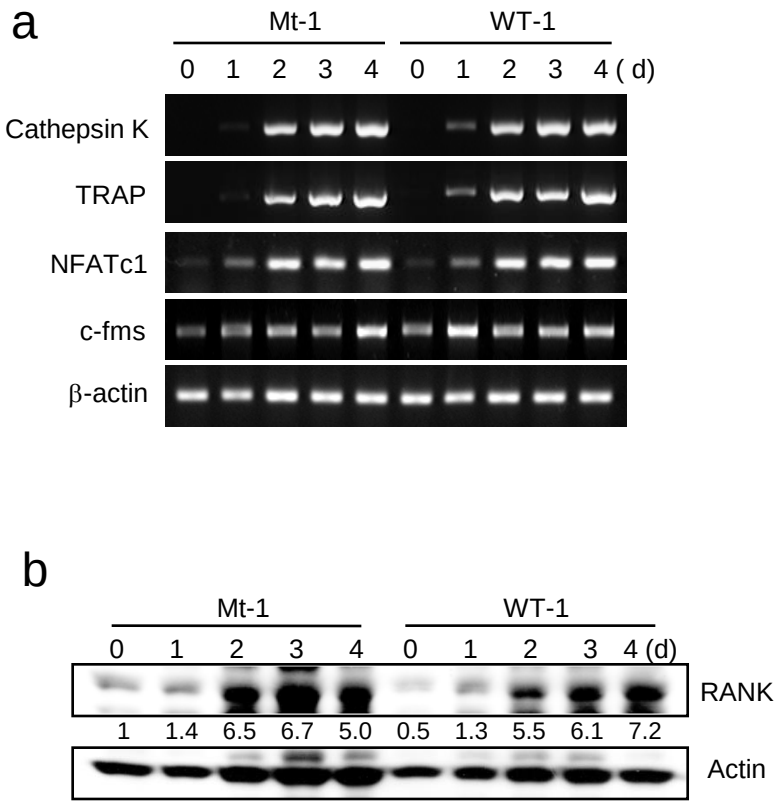

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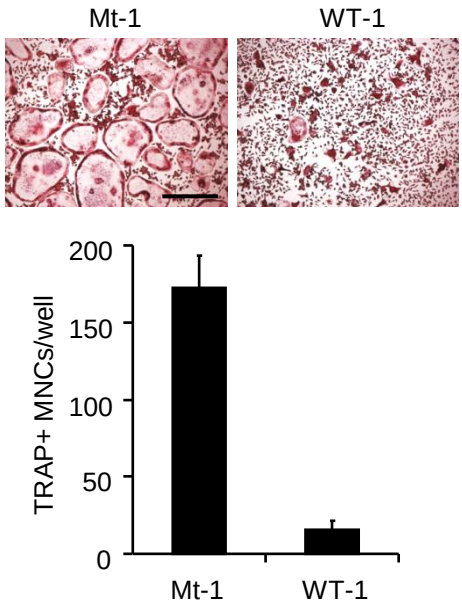

