Materials and methods

Cell culture and osteoclast differentiation

The murine monocyte/macrophage cell line RAW264.7 was obtained from American Type Culture Collection and maintained in 10% fetal bovine serum (FBS, Life Technologies) containing Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma) supplemented with penicillin and streptomycin (Corning). For osteoclast-like differentiation experiments, RAW264.7 cells were cultured in 10% FBS containing Minimum Essential Medium Eagle alpha modified media (α-MEM, Sigma) supplemented with penicillin and streptomycin, and cultured with or without 100 ng/mL of recombinant mouse RANKL (Peprotech) for three days. RANKL-treated RAW264.7 cells had a phenotype similar to osteoclasts as determined by tartrate-resistant acid phosphatase (TRAP) activity and TRAP staining (B-Bridge International). TRAP stained cell images were photographed using Eclipse TS100 microscope (Nikon) with SPOT idea digital camera and SPOT Basic Software (SPOT Imaging Solutions).

For bone osteoclast differentiation, bone marrow cells were harvested from femur of 8 week-old male C57BL/6 mice (Jackson Laboratory) under aseptic conditions and pooled. Cells were cultured overnight in 10% FBS containing α-MEM. Non-adherent cells were harvested, seeded in a 24 well plate at 1.0 x 10^5 cells/well and cultured with 50 ng/mL of mouse M-CSF (Peprotech) for three days, and followed with M-CSF and 50 ng/mL RANKL for seven days. Media was changed every 2 to 3 days.

Sample preparation, peptide labeling and fractionation

Three biological replicates for each 6-plex TMT-labeled RANKL-induced osteoclastogenesis experiment were conducted (Figure 1). In the two TMT experiments, the cells were lysed and
trypsinized using the in-solution urea+ RapiGest (Waters) strategy detailed previously. A modified protocol was used for the second TMT experiment where 8 M urea was substituted with 6 M urea/2 M thiourea. Tryptic peptides were labeled with TMT 6-plex reagent (Thermo Scientific), combined and desalted using Oasis Hlb 1cc (10mg) columns (Waters). The peptides were then fractionated into 24 fractions based on their isoelectric focusing point (pH range of 3 to 10) using the OFF-gel system (Agilent). The fractions were dried using a tabletop speed vacuum (Fisher Scientific), cleaned with the Oasis columns, and resuspended in 40 μL of 5 % acetonitrile (Fisher Scientific), 5 % formic acid (Sigma-Aldrich) for subsequent analysis by liquid chromatography/mass spectrometry (LC/MS).

**Mass spectrometry**

Peptide samples were analyzed with the high resolution/accuracy LTQ-Orbitrap (Elite model, Thermo Scientific) mass spectrometer fronted with a Nanospray FLEX ion source, and coupled to an Easy-nLC1000 HPLC pump (Thermo Scientific). The peptides were subjected to a dual column set-up: an Acclaim PepMap RSLC C18 trap column, 75 mm X 20 mm; and to an Acclaim PepMap RSLC C18 analytical column 50 mm X 150 mm or 75 mm X 250 mm (Thermo Scientific) for RAW264.7 osteoclast experiments 1 and 2 respectively (Thermo Scientific). The analytical gradient was run at 250 nL/min from 10 to 30 % Solvent B (acetonitrile/0.1 % formic acid) for 90 minutes, followed by five minutes of 95 % Solvent B. Solvent A was 0.1 % formic acid. All reagents were HPLC-grade. The instrument was set at 120 K resolution, and the top 20 precursor ions (within a scan range of 380-2000 m/z) were subjected to higher energy collision induced dissociation (HCD, collision energy 40%, isolation width 3 m/z, dynamic exclusion enabled) for peptide sequencing (MS/MS).

The MS/MS data were queried against the Mouse UniProt database (downloaded on May 27,
2012) using the SEQUEST search algorithm, via the Proteome Discoverer (PD) Package (version 1.3, Thermo Scientific), using a 10 ppm tolerance window in the MS1 search space, and a 0.02 Da fragment tolerance window for HCD. Methionine oxidation and 6-plex TMT labels were set as variable modifications, and carbamidomethylation of cysteine was set as a fixed modification. The peptide false discovery rate (FDR) was calculated using Percolator provided by PD: the FDR was determined based on the number of MS/MS spectral hits when searched against the reverse, decoy Mouse database. Peptides were filtered based on a 1 % FDR. Peptides assigned to a given protein group, and not present in any other protein group, were considered as unique. Consequently, each protein group was represented by a single master protein (PD Grouping feature). Master proteins with two or more unique peptides were used for TMT reporter ratio quantification.

**TMT quantification**

The relative change in protein abundance was calculated as follows: For individual peptide spectra, the osteoclast-macrophage TMT ratios were determined by following equation,

\[
\text{Ratio} = \frac{\sum_{i=1}^{n} X_i}{\sum_{i=1}^{n} J_i}
\]

where \(X\) and \(J\) represent the osteoclast and macrophage derived TMT reporter ions respectively, and \(n=3\). The relative change in protein abundance was then determined by the median log ratio of all peptides per protein. To calculate the p-value for each change in protein abundance, a normal distribution, \(N(0, 1)\) first was done using the NORMDIST function in Microsoft Excel. The Bonferroni-adjusted p-value threshold was set at \(\alpha = 0.05\). The protein abundances whose corresponding p-values below \(\alpha\) were investigated further as novel osteoclast-associated
proteins.

