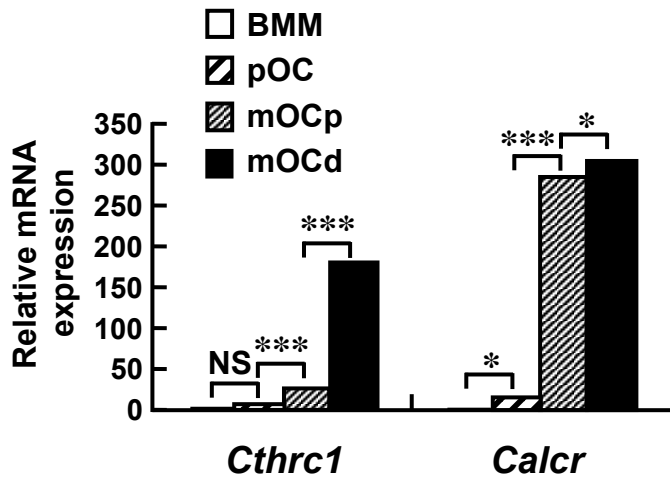


Supplemental Data

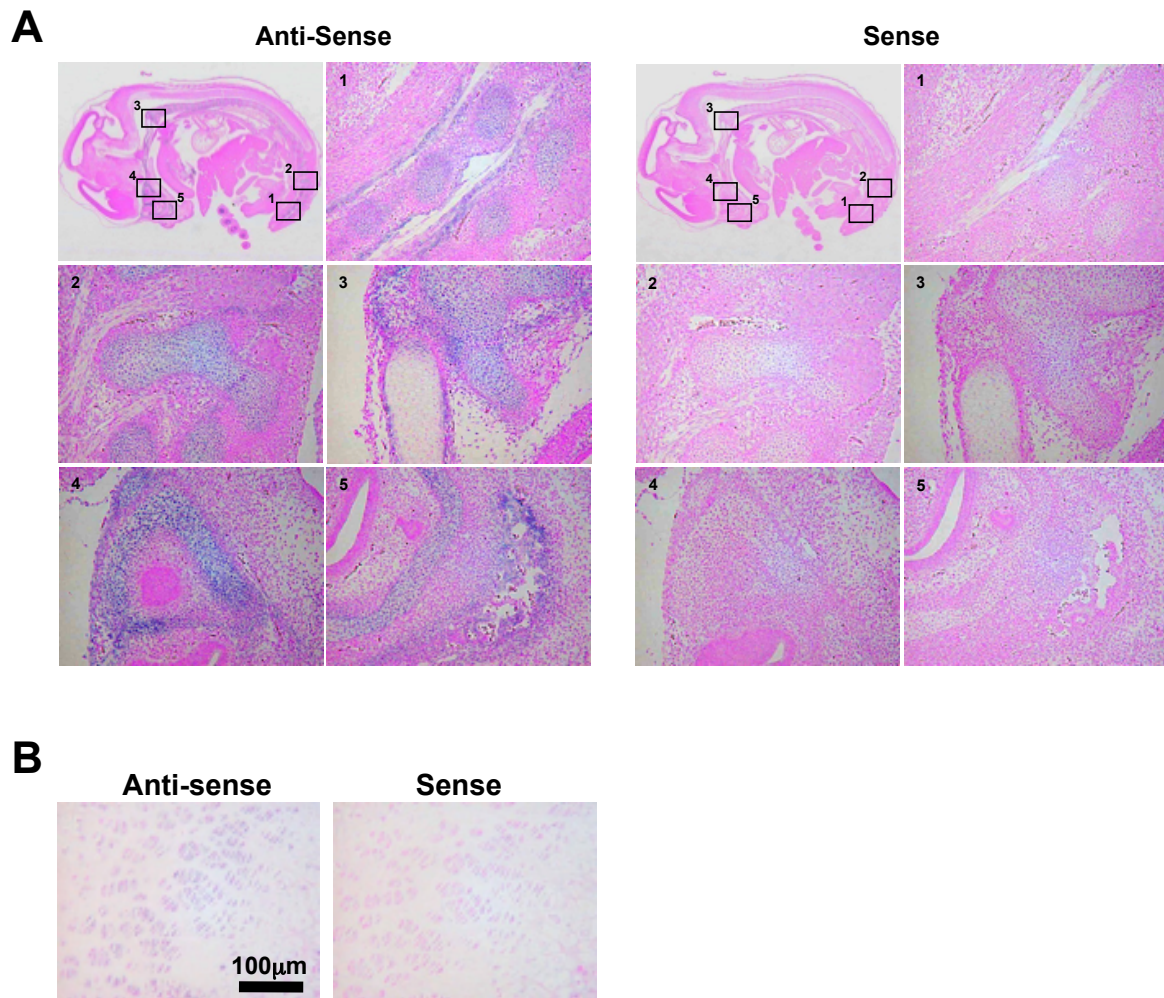
Osteoclast-secreted Cthrc1 in the coupling of bone resorption to formation

