Cystathionine γ-lyase Accelerates Osteoclast Differentiation
Identification of a Novel Regulator of Osteoclastogenesis by Proteomic Analysis

Takahiro Itou, Natalia Maldonado, Iwao Yamada, Claudia Goettsch, Jiro Matsumoto, Masanori Aikawa, Sasha Singh, Elena Aikawa

Objective—Clinical evidence has linked vascular calcification in advanced atherosclerotic plaques with overt cardiovascular disease and mortality. Bone resorbing monocyte-derived osteoclast-like cells are sparse in these plaques, indicating that their differentiation capability could be suppressed. Here, we seek to characterize the process of osteoclastogenesis by identifying novel regulators and pathways, with the aim of exploring possible strategies to reduce calcification.

Approach and Results—We used a quantitative mass spectrometry strategy, tandem mass tagging, to quantify changes in the proteome of osteoclast-like cells differentiated from RAW264.7 cells in response to, receptor activator of nuclear factor κ-B ligand induction, a common in vitro model for osteogenesis. More than 4000 proteins were quantified, of which 138 were identified as novel osteoclast-related proteins. We selected 5 proteins for subsequent analysis (cystathionine γ-lyase [Cth/CSE], EGF-like repeat and discoidin I–like domain–containing protein 3, integrin α FG-GAP repeat containing 3, adseverin, and Serpinb6b) and show that gene expression levels are also increased. Further analysis of the CSE transcript profile reveals an early onset of an mRNA increase. Silencing of CSE by siRNA and α-propyarglyglycine, a CSE inhibitor, attenuated receptor activator of nuclear factor κ-B ligand–induced tartrate-resistant acid phosphatase type 5 activity and pit formation, suggesting that CSE is a potent inducer of calcium resorption. Moreover, knockdown of CSE suppressed expression of osteoclast differentiation markers.

Conclusions—Our large-scale proteomics study identified novel candidate regulators or markers for osteoclastogenesis and demonstrated that CSE may act in early stages of osteoclastogenesis.

Key Words: macrophages ▪ osteoprotegerin ▪ RANKL protein ▪ vascular calcification

Western societies face a growing burden of cardiovascular calcification, a disease of disordered mineral metabolism.1,2 The interaction between prevalent epidemiological factors, such as age, hypercholesterolemia, and renal insufficiency, accelerates arterial and aortic valve calcification. Cardiovascular calcification causes devastating complications, including acute coronary events, because of plaque rupture and aortic valve stenosis.3-6 However, no medical therapies are currently available to retard or to reduce cardiovascular calcification,7 which has driven the exploration of underlying mechanisms.

Emerging evidence suggests that vascular calcification is an active process, rather than mere tissue degeneration, akin to that of bone formation that involves 2 major cell types: osteoblasts and osteoclasts. In the vascular wall, osteoblast-like cells, differentiated from vascular smooth muscle cells (SMCs), can generate calcium deposition, whereas osteoclast-like cells, differentiated from hematopoietic precursors of the mononuclear phagocyte lineage, might dissolve mineralized deposition.8 Histological studies have shown that calcified atherosclerotic lesions from humans and mice accumulate osteoblast-like cells, whereas a relatively fewer number of osteoclast-like cells are found.9

Our hypothesis posits that molecular regulators cause osteoclast or osteoblast dominance in atherosclerotic regions. To understand the mechanisms behind osteoclastogenesis, we conducted a proteomic analysis using receptor activator of nuclear factor κ-B ligand (RANKL)–induced osteoclast differentiation of the murine macrophage-like cell line RAW264.7, a commonly used model of osteoclastogenesis. Cellular proteins were analyzed by tandem mass tagging (TMT)–based quantitative mass spectrometry analysis.10 A selected set of proteins with increased activity and expression in differentiated RAW264.7 cells were studied further, leading to a novel osteoclast regulator, cystathionine γ-lyase (CSE). Our validation studies demonstrate that CSE accelerates monocyte-derived osteoclast-like differentiation and has a potent calcium resorption function. CSE was also shown by
to suppress osteoblast differentiation. Taken together, CSE may serve as a molecular switch regulating osteoclast–osteoblast balance in vascular wall.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

---

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSE</td>
<td>cystathionine γ-lyase</td>
</tr>
<tr>
<td>Edil3</td>
<td>EGF-like repeat and discoidin I-like domain-containing protein 3</td>
</tr>
<tr>
<td>Itfg3</td>
<td>integrin α FG-GAP repeat containing 3</td>
</tr>
<tr>
<td>MMP9</td>
<td>matrix metalloproteinase 9</td>
</tr>
<tr>
<td>RANKL</td>
<td>receptor activator of nuclear factor κ-B ligand</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>TMT</td>
<td>tandem mass tagging</td>
</tr>
<tr>
<td>TRAP</td>
<td>tartrate-resistant acid phosphatase type 5</td>
</tr>
</tbody>
</table>

---

**Results**

**TMT Strategy Applied to RANKL-Induced Osteoclastogenesis**

To correlate changes in the proteome with RANKL-induced osteoclastogenesis, we used the isobaric TMT approach (Figure 1). Treatment with RANKL induced the formation of TRAP stain positive multinucleated cells by day 3, a feature typical of osteoclasts (Figure 1A). Differentiation of RAW264.7 cells into osteoclast-like cells was also confirmed by TRAP activity assay in the cell culture supernatant (Figure 1B). Individual populations of tryptic peptides generated from 3 replicates of control and RANKL-stimulated RAW264.7 cells into osteoclast-like cells was also confirmed by TRAP activity assay in the cell culture supernatant (Figure 1C). As a consequence, all 6 TMT-modified peptide samples can be combined for subsequent fractionation and liquid chromatography/mass spectrometry analysis. In the first MS scan, the 6 peptide populations represent themselves as a single MS signal.

---

**Figure 1.** Tandem mass tagging (TMT)–based protein profiling of receptor activator of nuclear factor κ-B ligand (RANKL)–induced osteoclastogenesis of RAW264.7 cells. **A**, RAW264.7 cells were cultured with or without 100 ng/mL of RANKL for 3 days to induce osteoclast differentiation. **B**, Corresponding tartrate-resistant acid phosphatase type 5 activity from **A**, **C**, TMT labeling strategy: each biological replicate (3 controls and 3+RANKL) was harvested at day 3 of culture for subsequent cell lysis, proteolysis (trypsinization), and peptide labeling with 1 of 6 TMT chemicals (126–131, each uniquely colored). Labeled peptides (colored lines at step 3) were combined, fractionated by OFF-gel, and analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). **D**, Example tandem mass spectra. The intact peptide scan (MS1) is the combined signal of all 6 TMT-tagged peptides. The fragmentation scan (MS2) provides fragment ions for both peptide identification and TMT reporter ion intensities (red box) for relative peptide abundance profiling. **E**, Inset from **D** highlighting the possible outcomes for the relative TMT reporter intensities: no change or an increase or decrease (red arrows are indicated, respectively). The changes in abundance are reinforced by the 3 biological replicates. Scale bar, 500 μm. Data shown in mean±SD (n=4). *P<0.05.
(Figure 1D); however, during tandem MS (MS2), fragmentation of the TMT-tagged peptides generates ions not only for peptide sequencing but also ions in the lower m/z range that contains the reporter mass tags (126–131), whose respective intensities vary according to their respective peptide/protein relative abundances in the original samples (Figure 1D and 1E).