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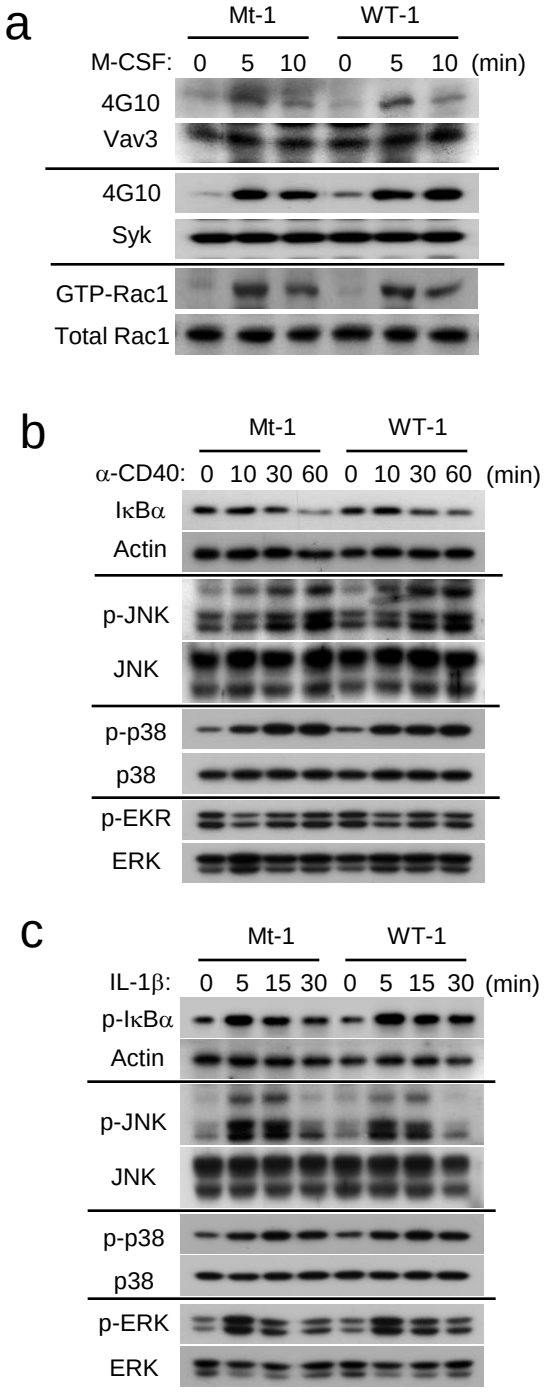
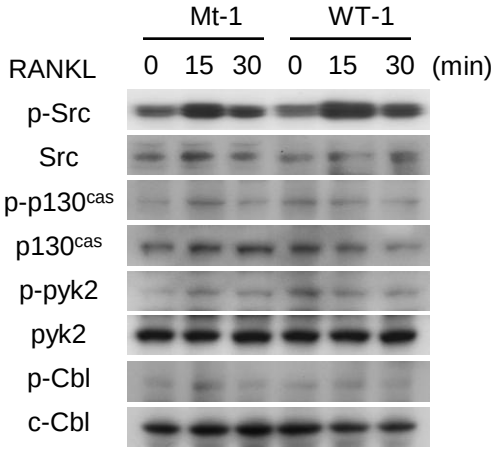


Supplemental Figure 8



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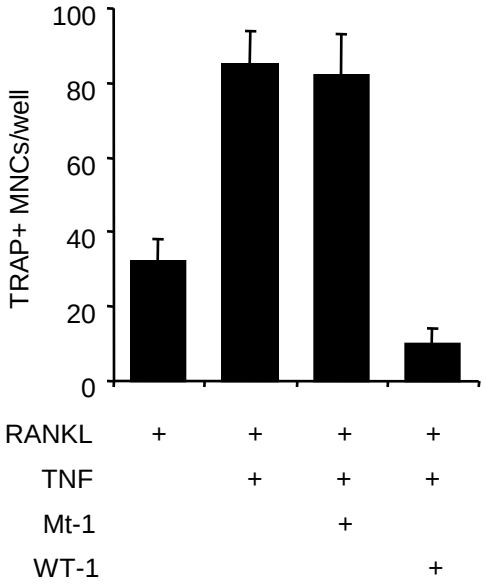