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Hiroyuki Aburatani, Shigeaki Kato, Masako Ito and Kyoji Ikeda



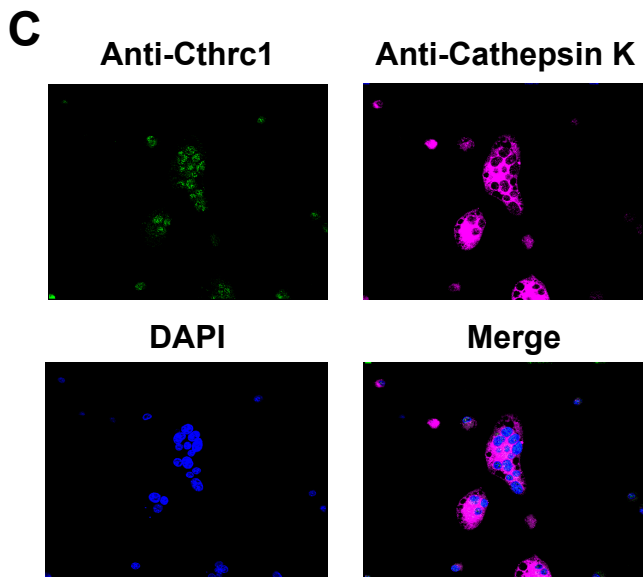
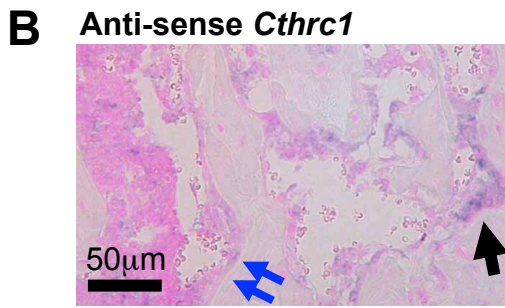
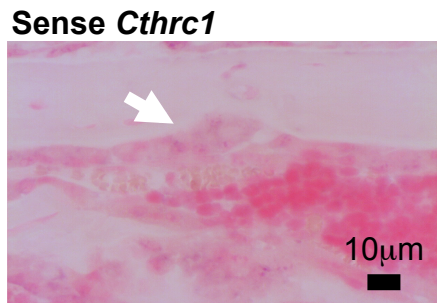
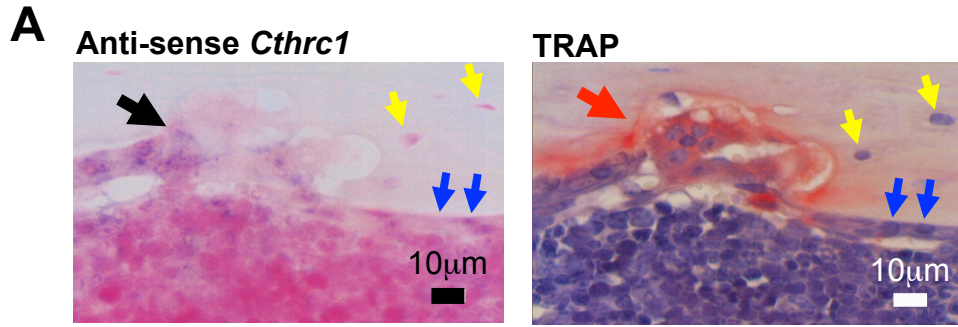
Supplemental Figure 1 *Cthrc1* expression in osteoclasts cultured on dentin