Evaluation of the Global Proteomics Data and Confirmation of Known Osteoclast Markers

We performed two 6-plex TMT experiments, each investigating 3 biological replicates of control (macrophage) versus RANKL-induced osteoclast cells, for a total of 6 biological replicates. The combined TMT experiments generated a total of 59,833 TMT-labeled peptides and 5,586 (3,729 overlapping) quantified proteins with ≥2 unique peptides (Figure 2A). When the combined data sets were filtered to include proteins whose summed unique peptides were ≥2 (where each TMT experiment provided a single unique peptide), we increased the final overlapping protein number to 4,244 proteins for the final analysis (Figure 2B). Overall, the 2 TMT experiments produced consistent quantification of protein ratios (Figure 2C).

MS2 spectrum of a known osteoclast-associated marker, TRAP/Acp5 is shown in Figure 2D. In addition to the fragment ion spectra required for peptide identification, the TMT reporter ion spectra provide the relative changes in peptide abundance between the 2 cell states. As expected for Acp5, for example, TMT spectrum displays a marked increase in its associated peptide abundance in all 3 osteoclast states with respect to those in the macrophage states (Figure 2D). This increase in abundance is in contrast to the TMT reporter ion profile for proteasome subunit β-type-3 whose reporter ion intensities remain constant across all 6 samples (Figure 2E). The TMT spectra from representative peptides of EGF-like repeat and discoidin I-like domain-containing protein 3 (Edil3; Figure 2F) and Cth/CSE (Figure 2G) reveal that these proteins increased as a function of osteoclastogenesis.

Proteins determined to increase (78 proteins) or to decrease (127 proteins) after Bonferroni correction significantly (threshold set at α=0.05) are indicated in the red boxes (Figure 2B). The Bonferroni correction adjusts the significance cut-off (α=0.05) as a function of sample number (n=4,244) and is considered a strict or conservative estimation of significance. Most known osteoclast and macrophage-specific markers are significant outliers after the Bonferroni correction; however, alternative and more relaxed cut-off methods, such as the standard ratio threshold cut-off at P=0.05 or the FDR calculated cut-off q=0.05, ensure that all marker proteins are considered as significantly regulated (not shown).

Known osteoclast markers cathepsin K, osteoclast-associated receptor, and TRAP/Acp5 increase in abundance as expected (Figure 2B), whereas known macrophage markers, lysozyme C-2 and C-1 and CD14, decrease in abundance as expected (Figure 2B). Table 1 displays a list of previously studied osteoclast-related proteins and their relative changes in abundance.

Identification of Novel Osteoclast-Specific Proteins

Once the expression profiles of known osteoclast-associated markers were verified, we screened the data set for putative novel osteoclast proteins (ie, proteins whose expression increased as a consequence of RANKL-induced osteoclastogenesis; Figure 2B). Using a 1.5-fold cut-off (P=0.01), 440 proteins remained, including a number of known markers (Table 1). We then cross-referenced the literature to reduce our list further to proteins not previously associated with osteoclastogenesis, resulting in a final list of 138 candidates (Table I in the online-only Data Supplement). Selected proteins are listed in Table 2, including Edil3, integrin α FG-GAP repeat containing 3 (Iftg3), Serpinb6b, adseverin, and Cth/CSE. These novel candidate osteoclast-associated proteins represent a variety of biological processes (Table 2; Table II in the online-only Data Supplement) for avenues for potential follow-up.

mRNA and Protein Levels for CSE, Edil3, Iftg3, Adseverin, and Serpinb6b Increased in Differentiated Osteoclast-Like Cells

To determine whether our candidates increase in expression in both RAW 264.7 cells and in mouse bone marrow–derived osteoclasts, we first performed Western blot analysis. Consistent with the proteomics data (Figure 2), Western blots in Figure 3A show an increase in Cth/CSE, adseverin, and cathepsin K for RANKL-induced osteoclasts. Western blot analysis for Edil3 demonstrated no difference in signal in the 2 conditions (not shown) and detected no signal for Iftg3 and Serpinb6b. Discrepancies between quantitative mass spectrometry results and Western blot analysis are not uncommon and can usually be resolved when investigated further.12

The mRNA levels for all 5 candidate proteins (CSE, Edil3, Iftg3, adseverin, and Serpinb6b) and controls (cathepsin K, TRAP, and matrix metalloproteinase 9 [MMP9]) significantly increased in differentiated RAW264.7 cells (Figure 3B). Time course analysis of differentiating RAW264.7 cells showed that CSE mRNA levels peak between days 1 and 2 of RANKL induction and decrease by day 3 (Figure I in the online-only Data Supplement). These data indicate that while CSE protein levels are higher in the differentiated state (day 3), peak activity is likely to have occurred earlier. However, Iftg3, adseverin, and serpinb6b showed steady increases in mRNA levels throughout differentiation, similar to controls (cathepsin K, TRAP, and MMP9), whereas Edil3 mRNA levels peak by day 1 and remained stable until day 3.

In mouse bone marrow cells stimulated with RANKL for 7 days, increased protein levels (CSE, adseverin, and cathepsin K) are detected by Western blot (Figure 3A). An increase in mRNA levels of the candidate proteins was also observed, with the exception of CSE (Figure 3C). However, a time course analysis revealed an increase in CSE mRNA levels at day 3 of RANKL stimulation, followed by a steady decrease of mRNA until day 7 (Figure II in the online-only Data Supplement). All but 1 gene (Iftg3) displays the most drastic increase mRNA levels by day 3; the variability in the Iftg3 measurements is likely because of the relative low transcription levels (Figure II in the online-only Data Supplement).

This analysis highlights the importance of investigating time-dependent regulation of mRNA and protein induction to ensure that key windows of expression are not missed. Overall, the transcript profiling is consistent with the quantitative proteomics data and suggests that increases in the protein levels were a result of increased transcription and translation.
CSE Is a Regulator of Osteoclastogenesis

An immune-localization study of the expression pattern of CSE in RANKL-induced osteoclasts (Figure 4A) shows that cytosolic expression of CSE is observed in control cells; however, expression is redistributed in differentiated osteoclasts, specifically around the nuclei and clearly contained within the F-actin boundary ring structure (Figure 4A, arrow), a hallmark for the osteoclast phenotype.13

To understand whether CSE is required for osteoclastogenesis, we performed siRNA experiments targeting CSE and as a control, RANK. siRNA was transfected 24 hours before cells were stimulated with RANKL, thus preventing CSE
activity early in RANKL-dependent signaling. By day 4 of culture, CSE silencing reduced osteoclast activity to a level comparable with that of RANK silencing, as determined by pit resorption (Figure 4B and 4D) and TRAP activity assays (Figure 4E). These data indicate that early inhibition of CSE levels is sufficient to inhibit osteoclast formation, consistent with the observation that CSE mRNA increases early after RANKL induction (Figure I in the online-only Data Supplement). CSE activity is also inhibited by the small molecule, \( \alpha \)-propargylglycine.\(^{14} \) RANKL-treated RAW264.7 cells were cultured in the presence or in the absence of 5 mmol/L.