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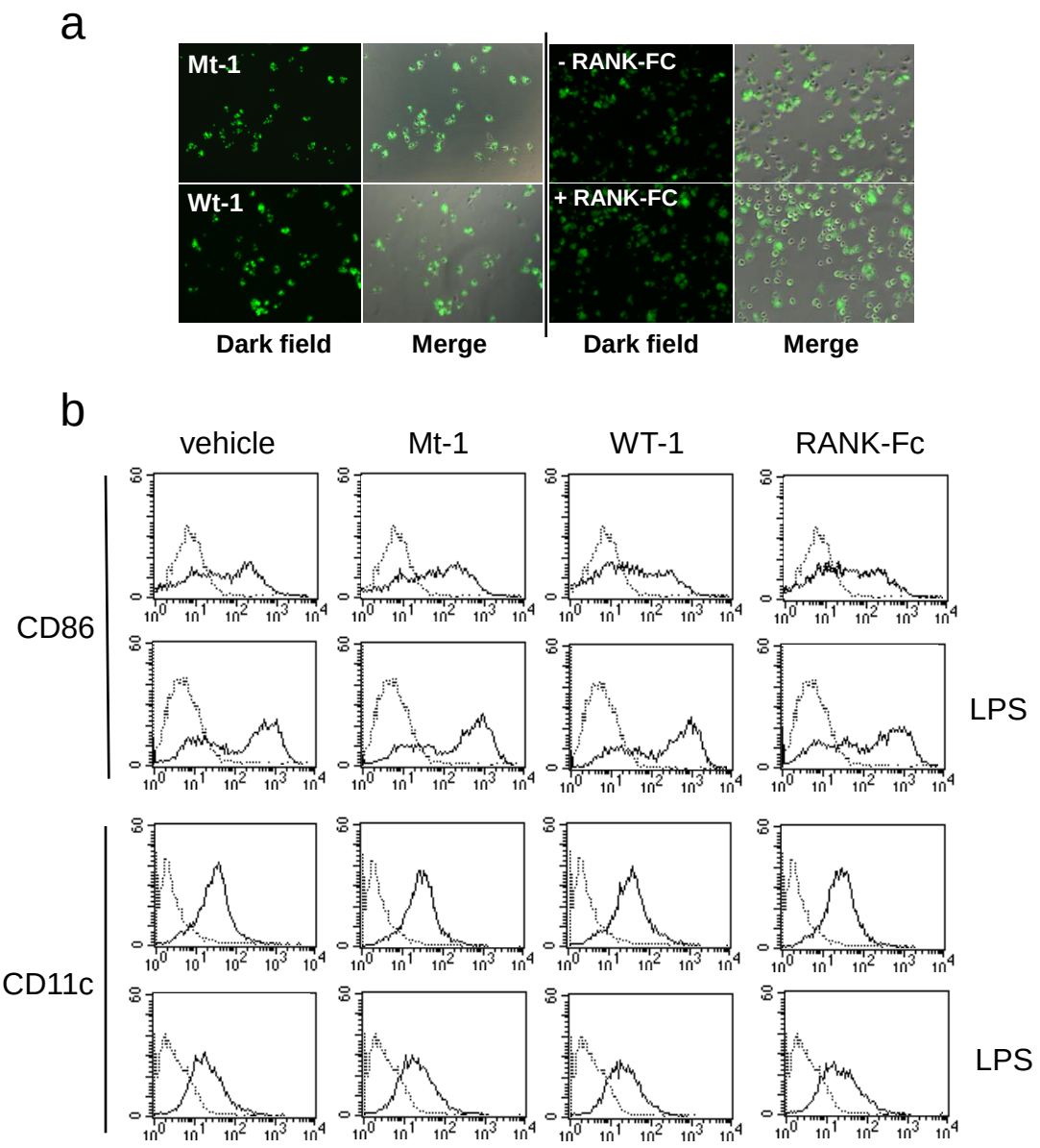

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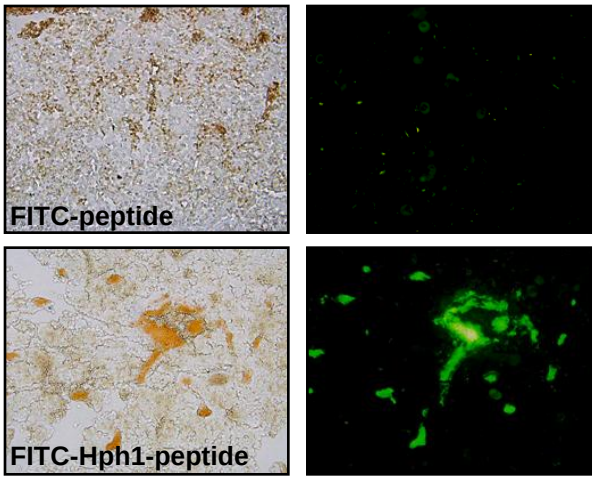
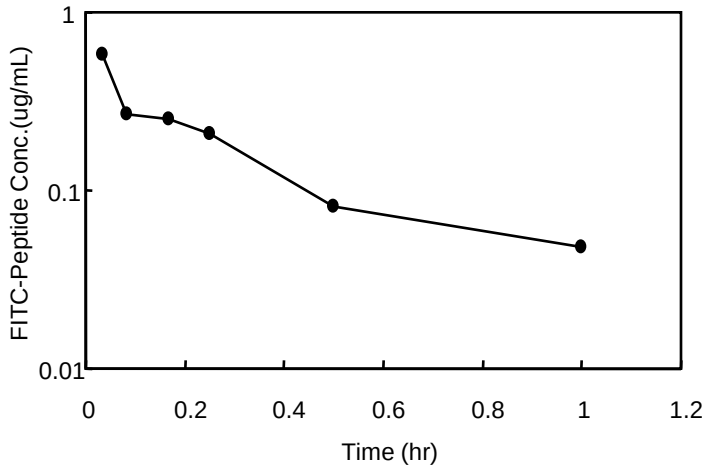


