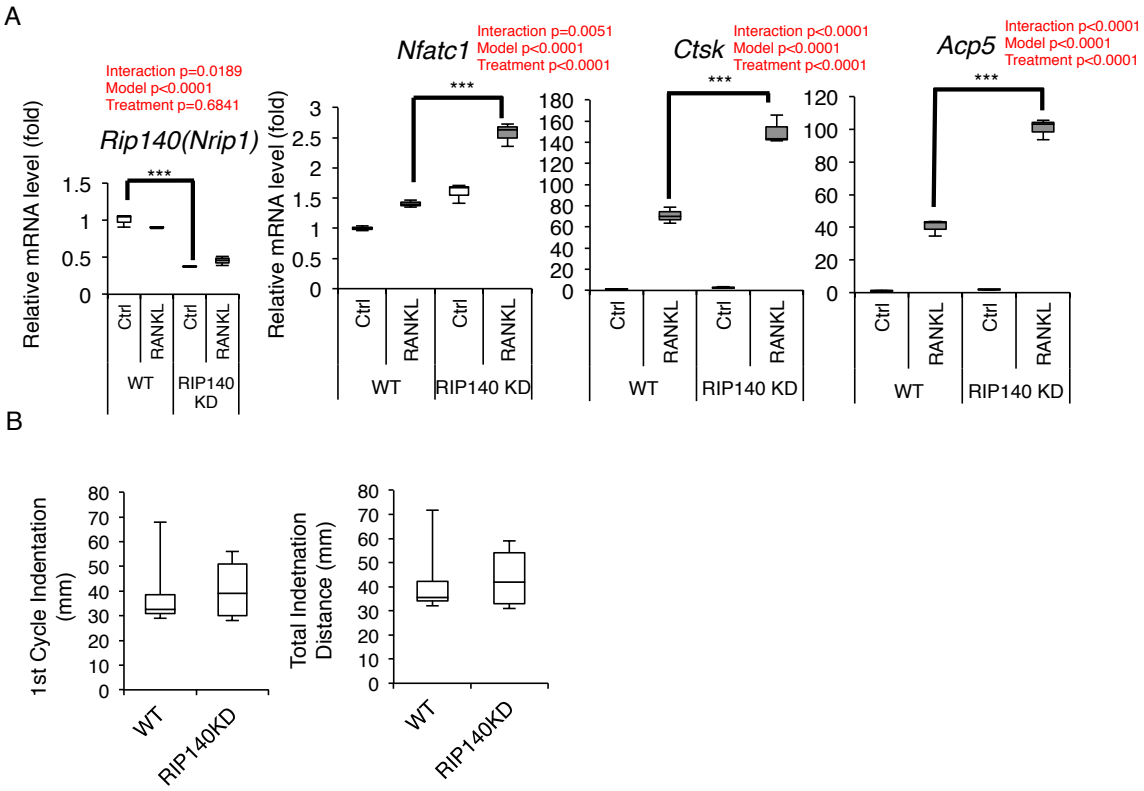
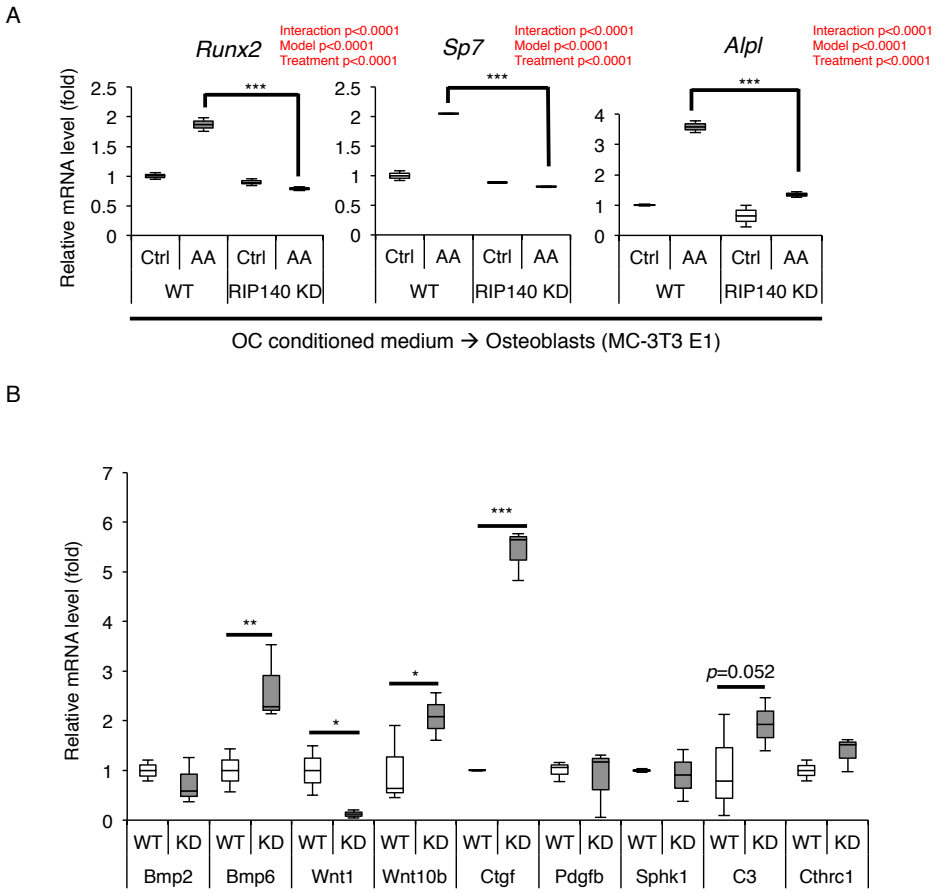
Supplemental figures and figure legends (90517-INS-RG-RV-2)