**Table 1. Selected Proteins Quantified in RAW264.7 RANKL-Induced Osteoclastogenesis Study**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Name</th>
<th>Normalized Log(_{10}) (RANKL/Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteoclast-associated receptor</td>
<td>Oscar</td>
<td>12.4</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Ctsk</td>
<td>11.4</td>
</tr>
<tr>
<td>C-C chemokine receptor type 1</td>
<td>Ccr1</td>
<td>11.0</td>
</tr>
<tr>
<td>V-type proton ATPase subunit d 2</td>
<td>Atp6v0d2</td>
<td>10.6</td>
</tr>
<tr>
<td>TRAP</td>
<td>Acp5</td>
<td>10.0</td>
</tr>
<tr>
<td>Matrix metalloproteinase 9</td>
<td>Mmp9</td>
<td>8.3</td>
</tr>
<tr>
<td>Unconventional myosin-ld</td>
<td>Myo1d</td>
<td>7.6</td>
</tr>
<tr>
<td>Creatine kinase B-type</td>
<td>Ckb</td>
<td>5.8</td>
</tr>
<tr>
<td>Integrin ( \beta )-3</td>
<td>Itgb3</td>
<td>3.3</td>
</tr>
<tr>
<td>Integrin ( \alpha ) V</td>
<td>Itgav</td>
<td>2.7</td>
</tr>
<tr>
<td>Monocyte differentiation antigen CD14</td>
<td>Cd14</td>
<td>−8.9</td>
</tr>
<tr>
<td>Lysosome C-2</td>
<td>Lys2</td>
<td>−15.8</td>
</tr>
</tbody>
</table>

**Table 2. Selected Novel Osteoclast Associated Proteins and Their Respective Gene Names Identified in This Study**

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene Name</th>
<th>Normalized Log(_{10}) (RANKL/Control)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF-like repeats and discoidin I-like domains 3</td>
<td>Edil3</td>
<td>10.6</td>
<td>22, 23, 24</td>
</tr>
<tr>
<td>NK13/Serpinb6b</td>
<td>Serpinb6b</td>
<td>10.0</td>
<td>27</td>
</tr>
<tr>
<td>RILP-like protein 1</td>
<td>Rilp1</td>
<td>8.3</td>
<td>…</td>
</tr>
<tr>
<td>Isoform 2 of lactadherin</td>
<td>Mtge8</td>
<td>8.2</td>
<td>…</td>
</tr>
<tr>
<td>CSE</td>
<td>Chth</td>
<td>7.7</td>
<td>30, 31</td>
</tr>
<tr>
<td>Protein ITFG3</td>
<td>Itfg3</td>
<td>7.4</td>
<td>25, 26</td>
</tr>
<tr>
<td>Aminocyclase-1</td>
<td>Acy1</td>
<td>6.8</td>
<td>…</td>
</tr>
<tr>
<td>Protein-glutamine ( \gamma )-glutamyltransferase 2</td>
<td>Tgm2</td>
<td>6.6</td>
<td>…</td>
</tr>
<tr>
<td>N-Acetylneuraminic acid lyase</td>
<td>Npl</td>
<td>6.6</td>
<td>…</td>
</tr>
<tr>
<td>Adseverin/Scin</td>
<td>Scin</td>
<td>6.2</td>
<td>28, 29</td>
</tr>
<tr>
<td>Peroxisomal trans-2-enoyl-CoA reductase</td>
<td>Pecr</td>
<td>6.0</td>
<td>…</td>
</tr>
<tr>
<td>Adenylate kinase isoenzyme 1</td>
<td>Ak1</td>
<td>5.9</td>
<td>…</td>
</tr>
<tr>
<td>Beta-enolase</td>
<td>Eno3</td>
<td>5.6</td>
<td>…</td>
</tr>
<tr>
<td>Gamma-enolase</td>
<td>Eno2</td>
<td>5.4</td>
<td>…</td>
</tr>
<tr>
<td>Lysophosphatidylcholine acyltransferase 1</td>
<td>Lpcat1</td>
<td>4.7</td>
<td>…</td>
</tr>
</tbody>
</table>

**Figure 3.** Protein and mRNA expression profiles of candidate osteoclast markers. A, Western blot analysis of cystathionine \( \gamma \)-lyase (Cth/CSE), adseverin, cathepsin K, and \( \beta \)-actin from control and receptor activator of nuclear factor \( \kappa \)-B ligand (RANKL)-induced RAW264.7 and mouse bone marrow cells (MBM). B, Real-time polymerase chain reaction (PCR) analysis of control (ctrl) vs RANKL-induced RAW264.7 cells. C, Real-time PCR analysis of MBM cells treated with M-CSF+RANKL (MR) vs controls treated with M-CSF only (M). mRNA levels were normalized to GAPDH, a house-keeping gene. Data are shown in mean±SD (n=3), \( *P<0.05 \). Edil3 indicates EGF-like repeat and discoidin I-like domain-containing protein 3; and Itfg3, integrin \( \alpha \) FG-GAP repeat containing 3.
Figure 4. Cystathionine γ-lyase (CSE) promotes receptor activator of nuclear factor κ-B ligand (RANKL)–induced osteoclastogenesis. A, Immunolocalization of CSE (red), F-actin (green), and nuclei (DAPI, blue) in control RAW264.7 cells vs RANKL-induced osteoclasts; bar, 100 μm. B, Pit formation assays for control and RANKL–induced, nontargeting siRNA (wild type [WT]/NT) RAW264.7 cells and siRNA (RANK or CSE)-transfected cells. C, Pit formation assays for control and RANKL–induced RAW264.7 cells with or without 5 mmol/L of dl-propargylglycine (PAG). D, Quantification of pit resorption from B. E, Relative tartrate-resistant acid phosphatase type 5 (TRAP) activity from B. F, Quantification of pit resorption from C. G, Relative TRAP activity from C; bars, 500 μm. Data are shown in mean±SD (n=4). H, mRNA expression of osteoclast markers from NT, RANK, or CSE siRNA-transfected RAW264.7 cells cultured with or without RANKL for 3 days. I, mRNA expression from RAW264.7 cells cultured with or without 100 ng/mL RANKL and 5 mmol/L PAG, respectively, for 3 days. Data are shown in mean±SD (n=3). *P<0.05. R indicates RANKL.
siRNA (Figure 4H). CSE knockdown significantly reduced levels of the osteoclast markers although not to the extent of RANK siRNA (Figure 4H). Likewise, reduction of CSE activity by the α-propargylglycine inhibitor reduced the mRNA levels of cathepsin K and MMP9 in RANKL-induced conditions; however, we could not confirm any significant change in the level of TRAP mRNA (Figure 4I).

We then investigated whether the α-propargylglycine–dependent inhibition modified the levels of macrophage marker genes, CD14, Lyz1, Lyz2, and F4/80. Indeed, a reduction in RANKL-induced suppression was observed for all macrophage markers although CD14 was not significant (Figure III in the online-only Data Supplement). This result suggests that CSE is an important regulator of osteoclastogenesis and not just an outcome of the process.

Our results indicate that CSE may regulate osteoclast formation through its product H2S. The addition of a H2S donor (GYY4137) significantly increased the number of TRAP positive osteoclasts, marked by their multinucleate phenotype, as judged by TRAP and pit resorption assays (Figure 5A–5C). Moreover, GYY4137 was able to rescue α-propargylglycine–dependent inhibition of osteoclastogenesis (Figure 5D), indicating that CSE activity involves H2S-dependent mechanisms.