The results of microarray analysis on the mRNA for calcitonin receptor (*Calcr*) and *Cthrc1*. RNA was isolated from bone marrow macrophages (BMM), mononuclear TRAP-positive pre-osteoclasts (pOC), multinucleated TRAP-positive mature osteoclasts on a plastic plate (mOCp) and multinucleated TRAP-positive mature osteoclasts on dentin (mOCd), and subjected to gene expression analysis using Affymetrix Gene Chip.



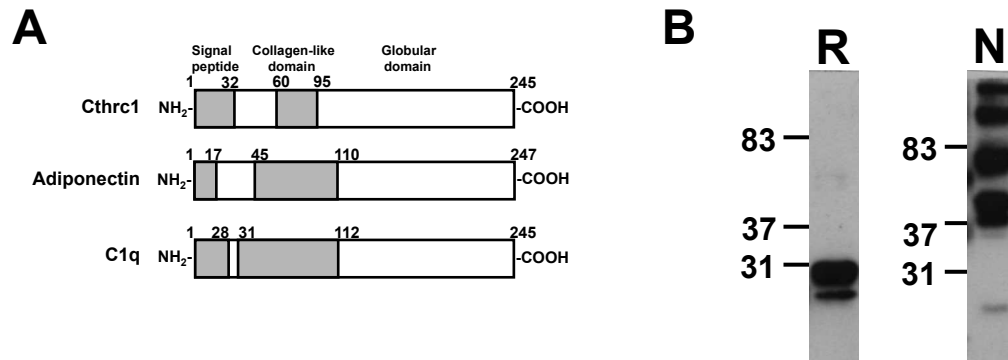
Supplemental Figure 2 *Cthrc1* expression in chondrocytes

In situ hybridization revealed prominent expression of the *Cthrc1* transcript in chondrocytes in the mouse embryo at E14.5 (**A**) and also in postnatal growth plate at 3 months old (**B**). In A, the areas indicated by the black boxes are magnified in panels 1-5. A sense probe served as the negative control.



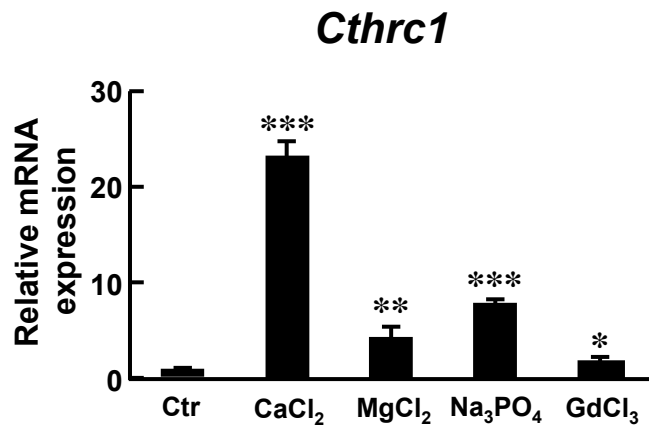
Supplemental Figure 3 *Cthrc1* is expressed in mature osteoclasts

(A) The *Cthrc1* transcript in multinucleated osteoclasts on trabecular bone (black arrow) in vivo by in situ hybridization (ISH) and co-staining with TRAP (red arrow). Sense *Cthrc1* probe served as the negative control. Note that the *Cthrc1* transcript was not detected in osteoblasts lining the bone surface (blue arrows) or osteocytes embedded inside the bone (yellow arrows). Scale bar; 10 μm . (B) *Cthrc1* was not detected in osteoblasts (blue arrows) on trabecular bone surface. Scale bar; 50 μm . (C) Immunocytochemical detection of CTHRC1 protein in bone resorbing osteoclasts. Multinucleated mature osteoclasts were cultured on dentin slice and were stained with rabbit anti-CTHRC1 antibody or mouse anti-cathepsin K monoclonal antibody, and then with FITC-conjugated anti-rabbit IgG or Cy3-conjugated anti-mouse IgG and DAPI.