Supplemental Figure 1.



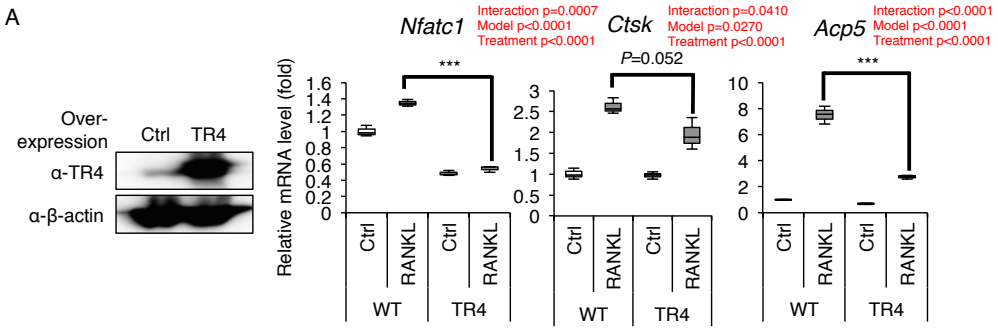
A. qRT-PCR analyses of osteoclast markers in bone marrow-derived osteoclasts from WT and mϕRIP140KD mice (n=3 mice/group) with or without RANKL treatment (100ng/ml). Data are representative of three experimental repeats (mean ± SD), and Student's t-test (n=3) was used (*P<0.05; **P<0.01; ***P<0.001). Additional statistical significance was determined by 2-way ANOVA. **B.** Reference point indentation of the tibia in WT (n=10) and mϕRIP140KD (n=9) mice at 9 weeks of age. Data are mean ± SD.

Supplemental Figure 2.



A. qRT-PCR analyses of osteoblast markers in MC-3T3 E1 cells incubated with conditioned medium of osteoclasts differentiated (5 days) from bone marrow cells of WT and mϕRIP140KD mice (n=4 mice/group). MC-3T3 E1 cells were incubated with conditioned medium from osteoclast culture, with or without ascorbic acid (AA, 50μg/ml) treatment for 3 days. Additional statistical significance was determined by 2-way ANOVA. **B.** qRT-PCR analyses of osteoclast coupling factors in primary osteoclasts differentiated (5 days) from bone marrow cells of WT and mϕRIP140KD mice (n=3 mice/group). For all graphs, data are representative of three experimental repeats (mean ± s.d), and Student's t-test was used unless otherwise specified (*P<0.05; **P<0.01; ***P<0.001).

Supplemental Figure 3.



A. Western blot analysis of TR4 overexpression in RAW264.7 cells (left). qRT-PCR analysis of osteoclast marker genes in control overexpressing (Ctrl) or TR4 overexpressing (TR4) RAW264.7 cells with or without RANKL treatment (50ng/ml) for 24 hrs (right). Data are representative of three experimental repeats (mean ± SD), and Student's t-test (n=3) was used (*P<0.05; **P<0.01; ***P<0.001). Additional statistical significance was determined by 2-way ANOVA.