**Plaque Macrophages and Osteoclast-Like Cells Expressed CSE**

To examine the expression of CSE in atherosclerotic plaques, we performed immunohistochemistry in the aortas of apoE-deficient mice and wild-type controls. We found strong CSE expression in intimal and medial SMCs. In addition, CSE coexpressed with the macrophage marker CD68 and the osteoclast marker osteoclast-associated receptor (Figure 6, arrows showing coexpressed cells). CSE expression was low in the aortas of wild-type mice.

**Discussion**

A major breakthrough in osteoclast biology occurred with the characterization of RANKL, its cell surface receptor (RANK), and its decoy receptor osteoprotegerin.15 Evidence suggests that monocytes/macrophages treated with RANKL differentiate into mature osteoclasts.16,17 Mice lacking RANKL or RANK also lack functional osteoclasts, indicating that RANKL signaling is necessary and sufficient for osteoclast survival and function. In our study, we turned to the RAW264.7 cell stimulated with RANKL and its decoy receptor osteoprotegerin. Unlike previous proteomics studies, which focused on either secreted or membrane proteins,20 we have sequenced deep into the RAW264.7 proteome to reveal novel players in the transition to the osteoclast-like state. The TMT strategy, in combination with the faster Orbitrap Elite platform, has improved the sensitivity and scope of our study when compared with approaches such as ICAT for the study of secreted proteins,19 and peptide counting for membrane proteins.20 As a result, we were able to monitor the relative abundances of >4000 proteins during RANKL-induced osteoclast differentiation of mouse macrophage-like cell line RAW264.7. We identified 138 candidate proteins previously unassociated with osteoclast differentiation and function. However, our particular global proteomics approach is only a snapshot of relative protein abundances between 2 distinct phenotypes; it serves as a solid starting point for subsequent in-depth follow-up of the mechanisms behind the changes in the proteome. Therefore, we investigated the time-dependent mRNA expression patterns of 5 novel osteoclast-associated proteins and found that the increase in protein abundances was because of a steady increase in transcription activity for Itfg3, adseverin, and serpinb6b for the 3-day differentiation period. However, the transcription activities for Edil3
and CSE both peaked by the first day, where mRNA levels for CSE decreased thereafter. The relative mRNA profiles of these 5 osteoclast-associated genes, although varied in their time-dependent profiles, are in accordance with their respective day 3 relative protein abundances. These observations underscore the sensitivity and accuracy of current proteomics approaches.

The selected proteins for follow-up studies, Edil3, Itfg3, Serpinb6b, adseverin, and CSE, represent diverse biological functions; however, they have not been previously associated with osteoclastogenesis. It has been demonstrated that Edil3 may contribute to angiogenesis, endochondral bone formation, and regulate inflammation. Itfg3 is a single pass transmembrane protein with confirmation as a glycoprotein. Serpinb6b is a member of the widely studied serine protease inhibitor family, the serpins, that inhibit their enzyme targets by covalent modification, an activity that is consequential to numerous and widespread biological processes. Adseverin belongs to the villin superfamily of proteins, which function in actin remodeling in response to calcium signaling. Mouse adseverin was first characterized in mouse T cells and mast cells but not observed in the mouse macrophage cell line PU5.8. Of the 5 novel osteoclast-associated proteins identified, CSE was of particular interest to us because of the recent findings that its product, H\textsubscript{2}S, suppresses vascular SMC-derived osteoblast-like cells. Moreover, silencing of CSE in human aortic SMC increases osteoblastogenesis and corresponding mineralization.

A detailed analysis of CSE revealed that loss of function by either siRNA or drug inhibition (\textalpha\textpropargylglycine) repressed osteoclast differentiation and that CSE mRNA expression could be an early marker. In addition, reduction of CSE activity also decreased the mRNA expression of canonical osteoclast markers (TRAP, cathepsin K, and MMP9). Our results suggest that CSE promotes osteoclast activity by regulating its associated enzymes and that this regulation of osteoclast formation occurs through H\textsubscript{2}S.

Moreover, macrophages and osteoclast-associated receptor-positive osteoclast-like cells express CSE protein in advanced murine atherosclerotic plaques. In addition, our data localized CSE to the intimal and medial SMCs of the plaques, consistent with the notion that CSE activity plays a role in osteoblast-like differentiation. CSE-deficient mice, for example, display marked hypertension, which may contribute to arterial wall remodeling and vascular calcification. Previous and current studies have led to the promising hypothesis: early induction of CSE activity in monocytes/macrophages promotes osteoclast differentiation, while simultaneously inhibiting osteoblast programming by neighboring SMCs. CSE dysfunction may thus have a dual role in vascular calcification: a loss of osteoclast-like potential and an increase in osteoblast-like dominance. Future studies may dissect the precise mechanisms behind this putative common molecular switch for osteoclast–osteoblast balance and may offer a novel therapeutic target for vascular calcification.

Acknowledgments

We thank Eri Kamura and Tyler Faits for their excellent technical assistance. Dr Piero Ricchiuto for his helpful comments on the
proteomics data analysis and Dr Katsumi Yabusaki for advice on imaging analysis.

Sources of Funding
This study was supported by a research grant from Kowa Company, Ltd. (Tokyo, Japan, to M. Aikawa) and the National Institutes of Health grants (R01HL114805 and R01HL109506 to E. Aikawa; R01HL107550 to M. Aikawa).

Disclosures
None.

References

Significance
Clinical evidence has linked vascular calcification in advanced atherosclerotic plaques with overt cardiovascular disease and mortality. Bone resorbing osteoclasts are sparse in atherosclerotic plaques, indicating that their differentiation capability could be suppressed. In this study, we identified novel regulators of osteoclastogenesis using state-of-the-art tandem mass tagging–based quantitative mass spectrometry analysis, with the aim of exploring new strategies to reduce cardiovascular calcification. Our validation analysis revealed a new regulator of osteoclastogenesis—cystathionine γ-lyase. Here, we show that CSE accelerates monocytes-derived osteoclast differentiation and has a potent calcium resorption function, resulting in an attractive anticalcification target.
Cystathionine γ-lyase Accelerates Osteoclast Differentiation: Identification of a Novel Regulator of Osteoclastogenesis by Proteomic Analysis
Takahiro Itou, Natalia Maldonado, Iwao Yamada, Claudia Goettsch, Jiro Matsumoto, Masanori Aikawa, Sasha Singh and Elena Aikawa

Arterioscler Thromb Vasc Biol. published online December 19, 2013;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2013/12/19/ATVBAHA.113.302576

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2013/12/19/ATVBAHA.113.302576.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**Figure I.** Time course expression of selected genes and markers. RAW264.7 cells were cultured with or without 100 ng/mL of RANKL for one to three days. Gene expression levels were examined by real-time QPCR normalized to GAPDH expression. Data are shown in mean ± SD (n=3). *p <0.05.
Figure II. Time course expression of selected genes and markers. MBM cells were cultured with M-CSF, with or without RANKL for one to seven days. Gene expression levels were examined by real-time QPCR normalized to GAPDH expression. M: M-CSF only, MR: M-CSF+RANKL. Data shown in mean ± SD (n=3). * p <0.05.
Figure III. Inhibition of CSE repressed RANKL-induced suppression of macrophage markers. RT-PCR from RAW264.7 cells cultured with or without 100 ng/mL of RANKL and 5 mM of PAG for three days. Data are shown in mean ± SD (n=3). * p <0.05. C:control, R:RANKL, P:PAG.