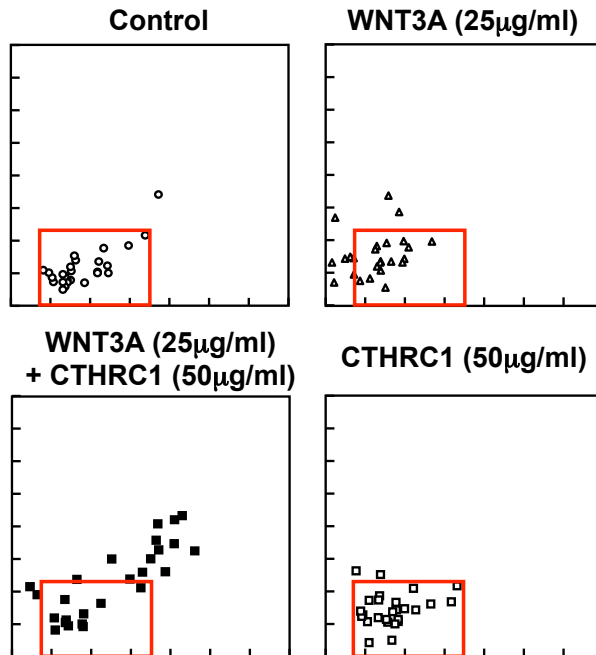
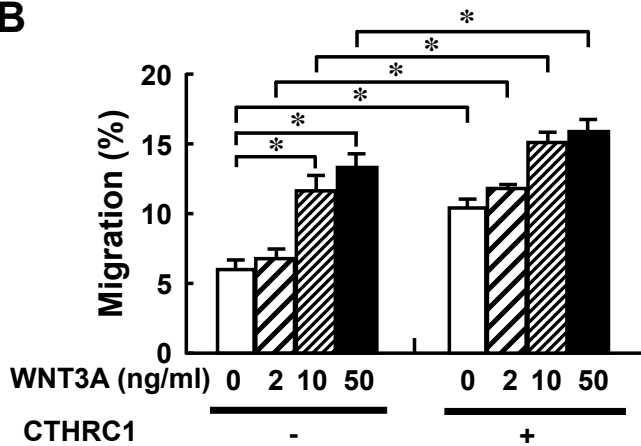
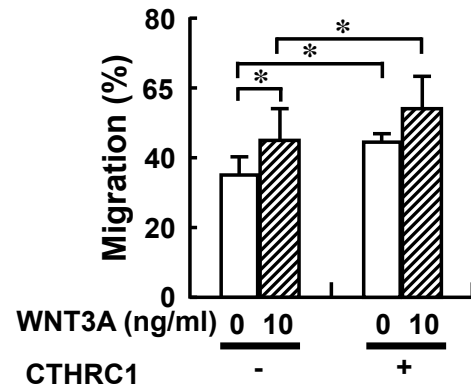
Supplemental Figure 4 Molecular structure of CTHRC1

(A) Molecular structure of CTHRC1 together with adiponectin and the complement C1q. (B) Western blotting analysis of CTHRC1. Recombinant CTHRC1 was loaded and analyzed under reducing (R) and non-reducing (N) conditions.