Supplemental Figure 12



Supplemental Table 1. Pharmacokinetic parameters of FITC-peptide following IV Injection of FITC-peptide at a dose of 5 mg/kg in mice.

PK Parameters	FITC-peptide
C_0 (μg/mL)	0.975
K_e (hr^{-1})	1.995
$t_{1/2}$ (hr)	0.348
V_d (mL)	276.9
Cl_t (mL/hr)	552.4
$AUC_{0-\infty}$ ($\mu_{g \cdot hr/mL}$)	0.181

C_0: Initial plasma concentration; Ke: Elimination rate constant; $t_{1/2}$: Elimination half-life; V_d: Volume of distribution; Cl_t: Total clearance; $AUC_{0-\infty}$: Area under the plasma concentration-time curve from 0 hr to infinity

Supplemental Information

Supplemental methods

Cell stimulation and analysis

Isolated BMMs or pre-osteoclasts (pre-OCs) from stromal cell-free BMMs were extensively washed to remove exogenous growth factors, cultured in media with low serum (0.5% FBS, 4 hours), and then stimulated by adding the anti-CD40 antibody (5 μg/ml), IL-1β (10 ng/ml), or RANKL (100 ng/ml) in the presence of the wild-type or mutant peptides as indicated. After stimulation, the cells were washed in ice-cold PBS, lysed, and subjected to immunoblot analysis using antibodies specific for phosphorylated c-Src (Tyr416), phosphorylated p130Cas (Tyr410), phosphorylated Syk (Tyr525/526) (Cell Signaling), phosphorylated Pyk2 (Tyr420) , p130Cas, c-Cbl, Pyk2, Syk (Santa Cruz), and β-actin (Sigma).

Dendritic cells (DCs) induction and flow cytometric analysis

DCs were prepared from BMMs with modification as previously described (1). BMMs were seeded in 24-well plates with granulocyte-macrophage (GM)-CSF (10 ng/ml). Cells were cultured for 4 more days with replacement of fresh medium containing the same concentration of GM-CSF on day 2. On day 4, 1 ml fresh medium containing 1 _g/ml of LPS (Sigma-Aldrich) was added to cultures to stimulate the maturation of DCs. The next day, the cells were harvested and analyzed with anti-CD86 and anti-CD11c antibodies.

RT-PCR analysis

RT-PCR analysis was performed using following primers: 5'-c-Fos, 5_-CTG GTG CAG CCC ACT CTG GTC-3_; 3'-c-Fos, 5_-CTTTCAGCAGATTGGCAATCTC-3_; 5_-Cathepsin K, 5_-CTT GTG GAC TGT GTG ACT-3_; 3_-Cathepsin K, 5_-AAC ACT GCA TGG TTC ACA-3_; 5_-TRAP, 5_-CTG GAG TGC ACG ATG CCA GCG ACA-3_; 3_-TRAP, 5_-TCC GTG CTC GGC GAT GGA CCA GA -3_; 5_-NFATc1, 5_-CTC GAA AGA CAG CAC TGG AGC AT-3_; 3_-NFATc1, 5_-CGG CTG CCT TCC GTC TCA TAG-3_; 5_-c-fms, 5_-AGT GTG GGT AAC AGC TCT CAG TAC-3_; and 3_-c-fms, 5_-TCC TAG AGT CTT ACC AAA CTG CAG-3_.