Table I. A list of novel candidate osteoclast proteins identified in this study. Uniprot accession number, protein and gene name, and normalized log ratios are provided. *: five selected proteins. See Pages (5 to 7)

Table II. Biological Functions of Selected Proteins

<table>
<thead>
<tr>
<th>Protein name</th>
<th>CSE</th>
<th>EDIL3</th>
<th>ITFG3</th>
<th>Adseverin</th>
<th>Serpinb6b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene name</td>
<td>Cth</td>
<td>Edil3, Del1</td>
<td>Itfg3</td>
<td>Scin</td>
<td>Serpinb6b</td>
</tr>
<tr>
<td></td>
<td>* Negative regulation of cell growth.</td>
<td>* Promotes Endothelial cells adhesion.</td>
<td></td>
<td>* Chondrocyte proliferation and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>* Positive regulation of NF-κB transcription factor activity.</td>
<td>*Regulated by hypoxia or vascular injury and has been implicated in vascular remodeling.</td>
<td></td>
<td>differentiation.</td>
<td></td>
</tr>
</tbody>
</table>
### Table III. TaqMan probes used for real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Assay ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Mm99999915_g1</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>TRAP</td>
<td>Mm00475698_m1</td>
<td>Tartrate-resistant acid phosphatase type 5, TRAP</td>
</tr>
<tr>
<td>Cathepsin K</td>
<td>Mm00484039_m1</td>
<td>Cathepsin K</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Mm00442991_m1</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>Cth/CSE</td>
<td>Mm00461247_m1</td>
<td>Cystationine gamma-lyase</td>
</tr>
<tr>
<td>Edil3</td>
<td>Mm01291247_m1</td>
<td>EGF-like repeats and discoidin l-like domain 3,</td>
</tr>
<tr>
<td>Itfg3</td>
<td>Mm01309795_m1</td>
<td>Integrin alpha FG-GAP repeat containing 3</td>
</tr>
<tr>
<td>Adseverin</td>
<td>Mm00485972_m1</td>
<td>Gelsolin-like protein, Scinderin</td>
</tr>
<tr>
<td>Serpinb6b</td>
<td>Mm00488413_m1</td>
<td>NK13, Protein Serpinb6b, Serine (Or cysteine) peptidase inhibitor, clade B, member 6b</td>
</tr>
<tr>
<td>RANK</td>
<td>Mm00437132_m1</td>
<td>Tumor necrosis factor receptor superfamily member 11A, Tnfrsf11a</td>
</tr>
<tr>
<td>CD14</td>
<td>Mm00438094_g1</td>
<td>Monocyte differentiation antigen CD14</td>
</tr>
<tr>
<td>Lyz1</td>
<td>Mm00657323_m1</td>
<td>Lysosome C-1, type P</td>
</tr>
<tr>
<td>Lyz2</td>
<td>Mm01612741_m1</td>
<td>Lysosome C-2, type M</td>
</tr>
<tr>
<td>F4/80</td>
<td>Mm00802529_m1</td>
<td>EGF-like module-containing mucin-like hormone receptor-like 1, Emr1</td>
</tr>
</tbody>
</table>

### Table IV. Target sequences of siRNA oligos

<table>
<thead>
<tr>
<th>Gene</th>
<th>Target sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RANK</td>
<td>CAAGAAGUGUGUGAAGGUA</td>
</tr>
<tr>
<td></td>
<td>CCAAGGAGGCCAGGCUUA</td>
</tr>
<tr>
<td></td>
<td>GCGCAGACUUCACUCCAUA</td>
</tr>
<tr>
<td></td>
<td>GAGCAGAACUGACUCUAUG</td>
</tr>
<tr>
<td>CSE</td>
<td>GAGCAGUUCCUCUCCUUUU</td>
</tr>
<tr>
<td></td>
<td>GAUAACCUUCUCAUGUCUG</td>
</tr>
<tr>
<td></td>
<td>GCCCAGGAUGUCCAGUUU</td>
</tr>
<tr>
<td></td>
<td>GAAUUUGACUGAAGAUUU</td>
</tr>
<tr>
<td>Non-Targeting</td>
<td>UAGCGACUAAACACAUCAA</td>
</tr>
<tr>
<td></td>
<td>UAAGGCUGAAGAGAGAUAC</td>
</tr>
<tr>
<td></td>
<td>AUGUAUUGCCUGUAUUGAG</td>
</tr>
<tr>
<td></td>
<td>AUGAAGUGAAUUGCUCAA</td>
</tr>
</tbody>
</table>
Table I  A list of novel candidate osteoclast proteins identified in this study. Uniprot accession number, protein and gene name, and normalized log ratios are provided.

*: five selected proteins.