Supplemental Table 1. Primer information

	qPCR Primer, 5' - 3'	
Gene	Forward	Reverse
OSTEOBLAST		
Runx2	GCCGGGAATGATGAGAACTA	GGTGAAACTCTTGCCTCGTC
Osterix	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAAGGGGACAGA
Alk. Phos.	TGAGCGACACGGACAAGA	GGCCTGGTAGTTGTTGTGAG
OSTEOCLAST		
Nfatc1	CCCCATCCGCCAGGCTACA	GGTTGTCTGCACTGAGCCAACTCC
Ctsk	AATACGTGCAGCAGAACGGAGGC	CTCGTTCCCCACAGGAATCTCTCTGTAC
Acp5	CACTCCCACCCTGAGATTTGTG	ACGGTTCTGGCGATCTCTTTG
Coupling factors		
Bmp2	GCTCAGCATGTTTGGCCTGA	TCCTCCACGGCTTCTTCGTG
Bmp6	CTCGGGATGGACTCCACGTC	GTGGACCTCGCTCACCTTGA
Wnt1	CGGGACCTACGCTTCCTCAT	CGGACATCCCGTGGCATTTG
Wnt10b	ACGCCAGGTGGTAACGGAAA	GCTGCCCTCCAACAGGTCTT
Pdgfb	ATCCGCTCCTTTGATGATCT	GAGCTTTCCAACTCGACTCC
Ctgf	CCTGCCCTAGCTGCCTACC	GAACAGGCGCTCCACTCTGT
C3	GCTGGAGAGCGAAGAGACCA	TCACTGGTCAGCACTTGCCT
Cthrc1	ATCAGCGCCTCTGAGAACCC	CATCACGACCGGGAACTCCT
Sphk1	CCCGTCGACACACACCTTGT	CCCATGGGTGCTGCAAACAG

Supplemental Materials and Methods

Reagents

Reagent sources: mouse RANKL (Biolegend, #577102), mouse M-CSF (Sigma-Aldrich, #M9170). Antibody sources: α-HA (F-7, sc-7392), α-NFATc1 (H-110, sc-13033), α-TR4 (M-76, sc-9086), α-pMEK1/2 (sc-7995), α-MEK1/2 (12-B, sc-436), α-p65 (C-20, sc-372), α-β-actin (C4, sc-47778) from Santa Cruz. α-FLAG (F3165) from Sigma-Aldrich. α-RIP140 (ab42126) from Abcam. α-pERK1/2 (#9101), α-ERK1/2 (#9102) and α-pp65 (93H1, #3033) are from Cell signaling.

RNA isolation and gene expression analysis

Total RNA was isolated using TRIzol (Invitrogen). Reverse transcription (RT) was performed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (qPCR) was performed with SYBR enzyme mix (Thermo Scientific, #K0253). Each gene expression experiment was performed triplicate. Expression levels were normalized to β-actin mRNA level.

Western blot analysis

RAW264.7 cells were lysed with cell lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 2.5mM Sodium Pyrophosphate, 1mM glycerophosphate, 1% NP40, 1% deoxy cholate), and cell lysates were mixed with SDS loading dye and loaded to a SDS-PAGE gel to separate proteins after protein quantification using a Bradford method. Separated proteins in a SDS-PAGE gel are transferred to a western blot membrane and blotted with primary and secondary antibodies.

Chromatin-immunoprecipitation (ChIP) assay

RAW264.7 cells with control expression or doxycycline-inducible FLAG-HA-RIP140 overexpression were crosslinked with 1% formaldehyde, lysed, sonicated and immunoprecipitated with antibodies overnight. Magnetic beads (EDM Millipore, #16-663) were added to pull down antibody-protein-DNA complex. After 4 hr incubation, beads were washed with low salt, high salt, LiCl buffer and TE buffer according to the instruction from EZ-ChIP (EMD Millipore, #17-371). Following reverse-crosslinking, DNA was isolated and analyzed by qPCR.