Phagocytosis assay

Phagocytosis assay was performed as described (2). Briefly, fluorescein-conjugated zymosan A (*Saccharomyces cerevisiae*) Bio Particle (Molecular Probes, Eugene, OR) was added to BMMs in 96-well culture plates (20 _g/0.2 ml/well). After 1 hour of incubation, cells were washed with PBS to remove the particles that were not incorporated by the cells. Cells were fixed and observed with ultraviolet (UV) illumination under the microscope (Leica, Wetzlar, Germany).

MTT assay

BMMs were cultured with either wild-type or mutant peptides in the presence of RANKL and M-CSF for 2-3 days. The MTT reagent was added to the cells to measure the survival of pOCs. After incubation for 30 minutes at 37°C, precipitates were solubilized by incubation with DMSO for 10 minutes at room temperature, and the absorbance was measured with a microplate reader.

Local administration of FITC-Hph1-peptide

The tibial bone was removed 2 hours after intramuscular injection of FITC-Hph-1 or control peptides. After fixation with 10% neutral formalin for 24 hours at room temperature, both ends of the diaphysis were cut off with a disc, and the marrow tissue was carefully removed. 10 μm-thick cyrosections were prepared with a cryostat (Leica CM 1850), and the unstained sections were examined under a fluorescent microsope (Olympus).

Pharmacokinetic analysis

Male BALC/c mice (20 g) were housed in a light controlled room that was maintained at a temperature of 23 ± 3°C and a relative humidity of 50 ± 5% with free access to food and water. The FITC-peptide dissolved in DMSO (2 mg/ml) was administered to each mouse intravenously at a dose of 5 mg/kg. Blood was removed from the heart at 2, 5, 10, 15, 30, 60, 120, and 240 minutes (n=4 at each blood sampling time) after intravenous injection. Serum samples were prepared from the drawn blood by centrifugation at 13,000 rpm for 15 minutes. Florescence of the serum sample (0.1 ml) was measured at 518 nm using a fluorescence microplate reader (MDS Analytical Technologies). Pharmacokinetic parameters were estimated from plasma concentration-time profile using the WinNonlin program (Pharsight Co.) with non-compartmental analysis.

References:

1. Inaba, K. et al. 2000. The formation of immunogenic major histocompatibility complex class II-peptide ligands in lysosomal compartments of dendritic cells is regulated by inflammatory stimuli. *J. Exp. Med*. 191:927-936.
2. Lee, J. et al. 2006. Id helix-loop-helix proteins negatively regulate TRANCE-mediated osteoclast differentiation. *Blood*. 107:2686-2693.

Supplemental Figure legends

Supplemental Figure 1. Transduced BMMs with RANK WT or RANK Mt were serum-starved for 1 hour, and stimulated with an anti-Flag antibody (10 μg/ml) for the indicated period. RT-PCR was performed for c-Fos and β-actin. The fold induction of c-Fos was determined by normalizing to actin expression. Data are expressed as mean +/- s.d.

Supplemental Figure 2. (a) Pre-OCs isolated by co-culture of bone marrow cells with osteoblasts for 6 days. Cells were stained for TRAP (left). Number of $TRAP^+$ pre-OCs was counted (right). Data are expressed as mean +/- s.d. (b) Pre-OCs from stromal-cell free BMM cultures for 2 days. Cells were stained for TRAP (left). Number of $TRAP^+$ pre-OCs was counted (right). Data are expressed as mean +/- s.d. Scale bar, 50 μm.

Supplemental Figure 3. Effect of the RANK inhibitor peptide on pre-OCs survival. BMMs were cultured with either wild-type or mutant peptides in the presence of RANKL and M-CSF for 2-3 days. The MTT reagent was used to measure cell survival of pre-OCs. Data are expressed as mean +/- s.d.

Supplemental Figure 4. Effect of alanine-scanning of the 6-mer RANK inhibitor peptide on osteoclast formation. (a) Sequences of alanine-scanning of the 6-mer RANK inhibitor peptide conjugated with Hph-1 PTD. (b) In vitro differentiation of osteoclasts from BMMs treated with each RANK inhibitor peptides. TRAP-positive multinucleated cells (MNCs) containing more than 5 nuclei were counted 4 days after RANKL stimulation. Data are expressed as mean +/- s.d.