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Protein Name</th>
<th>Gene Name</th>
<th>Normalized log10 ratio (RANKL/control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q8C4U8</td>
<td>EGF-like repeats and discoidin I-like domains 3</td>
<td>Edil3</td>
<td>10.6</td>
</tr>
<tr>
<td>Q08804</td>
<td>NK13 (Protein Serpinb6b)</td>
<td>Serpinb6b</td>
<td>10.0</td>
</tr>
<tr>
<td>Q9JJC6</td>
<td>RILP-like protein 1 (Rab-interacting lysosomal-like protein 1)</td>
<td>Rilpl1</td>
<td>8.3</td>
</tr>
<tr>
<td>P21956-2</td>
<td>Isoform 2 of Lactadherin</td>
<td>Mfge8</td>
<td>8.2</td>
</tr>
<tr>
<td>Q8VCN5</td>
<td>Cystathionine gamma-lyase</td>
<td>Cth</td>
<td>7.7</td>
</tr>
<tr>
<td>Q8COZ1</td>
<td>Protein ITFG3</td>
<td>Itfg3</td>
<td>7.4</td>
</tr>
<tr>
<td>F8WJD9</td>
<td>Glutathione S-transferase pi 1 (Fragment)</td>
<td>Gstp1</td>
<td>7.3</td>
</tr>
<tr>
<td>Q80XN0</td>
<td>D-beta-hydroxybutyrate dehydrogenase, mitochondrial</td>
<td>Bdh1</td>
<td>7.2</td>
</tr>
<tr>
<td>D3YUS5</td>
<td>RAS protein activator-like 2</td>
<td>Rasal2</td>
<td>7.0</td>
</tr>
<tr>
<td>Q99JW2</td>
<td>Aminoacylase-1</td>
<td>Acy1</td>
<td>6.8</td>
</tr>
<tr>
<td>Q91X50</td>
<td>Glutathione S-transferase, theta 1</td>
<td>Gstt1</td>
<td>6.7</td>
</tr>
<tr>
<td>Q9DCJ9</td>
<td>N-acetylneuramine lyase</td>
<td>Npl</td>
<td>6.6</td>
</tr>
<tr>
<td>P21981</td>
<td>Protein-glutamine gamma-glutamyltransferase 2</td>
<td>Tgm2</td>
<td>6.6</td>
</tr>
<tr>
<td>Q60604</td>
<td>Adseverin (Gelsolin-like protein) (Scinderin)</td>
<td>Scin</td>
<td>6.2</td>
</tr>
<tr>
<td>E9Q251</td>
<td>RIKEN cDNA 4632428N05 gene (Fragment)</td>
<td>4632428N05Rik</td>
<td>6.2</td>
</tr>
<tr>
<td>GSEBT9</td>
<td>Hydroxyacyl glutathione hydrolase</td>
<td>Hagh</td>
<td>6.1</td>
</tr>
<tr>
<td>Q60936-2</td>
<td>Isoform 2 of Chaperone activity of bc1 complex-like, mitochondrial</td>
<td>Adck3</td>
<td>6.1</td>
</tr>
<tr>
<td>D6RHN4</td>
<td>peroxisomal trans-2-enoyl-CoA reductase</td>
<td>Pecr</td>
<td>6.0</td>
</tr>
<tr>
<td>Q9R0Y5</td>
<td>Adenylation kinase isoenzyme 1</td>
<td>Ak1</td>
<td>5.9</td>
</tr>
<tr>
<td>P30275</td>
<td>Creatine kinase U-type, mitochondrial</td>
<td>Ckmt1</td>
<td>5.9</td>
</tr>
<tr>
<td>P97449</td>
<td>Aminopeptidase N</td>
<td>Anpep</td>
<td>5.7</td>
</tr>
<tr>
<td>Q9ESW8</td>
<td>Pyroglutamyl-peptidase 1</td>
<td>Pgpep1</td>
<td>5.7</td>
</tr>
<tr>
<td>Q3UOJ8</td>
<td>TBC1 domain family member 2B</td>
<td>Tbc1d2b</td>
<td>5.7</td>
</tr>
<tr>
<td>P21550</td>
<td>Beta-enolase</td>
<td>Eno3</td>
<td>5.6</td>
</tr>
<tr>
<td>Q9CQW2</td>
<td>ADP-ribosylation factor-like protein 8B</td>
<td>Arl8b</td>
<td>5.5</td>
</tr>
<tr>
<td>G3UWG1</td>
<td>MCG115977</td>
<td>Gm10108</td>
<td>5.4</td>
</tr>
<tr>
<td>P17183</td>
<td>Gamma-enolase</td>
<td>Eno2</td>
<td>5.4</td>
</tr>
<tr>
<td>Q9EP71</td>
<td>Ankycorbin</td>
<td>Rai14</td>
<td>5.3</td>
</tr>
<tr>
<td>Q6PNCO</td>
<td>DmX-like protein 1 (X-like 1 protein)</td>
<td>DmXl1</td>
<td>5.2</td>
</tr>
<tr>
<td>E9Q2E1</td>
<td>Ankyrin 2, neuronal</td>
<td>Ank2</td>
<td>5.2</td>
</tr>
<tr>
<td>Q9DBW0</td>
<td>Cytochrome P450 4V2</td>
<td>Cyp4v2</td>
<td>5.1</td>
</tr>
<tr>
<td>Q8R4N0</td>
<td>Citrate lyase subunit beta-like protein, mitochondrial</td>
<td>Clybl</td>
<td>5.0</td>
</tr>
<tr>
<td>Q91UZ1</td>
<td>Phospholipase C beta 4</td>
<td>Ptb4</td>
<td>4.9</td>
</tr>
<tr>
<td>Q99KI0</td>
<td>Aconitate hydratase, mitochondrial</td>
<td>Aco2</td>
<td>4.9</td>
</tr>
<tr>
<td>Q8BWT1</td>
<td>3-ketoacyl-CoA thiolase, mitochondrial</td>
<td>Aca2</td>
<td>4.9</td>
</tr>
<tr>
<td>Q7TN98-4</td>
<td>Isoform 4 of Cytoplasmic polyadenylation element-binding protein 4</td>
<td>Cpeb4</td>
<td>4.9</td>
</tr>
<tr>
<td>Q9CZS1</td>
<td>Aldehyde dehydrogenase X, mitochondrial</td>
<td>Aldh1b1</td>
<td>4.8</td>
</tr>
<tr>
<td>Q61578</td>
<td>NADPH:adenodoxin oxidoreductase, mitochondrial</td>
<td>Fdxr</td>
<td>4.8</td>
</tr>
<tr>
<td>P35B31</td>
<td>Tyrosine-protein phosphatase non-receptor type 12</td>
<td>Ptpn12</td>
<td>4.8</td>
</tr>
<tr>
<td>P29352</td>
<td>Tyrosine-protein phosphatase non-receptor 22</td>
<td>Ptpn22</td>
<td>4.8</td>
</tr>
<tr>
<td>Q3TFD2</td>
<td>Lysothosphatidylcholine acyltransferase 1</td>
<td>Lpcat1</td>
<td>4.7</td>
</tr>
<tr>
<td>Q91XC8</td>
<td>Death-associated protein 1</td>
<td>Dap</td>
<td>4.6</td>
</tr>
<tr>
<td>Q9WTP6</td>
<td>Adenylate kinase 2, mitochondrial</td>
<td>Ak2</td>
<td>4.6</td>
</tr>
<tr>
<td>A2AQZ2</td>
<td>Phytanoyl-CoA dioxygenase domain containing 1 (Fragment)</td>
<td>Phyhd1</td>
<td>4.6</td>
</tr>
<tr>
<td>Q9Z210</td>
<td>LETM1 and EF-hand domain-containing protein 1, mitochondrial</td>
<td>Letm1</td>
<td>4.6</td>
</tr>
<tr>
<td>Accession No.</td>
<td>Protein Name</td>
<td>Gene Name</td>
<td>Normalized log10 ratio (RANKL/control)</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------------------</td>
<td>-----------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>Q7TPR4</td>
<td>Alpha-actinin-1</td>
<td>Actn1</td>
<td>4.5</td>
</tr>
<tr>
<td>Q8VHX6-2</td>
<td>Isoform 2 of Filamin-C</td>
<td>Flnc</td>
<td>4.5</td>
</tr>
<tr>
<td>P50396</td>