In vitro TRAP (tartrate-resistant acid phosphatase) assay/ALP (alkaline phosphatase)

TRACP and ALP assay kit was purchased from Takara (#MK301). For TRAP assay, bone marrow-derived primary osteoclasts or RAW264.7 cells were plated in a 96 well plate and lysed with extraction solution. Cells were immediately incubated with pNPP-contained substrate solution for 60 min, and then stop solution (0.5N NaOH) was added to measure the absorbance at 405nm. For ALP assay, serum was collected from mice blood, and then samples were incubated with substrate, as similar to TRAP assay except for the buffer condition.

Microcomputed Tomography

Microcomputed tomography (μCT) was used for nondestructive 3-dimensional evaluation of bone volume and architecture. Femora were scanned in 70% ethanol using a Scanco μCT40 scanner (Scanco Medical AG, Basserdorf, Switzerland) at a voxel size of 12 x 12 x 12 μm (55 kVp x-ray voltage, 145 μA intensity, and 200 ms integration time). Filtering parameters sigma and support were set to 0.8 and 1, respectively. Bone segmentation was conducted at a threshold of 245 (scale, 0–1000) determined empirically. Total femora (cancellous + cortical bone) were evaluated followed by

evaluation of cortical bone in the mid femur diaphysis and cancellous bone in the distal femur metaphysis. For the femoral diaphysis, 20 consecutive slices (240 μm) of bone were evaluated and cross-sectional volume (cortical and marrow volume, mm^3), cortical volume (mm^3), marrow volume (mm^3), and cortical thickness (μm) measured. Polar moment of inertia (mm^4) was determined as a surrogate measure of bone strength in torsion. For the femoral metaphysis, 42 consecutive slices (504 μm) of cancellous bone, 75 slices (1,050 μm) proximal to the growth plate, were evaluated. Analysis of the lumbar vertebra included the entire region of cancellous bone between the cranial and caudal growth plates (151 ± 2 slices, 1,812 ± 24 μm). Direct cancellous bone measurements included cancellous bone volume fraction (bone volume/tissue volume, BV/TV, %), connectivity density (mm^{-3}), trabecular number (mm^{-1}), and trabecular thickness (μm).

Histomorphometry

Methods used for measuring static and dynamic bone histomorphometry have been described[43]. In brief, distal femora were dehydrated in a graded series of ethanol and xylene, and embedded undecalcified in modified methyl methacrylate. Longitudinal sections (4 μm thick) were cut with a vertical bed microtome (Leica 2065) and affixed to slides precoated with 1% gelatin solution. One section/animal was mounted unstained for measurement of fluorochrome labels. One section/animal was stained for tartrate-resistant acid phosphatase and counterstained with toluidine blue (Sigma) and used for cell-based measurements. All data were collected using the OsteoMeasure System (OsteoMetrics, Inc.). The sampling site for the distal femoral metaphysis was located 0.25-1.25 mm proximal to the growth plate. Fluorochrome-based measurements of bone formation

included mineralizing perimeter (mineralizing perimeter/bone perimeter: cancellous bone perimeter covered with double plus half single label normalized to bone perimeter, %), 2) mineral apposition rate (the distance between two fluorochrome markers that comprise a double label divided by the 3 day interlabel interval, μm/d), and 3) bone formation rate (bone formation rate/bone perimeter: calculated by multiplying mineralizing perimeter by mineral apposition rate normalized to bone perimeter, μm2/μm/y). Cell-based measurements included osteoblast perimeter (osteoblast perimeter/bone perimeter, %) and osteoclast perimeter (osteoclast perimeter/bone perimeter, %). Osteoblasts were identified morphologically as plump cuboidal cells immediately adjacent to the thin layer of osteoid in direct contact with the bone perimeter. Osteoclasts were identified as multinucleated (two or more nuclei) cells with acid phosphatase-positive (red-stained) cytoplasm in contact with the bone surface. All bone histomorphometric data are reported using standard nomenclature[44].

Reference point indentation

The material properties of the anterior surface of the tibia mid-diaphysis were assessed using ex vivo reference point indentation (RPI) (BioDent Hfc; Active Life Scientific). Tibias were rehydrated in PBS prior to testing, and all tests were conducted in PBS. A reference force of ~550 N was applied to each femur. Measurements consisted of 10 cycles at 4 N. 3 to 5 measurements were collected per tibia sample, with a distance of at least 250 μm between sampling sites. RPI metrics, including first cycle indentation distance, total indentation distance, first cycle energy dissipated, and first cycle unloading slope, were obtained using the manufacturer's software.