Supplemental Figure 5 *Cthrc1* expression in osteoclasts

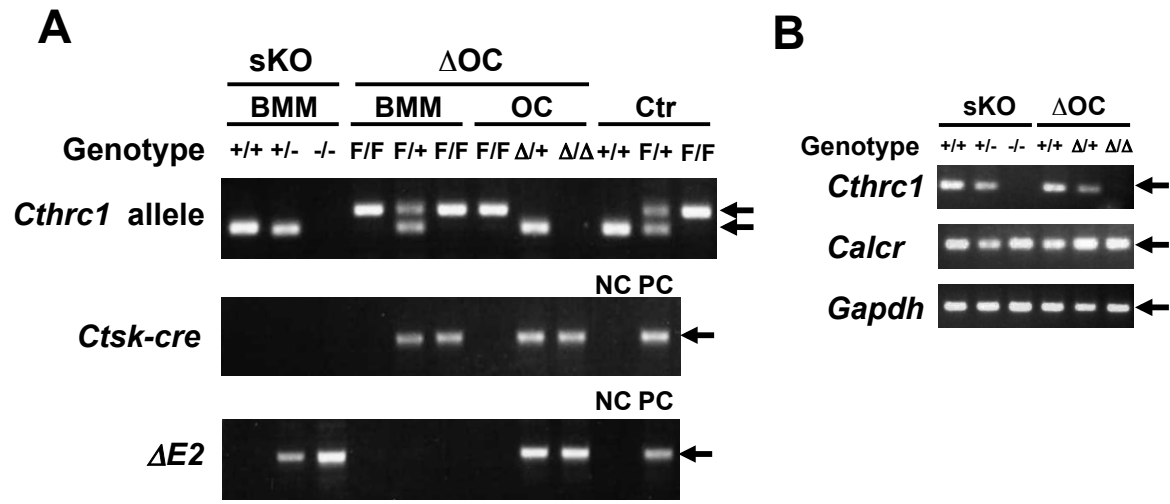
Osteoclasts were cultured in the presence of 40 mM CaCl₂, 32 mM MgCl₂, 20 mM Na₃PO₄, or 200 μM GdCl₃ for 24 hours. RNAs were extracted and real time PCR was performed for *Cthrc1* expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n=3 each group)

A**B****C**

Supplemental Figure 6 Cthrc1 stimulates the chemotaxis of stromal and osteoblastic cells

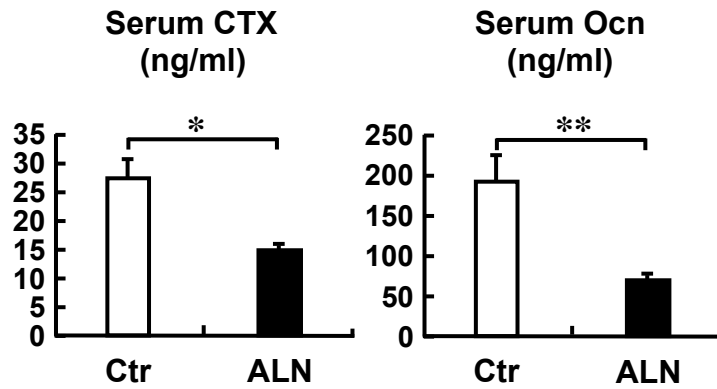
(A) Chemotaxis of stromal ST2 cells toward CTHRC1 and/or WNT3A was assessed using an EZ-TAXIScan™ (Effector Cell Institute, Tokyo), as described in the Methods section. (B,C) The Cultrex 96 Well Cell Migration Assay Kit (Trevigen,

Inc. Gaithersburg, MD, USA) was used to assess the migration of ST2 cells (**B**) and calvaria-derived primary osteoblasts (**C**), as described in the Methods and Methods. Recombinant CTHRC1 protein was applied at 2 $\mu\text{g/ml}$ and 80 ng/ml in (**B**) and (**C**), respectively. * $p < 0.05$ (n=3 each group)



Supplemental Figure 7 Generation of osteoclast-specific *Cthrc1* knockout mice

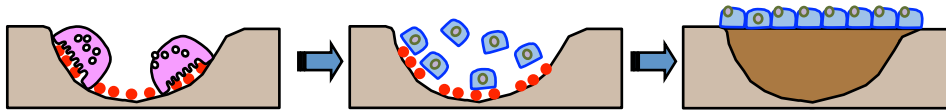
(A) Genotyping of the flox and excised (exon 2) allele ($\Delta E2$) at the *Cthrc1* gene locus. Ctr indicates the control tail DNAs from the wild-type (+/+), flox/+ (F/+) and flox/flox (F/F) mice. NC and PC indicate the negative and positive controls, respectively. Bone marrow macrophages (BMM) and osteoclasts (OC) were prepared from each mice and genomic DNAs were extracted. PCR was performed using primers listed in Table 1S. (B) Demonstration of the specific deficiency of the expression of the *Cthrc1* gene, but not the calcitonin receptor (*Calcr*), in osteoclasts generated ex vivo from conditional KO in osteoclasts (ΔOC) as well as systemic KO (sKO) mice.