<td>Rab GDP dissociation inhibitor alpha</td>
<td>Gdi1</td>
<td>4.5</td>
</tr>
<tr>
<td>P03921</td>
<td>NADH-ubiquinone oxidoreductase chain 5</td>
<td>Mnd5</td>
<td>4.5</td>
</tr>
<tr>
<td>P35486</td>
<td>Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial</td>
<td>Pdha1</td>
<td>4.5</td>
</tr>
<tr>
<td>Q9CQB4</td>
<td>Cytochrome b-c1 complex subunit 7</td>
<td>Uqcrb</td>
<td>4.4</td>
</tr>
<tr>
<td>Q9Z2D6</td>
<td>Methyl-CpG-binding protein 2</td>
<td>Mecp2</td>
<td>4.4</td>
</tr>
<tr>
<td>A2AEV0</td>
<td>WD repeat domain 45 (Fragment)</td>
<td>Wdr45</td>
<td>4.4</td>
</tr>
<tr>
<td>Q8R111</td>
<td>Cytochrome b-c1 complex subunit 9</td>
<td>Uqcr10</td>
<td>4.4</td>
</tr>
<tr>
<td>O08749</td>
<td>Dihydropyridyl dehydrogenase, mitochondrial</td>
<td>Dld</td>
<td>4.4</td>
</tr>
<tr>
<td>Q99K51</td>
<td>Plastin-3 (T-plastin)</td>
<td>Pls3</td>
<td>4.3</td>
</tr>
<tr>
<td>P47856-2</td>
<td>Isoform 2 of Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1</td>
<td>Gfpt1</td>
<td>4.3</td>
</tr>
<tr>
<td>G3X9N6</td>
<td>Oxysterol-binding protein</td>
<td>Osbp18</td>
<td>4.2</td>
</tr>
<tr>
<td>Q9CQ69</td>
<td>Cytochrome b-c1 complex subunit 8</td>
<td>Uqcrq</td>
<td>4.2</td>
</tr>
<tr>
<td>P08249</td>
<td>Malate dehydrogenase, mitochondrial</td>
<td>Mdh2</td>
<td>4.2</td>
</tr>
<tr>
<td>Q9DB77</td>
<td>Cytochrome b-c1 complex subunit 2, mitochondrial</td>
<td>Uqcr2</td>
<td>4.1</td>
</tr>
<tr>
<td>Q9D0M3-2</td>
<td>Isoform 2 of Cytochrome c1, heme protein, mitochondrial</td>
<td>Cyc1</td>
<td>4.1</td>
</tr>
<tr>
<td>Q9CZB0</td>
<td>Succinate dehydrogenase cytochrome b560 subunit, mitochondrial</td>
<td>Sdhc</td>
<td>4.1</td>
</tr>
<tr>
<td>Q9D0K2</td>
<td>Succinyl-CoA:3-ketoacid-coenzyme A transferase 1, mitochondrial</td>
<td>Oxt1</td>
<td>4.1</td>
</tr>
<tr>
<td>Q8K2Q9</td>
<td>Shootin-1</td>
<td>Kiaa1598</td>
<td>4.0</td>
</tr>
<tr>
<td>Q76N33</td>
<td>AMSH-like protease</td>
<td>Stambpl1</td>
<td>4.0</td>
</tr>
<tr>
<td>D3Z6B9</td>
<td>Mitochondrial 10-formyltetrahydrofolate dehydrogenase</td>
<td>Aldh12</td>
<td>4.0</td>
</tr>
<tr>
<td>Q8BR20-2</td>
<td>Isoform 2 of UPF0600 protein C5orf51 homolog</td>
<td>C5orf51</td>
<td>4.0</td>
</tr>
<tr>
<td>Q61B23</td>
<td>Programmed cell death protein 4</td>
<td>Pdcd4</td>
<td>3.9</td>
</tr>
<tr>
<td>Q9CQA3</td>
<td>Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial</td>
<td>Sdhb</td>
<td>3.9</td>
</tr>
<tr>
<td>Q6PHZ2-2</td>
<td>Isoform 2 of Calcium/calmodulin-dependent protein kinase type II subunit delta</td>
<td>Camk2d</td>
<td>3.9</td>
</tr>
<tr>
<td>Q8BN82-2</td>
<td>Isoform 2 of Sialin</td>
<td>Scl17a5</td>
<td>3.8</td>
</tr>
<tr>
<td>E9PUK3</td>
<td>Formin-binding protein 1-like</td>
<td>Fnbp1l</td>
<td>3.8</td>
</tr>
<tr>
<td>Q9D0D1</td>
<td>Pyruvate dehydrogenase E1 component subunit beta, mitochondrial</td>
<td>Pdhb</td>
<td>3.8</td>
</tr>
<tr>
<td>P50429</td>
<td>Arylsulfatase B</td>
<td>Arsb</td>
<td>3.7</td>
</tr>
<tr>
<td>Q9DBJ1</td>
<td>Phosphoglycerate mutase 1</td>
<td>Pgam1</td>
<td>3.7</td>
</tr>
<tr>
<td>P97478</td>
<td>Ubiquinone biosynthesis protein C</td>
<td>Cog7</td>
<td>3.7</td>
</tr>
<tr>
<td>Q8CGK3</td>
<td>Lon protease homolog, mitochondrial</td>
<td>Lonp1</td>
<td>3.7</td>
</tr>
<tr>
<td>Q8KR2B3</td>
<td>Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial</td>
<td>Sdhb</td>
<td>3.7</td>
</tr>
<tr>
<td>Q6A028</td>
<td>Switch-associated protein 70 (SWAP-70)</td>
<td>Swap70</td>
<td>3.6</td>
</tr>
<tr>
<td>Q91VT4</td>
<td>Carbonyl reductase family member 4</td>
<td>Cbr4</td>
<td>3.6</td>
</tr>
<tr>
<td>Q8BGGR9</td>
<td>Ubiquitin-like domain-containing CTD phosphatase 1</td>
<td>Ublcp1</td>
<td>3.6</td>
</tr>
<tr>
<td>Q9D2G2</td>
<td>Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial</td>
<td>Dlst</td>
<td>3.6</td>
</tr>
<tr>
<td>Q9ES46</td>
<td>Beta-parvin</td>
<td>Parvb</td>
<td>3.5</td>
</tr>
<tr>
<td>P23298</td>
<td>Protein kinase C eta type</td>
<td>Prkch</td>
<td>3.5</td>
</tr>
<tr>
<td>B1ATS4</td>
<td>ATPase, Ca++ transporting, ubiquitous</td>
<td>Atp2a3</td>
<td>3.5</td>
</tr>
<tr>
<td>Q8CI78</td>
<td>Required for meiotic nuclear division protein 1 homolog</td>
<td>Rmnd1</td>
<td>3.5</td>
</tr>
<tr>
<td>Q8BGT5</td>
<td>Alanine aminotransferase 2</td>
<td>Gpl2</td>
<td>3.4</td>
</tr>
<tr>
<td>Q60770</td>
<td>Syntaxin-binding protein 3</td>
<td>Stxbp3</td>
<td>3.4</td>
</tr>
<tr>
<td>P05201</td>
<td>Aspartate aminotransferase, cytoplasmic</td>
<td>Got1</td>
<td>3.4</td>
</tr>
<tr>
<td>Accession No.</td>
<td>Protein Name</td>
<td>Gene Name</td>
<td>Normalized log10 ratio (RANKL/control)</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>--------------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>P35821</td>
<td>Tyrosine-protein phosphatase non-receptor type 1</td>
<td>Ptpn1</td>
<td>3.4</td>
</tr>
<tr>
<td>P17095-1</td>
<td>Isoform HMG-Y of High mobility group protein HMG-I/HMG-Y</td>
<td>Hmga1</td>
<td>3.4</td>
</tr>
<tr>
<td>Q9D6R2</td>
<td>Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial</td>
<td>Idh3a</td>
<td>3.4</td>
</tr>
<tr>
<td>Q9WUM5</td>
<td>Succinyl-CoA-ligase [ADP/GDP-forming] subunit alpha, mitochondrial</td>
<td>Sullg1</td>