**Western blot**

Cellular protein was isolated with RIPA buffer (Cell Signaling Technology) containing protease inhibitor cocktail (complete-mini, Roche Applied Science). Protein concentration was determined by BCA protein assay kit (Pierce) and equal amount of protein (10 μg) was separated by a 10% SDS-polyacrylamide gel. Separated proteins were transferred onto a PVDF membrane (Pall Life Sciences) and the membrane was blocked with 3% BSA (Sigma) containing TBS-T (50mM Tris-HCl, pH 7.4, 150mM NaCl and 0.05% Tween-20). The membrane was incubated with anti-CSE (Sigma), anti-Adseverin (Santa Cruz), anti-Cathepsin K (Abcam) or β-actin (Novus Biologicals) primary antibodies for 90 minutes at room temperature (RT) and followed by HRP-conjugated anti-mouse or anti-rabbit IgG antibody (GE Healthcare) for 60 minutes at RT. The signals were detected by ECL Western Blotting Substrate (Pierce) and visualized by ImagQuant LAS 4000 biomolecular imager (GE Healthcare).

**Immunocytochemistry**

Cells were seeded in 8 well Lab-Tek chamber slides (Nunc) at 2.0 x 10^4/well and cultured with 100 ng/mL of RANKL for three days. Cells were washed with PBS (phosphate buffered saline) and fixed by 4% formalin for 10 minutes at RT. After blocking with 2% BSA and 0.05% Tween-20 containing PBS, cells were incubated with AlexaFluor 488 labeled Phalloidin (Molecular Probes) for 30 minutes at RT. Cells were incubated with anti-CSE antibody (1:250, Sigma) for 90 minutes at RT, followed with Alexa Fluor 594-conjugated anti-rabbit IgG antibody (1:250, Invitrogen) for 60 minutes at RT. Cells were washed three times with PBS and embedded in mounting medium containing DAPI (VECTOR Laboratories). Slides were examined using the Eclipse 80i
microscope (Nikon) and images were processed with the Elements 3.20 software (Nikon).

**Real-time quantitative PCR analysis**

Total RNA was isolated by TRIZOL reagent (Invitrogen) and reverse transcribed with high capacity cDNA transcription kit (Applied Biosystems). Real-time quantitative PCR was performed with TaqMan Gene Expression Assay probes (Applied Biosystems, see Supplemental Table III for assay ID’s) and TaqMan fast universal PCR kit (Applied Biosystems) by 7900 HT Fast Real Time PCR System (Applied Biosystems) and normalized by GAPDH.

**Immunohistochemistry**

Aortic arches of 20 week-old male C57BL/6J mice (WT, Jackson Laboratory, n=3) and ApoE deficient mice (ApoE-/-, Jackson Laboratory, n=3) fed with normal chow diet (WT) or high-fat diet for 10 weeks, were cut at 6 μm and cryo-sections were fixed in 4% paraformaldehyde. The animal protocols were reviewed and approved by the Beth Israel Deaconess Medical Center (BIDMC) Animal Care and Use Committee (IACUC approved protocol 017-2011).

After blocking in 4% of horse serum, sections were incubated with anti-CSE antibody (1:250, Sigma) for 90 min at RT, followed by biotin-labeled secondary antibody (1:200, VECTOR Laboratories) for 45 min at RT, and streptavidin-conjugated Alexa Fluor 488 antibody (Invitrogen) for 20 min at RT. After Avidin/Biotin blocking (VECTOR Laboratories) step, the second primary antibodies (OSCAR (1:50), Santa Cruz or CD68 (1:1,000, BD Bioscience) were applied overnight at 4°C followed by biotin-labeled secondary antibody for 45 min at RT, and streptavidin-coupled Alexa Fluor 594 antibody (Invitrogen) for 20 min at RT. Sections were washed three times in PBS for 5 min and embedded in mounting medium containing DAPI (VECTOR Laboratories). Slides were examined using the confocal microscope A1 (Nikon), and images were processed with the
Elements 3.20 software (Nikon).

**Pit formation Assay for osteoclast functional analysis**

After 24 hours culture, RAW264.7 cells were harvested, seeded in an Osteo Assay Surface 24 well plate (Corning) at 4.0 x 10^4/well, and treated with or without 100 ng/mL of RANKL (taken as Day 0). The culture medium (+/- RANKL) was replaced at day 2 and then collected at day 4 for TRAP activity and the corresponding cells were removed with 10 % bleach for 10 minutes at RT. Pit images were assessed under a light microscope. An average of three random visual fields per well were obtained and the percentage of resorbed area was calculated using the ImageJ software ([http://rsb.info.nih.gov/ij/](http://rsb.info.nih.gov/ij/)).

**siRNA experiments**

Mouse siRNAs (RANK and CSE) and non-targeting control siRNA (NT) were purchased from Fisher Scientific (SMARTpool siGENOME siRNA). Sequences are listed on Supplemental Table IV. Cells were seeded in 60 mm dish at 1.0x10^6 and cultured overnight; 10 nM of siRNA was transfected with SilenceMag siRNA delivery reagent (Boca Scientific) according to instructions.

**Effect of CSE inhibitor and H\textsubscript{2}S donor on RANKL-induced osteoclastogenesis**

RAW264.7 cells were seeded onto an Osteo Assay Surface plate at 4.0x10^4/well and cultured with 100 ng/mL of RANKL for four days. CSE inhibitor DL-propargylglycine (PAG, Sigma) was added in concentrations of 0.25 to 5 mM on day 0. The H\textsubscript{2}S donor, GYY4137 (Sigma), was added in concentrations of 50 to 200 µM. At the end of experiment, TRAP and pit resorption assays were examined.
**Statistical analysis**

Data are shown in mean ± SD. Comparisons between two groups were performed by unpaired Student’s t test. Comparisons of multiple groups were made by one-way ANOVA, followed by the Bonferroni test. \( P \) values <0.05 were considered statistically significant.

**References**