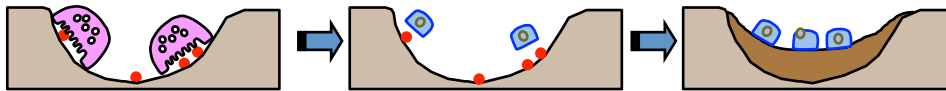
Supplemental Figure 8 Alendronate reduces bone turnover

The serum CTX and osteocalcin (Ocn) concentrations were measured after 12-week-old male C57BL/6 mice were treated with alendronate (ALN) (1 mg/kg BW) 5 times a week for 4 weeks. * $p < 0.05$, ** $p < 0.01$ (n=3 each group).

normal



with aging



Supplemental Figure 9 Model for osteoclast-derived *Cthrc1* in the coupling from bone resorption to formation

Cthrc1 production is reduced with aging or following alendronate treatment, which impairs the differentiation and/or recruitment of osteoblastic/stromal cells, thereby resulting in insufficient bone formation and reduced bone mass.

Supplemental Table 1 Primers for PCR

| For general PCR | |
|---------------------------|--|
| Gene | Forward primer (5'-3') Reverse primer (5'-3') |
| <i>Calcr</i> | CATTCCTGTACTTGGTTGGC AGCAATCGACAAGGAGTGAC |
| <i>Cthrc1</i> | AAGCAGTGTTTCGTGGAGT GTCCTTCCACAGAGGAAGT |
| <i>Ctsk</i> | GGAAGAAGACTCACCAGAAGC GTCATATAGCCGCCTCCACAG |
| <i>Ctsk-cre</i> | TTATTCCTTCCGCCAGGATG TAGTTTTTACTGCCAGACCG |
| $\Delta E2$ <i>Cthrc1</i> | CGTTGGGTGAGGGAATTTGAGC GTCAGCACATTACCTATCCTG |
| <i>Flox Cthrc1</i> | GGGAAAATTGCGGTAAGTAAAGCCC GTCAGCACATTACCTATCCTG |
| <i>Gapdh</i> | ACTTTGTCAAGCTCATTTC TGCAGCGAACTTTATTGATG |
| For real-time PCR | |
| <i>Acp5</i> | CGTCTCTGCACAGATTGCAT AAGCGCAAACGGTAGTAAGG |
| <i>Alp</i> | GAGTGAGCGCAGCCACAGA TGTGACCTCATTGCCCTGAGT |
| <i>Ap2</i> | AAGAGAAAACGAGATGGTGACAA CTTGTGGAAGTCACGCCTTT |
| <i>Bglap</i> | CTGACAAAGCCTTCATGTCCAA GGTAGCGCCGGAGTCTGTT |
| <i>Calcr</i> | CCTTCCAGAGGAGAAGAAACC GGAGATTCCGCCTTTTAC |
| <i>Col1a1</i> | CGTCTGGTTTGGAGAGAGCAT GAGCCCTCGCTTCCGTACT |
| <i>Cthrc1</i> | AAGCAAAAAGCGCTGATCC CCTGCTGGTCCTTGTAGACAC |
| <i>Ctsk</i> | CTCCATCGACTATCGAAAGAAAG AAAGCCCAACAGGAACCAC |
| <i>Gapdh</i> | AGCTTGTCAACGGGAAG TTTGATGTTAGTGGGGTCTCG |

| | |
|---------------------|---|
| <i>Lpl</i> | CTCGCTCTCAGATGCCCTAC GGTTGTGTTGCTTGCCATT |
| <i>Pparg</i> | GAAAGACAACGGACAAATCACC GGGGGTGATATGTTTGA ACTTG |
| <i>Runx2</i> | TGCCCAGGCGTATTT CAG TGCCTGGCTCTTCTTACTGAG |
| <i>Sp7</i> | TCCATTCTCCCTCCCTCT GGACTGGAGCCATAGTGAGC |