<td>3.4</td>
</tr>
<tr>
<td>Q9Z0U1</td>
<td>Tight junction protein ZO-2</td>
<td>Tjp2</td>
<td>3.3</td>
</tr>
<tr>
<td>A2A839</td>
<td>Erythrocyte protein band 4.1</td>
<td>Epb4.1</td>
<td>3.3</td>
</tr>
<tr>
<td>Q80Y55</td>
<td>BSD domain-containing protein 1</td>
<td>BsdC1</td>
<td>3.3</td>
</tr>
<tr>
<td>Q8RSL3-2</td>
<td>Isoform 2 of Vam6/Vps39-like protein</td>
<td>Vps39</td>
<td>3.3</td>
</tr>
<tr>
<td>Q64433</td>
<td>10 kDa heat shock protein, mitochondrial</td>
<td>Hspe1</td>
<td>3.3</td>
</tr>
<tr>
<td>Q8BMS4</td>
<td>Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial</td>
<td>Coq3</td>
<td>3.3</td>
</tr>
<tr>
<td>P62046</td>
<td>Leucine-rich repeat and calponin homology domain-containing protein 1</td>
<td>Lrch1</td>
<td>3.3</td>
</tr>
<tr>
<td>A8DUK4</td>
<td>Hemoglobin subunit beta-1</td>
<td>Hbb-b1</td>
<td>3.2</td>
</tr>
<tr>
<td>E9QJS2</td>
<td>Phosphoserine phosphatase</td>
<td>Psph</td>
<td>3.2</td>
</tr>
<tr>
<td>Q8BTU6</td>
<td>Eukaryotic initiation factor 4A-II</td>
<td>Eif4a2</td>
<td>3.2</td>
</tr>
<tr>
<td>P53702</td>
<td>Cytochrome c-type heme lyase</td>
<td>Hccs</td>
<td>3.2</td>
</tr>
<tr>
<td>Q3UFY8</td>
<td>Mitochondrial ribonuclease P protein 1</td>
<td>Rg9mtd1</td>
<td>3.2</td>
</tr>
<tr>
<td>Q9CXT7-2</td>
<td>Isoform 2 of Transmembrane protein 192</td>
<td>Tmem192</td>
<td>3.2</td>
</tr>
<tr>
<td>Q4KIM3-3</td>
<td>Isoform 3 of Oxidation resistance protein 1</td>
<td>Oxr1</td>
<td>3.2</td>
</tr>
<tr>
<td>Q8RSL0-3</td>
<td>Isoform 3 of SH3 domain-containing kinase-binding protein 1</td>
<td>Sh3kbp1</td>
<td>3.2</td>
</tr>
<tr>
<td>Q8R4O4</td>
<td>Protein QIL1</td>
<td>Qil1</td>
<td>3.1</td>
</tr>
<tr>
<td>Q8CEE7</td>
<td>Retinol dehydrogenase 13</td>
<td>Rdh13</td>
<td>3.1</td>
</tr>
<tr>
<td>Q8ROX7</td>
<td>Sphingosine-1-phosphate lyase</td>
<td>Sgl1</td>
<td>3.1</td>
</tr>
<tr>
<td>Q3TKM5</td>
<td>Isocitrate dehydrogenase 3 (NAD+), gamma, isoform CRA_c</td>
<td>Idh3g</td>
<td>3.1</td>
</tr>
<tr>
<td>P20108</td>
<td>Thioredoxin-dependent peroxide reductase, mitochondrial</td>
<td>Prdx3</td>
<td>3.1</td>
</tr>
<tr>
<td>Q9CQZ5</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6</td>
<td>Ndufa6</td>
<td>3.1</td>
</tr>
<tr>
<td>Q8VCL2</td>
<td>Protein SCO2 homolog, mitochondrial</td>
<td>Sco2</td>
<td>3.0</td>
</tr>
<tr>
<td>Q8R180</td>
<td>ERO1-like protein alpha</td>
<td>Ero1l</td>
<td>3.0</td>
</tr>
<tr>
<td>E9QPX3</td>
<td>NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial</td>
<td>Ndufs4</td>
<td>3.0</td>
</tr>
<tr>
<td>P00405</td>
<td>Cytochrome c oxidase subunit 2</td>
<td>Mco2</td>
<td>3.0</td>
</tr>
<tr>
<td>Q9CPQ1</td>
<td>Cytochrome c oxidase subunit 6C</td>
<td>Cox6c</td>
<td>3.0</td>
</tr>
<tr>
<td>Q7TMQ7</td>
<td>WD repeat-containing protein 91</td>
<td>Wdr91</td>
<td>3.0</td>
</tr>
<tr>
<td>Q8K1Z0</td>
<td>Ubiquinone biosynthesis protein C</td>
<td>Coq9</td>
<td>2.9</td>
</tr>
<tr>
<td>P49586</td>
<td>Choline-phosphate cytidylyltransferase A</td>
<td>Pcyt1a</td>
<td>2.9</td>
</tr>
<tr>
<td>Q9CQ91</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3</td>
<td>Ndufa3</td>
<td>2.9</td>
</tr>
<tr>
<td>Q8BGU5</td>
<td>Cyclin-Y (Cyclin fold protein 1)</td>
<td>Ccny</td>
<td>2.8</td>
</tr>
<tr>
<td>P51125-3</td>
<td>Isoform 3 of Calpastatin</td>
<td>Cast</td>
<td>2.8</td>
</tr>
<tr>
<td>G5EBR2</td>
<td>Tropomyosin 1, alpha, isoform CRA_k</td>
<td>Tpm1</td>
<td>2.8</td>
</tr>
<tr>
<td>Q5BA65-2</td>
<td>Isoform 2 of C-Jun-amino-terminal kinase-interacting protein 4</td>
<td>Spag9</td>
<td>2.8</td>
</tr>
<tr>
<td>G3X9L6</td>
<td>MCG55033</td>
<td>Gm10250</td>
<td>2.7</td>
</tr>
<tr>
<td>D3Z479</td>
<td>Sorting nexin 15</td>
<td>Snx15</td>
<td>2.7</td>
</tr>
<tr>
<td>Q5C88</td>
<td>von Willebrand factor A domain-containing protein 8</td>
<td>Kiaa0564</td>
<td>2.7</td>
</tr>
<tr>
<td>Q5DCV4</td>
<td>Regulator of microtubule dynamics protein 1</td>
<td>Fam82b</td>
<td>2.7</td>
</tr>
<tr>
<td>Q9DBU3</td>
<td>Serine/threonine-protein kinase RI</td>
<td>Rck3</td>
<td>2.6</td>
</tr>
<tr>
<td>Q8K4F5</td>
<td>Abhydrolase domain-containing protein 11</td>
<td>Abhd11</td>
<td>2.6</td>
</tr>
<tr>
<td>P09671</td>
<td>Superoxide dismutase [Mn], mitochondrial</td>
<td>Sod2</td>
<td>2.6</td>
</tr>
<tr>
<td>Q6Y5D8-3</td>
<td>Isoform 3 of Rho GTPase-activating protein 10</td>
<td>Arhgap10</td>
<td>2.6</td>
</tr>
<tr>
<td>Q99JY0</td>
<td>Trifunctional enzyme subunit beta, mitochondrial</td>
<td>Hadhb</td>
<td>2.6</td>
</tr>
<tr>
<td>Q9D1H6</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 4</td>
<td>Ndufa4</td>
<td>2.6</td>
</tr>
</tbody>
</table>
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),\(^4\) 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.\(^5\),\(^6\) 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 10⁴/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
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 microscope (Nikon) and images were processed with the Elements 3.20 software (Nikon).
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 H₂S 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 H₂S 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