Supplemental Figure 5. (a) BMMs were cultured for the indicated days with M-CSF and RANKL in the presence of either wild-type or mutant peptides. RT-PCR was performed for the expression of cathepsin K, TRAP, NFATc1, c-fms, and β-actin. (b) As in (a), except that expression of RANK was determined western blot analysis. The fold increase of RANK was determined by normalizing to the density of actin control.

Supplemental Figure 6. Effect of NFATc1 expression on the inhibitory effect of the RRI peptide. A constitutive active form of NFATc1 was retrovirally overexpressed in BMMs and cultured for 5 days with M-CSF and RANKL in the presence of either wild-type or mutant peptides. A representative TRAP staining is shown (upper). $TRAP^+$ MNCs were counted and compared (lower). Scale bar, 100 μm. Data are expressed as mean +/- s.d.

Supplemental Figure 7. Effects of the RANK inhibitor peptide on M-CSF, CD40, and IL-1β signaling. (a) Pre-OCs were stimulated with M-CSF for the indicated period. Vav3 or Syk was immunoprecipitated and subjected to immunoblot analysis using anti-phosphotyrosine Ab (4G10). The activated forms of Rac1 were detected by GST pull-down assays. Vav3, Syk, or Rac1 levels in total lysates were used as the loading control. (b) BMMs were treated with anti-CD40 Ab (5 μg/ml) together with wild-type or mutant peptides for the indicated period**.** IκBα degradation and phosphorylation of MAP kinases including JNK, p38, and ERK were determined by immunoblot analysis. (c) Same as (b), except for stimulation with IL-1β (10 ng/ml). Phosphorylation of IκBα or MAP kinasaes were determined by immunoblot analysis.

Supplemental Figure 8. Effect of the RANK inhibitor peptide on RANKL-induced c-Src phosphorylation. Pre-OCs were stimulated with RANKL for the indicated period. Phosphorylation of c-Src was determined by immunoblot analysis. c-Src levels were used as the loading control.

Supplemental Figure 9. The RANK inhibitor peptide suppresses the stimulatory effect of TNF on RANKL-induced OC formation. BMMs were cultured in the presence of RANKL (10 ng/ml) and M-CSF. 1 day after, the cultures were stimulated with TNF (10 ng/ml) together with the WT-1 or Mt-1 peptides for an additional 3 days. $TRAP^+$ MNCs were counted. Data are expressed as mean +/- s.d.

Supplemental Figure 10. The RANK inhibitor peptide does affect neither phagocytosis of BMMs nor differentiation of dendritic cells. (a) BMMs were cultured for 1 day with M-CSF and RANKL in the presence of either the peptides (wild-type or mutant peptides) or RANK-Fc (5 μg/ml). Cultured cells were incubated with fluorescein-conjugated zymosan particles for 1 hour and washed with PBS. Cells were fixed and observed with UV illumination under a microscope. Fluorescein-conjugated zymosan particles incorporated by the cells appear as green dots (original magnification, _100). (b) RANK inhibitor peptide does not affect the expression level of characteristic dendritic-cell markers. BMMs were cultured for 4 days with GM-CSF in the presence of either the peptides or RANK-Fc to generate dendritic cells. LPS (1 _g/ml) was added to cultures to induce dendritic cell maturation. The cells were harvested the next day and stained for FACS analysis with anti-CD86, anti-CD11c antibodies (solid line), or control IgG (dotted line).

Supplemental Figure 11. In vivo delivery of the FITC-Hph1-RRI peptide through the intramuscular route. The FITC-Hph1-RRI or FITC-RRI peptide (20 mg peptide per kg body weight) was injected through the intramuscular route. The delivered peptides in the bone marrow tissue were analyzed 2 hours later by fluorescent microscopy. Magnification, x200.

Supplemental Figure 12. Serum concentration-time profile of the FITC-peptide following intravenous injection of the FITC-Hph1-peptide at a dose of 5 mg/kg in mice.