Hdac3 regulates bone modeling by suppressing osteoclast responsiveness to RANKL

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Hdac3 is a lysine deacetylase that removes acetyl groups from histones and additional proteins. Although Hdac3 functions within mesenchymal lineage skeletal cells are defined, little is known about Hdac3 activities in bone-resorbing osteoclasts. In this study we conditionally deleted Hdac3 within Ctsk-expressing cells and examined the effects on bone modeling and osteoclast differentiation in mice. Hdac3 deficiency reduced femur and tibia periosteal circumference and increased cortical periosteal osteoclast number. Trabecular bone was likewise reduced and was accompanied by increased osteoclast number per trabecular bone surface. We previously showed that Hdac3 deacetylates the p65 subunit of the NF-κB transcriptional complex to decrease DNA-bound and transcriptional activity. Hdac3-deficient osteoclasts demonstrate increased K310 NF-κB acetylation and NF-κB transcriptional activity. Hdac3-deficient osteoclast lineage cells were hyper-responsive to RANKL and showed elevated ex vivo osteoclast number and size and enhanced bone resorption in pit formation assays. Osteoclast-directed Hdac3 deficiency decreased cortical and trabecular bone mass parameters, suggesting that Hdac3 regulates coupling of bone resorption and bone formation. We surveyed a panel of osteoclast-derived coupling factors and found that Hdac3 suppression diminished sphingosine-1-phosphate production. Osteoclast-derived sphingosine-1-phosphate acts in paracrine to promote bone mineralization. Mineralization of WT bone marrow stromal cells cultured with conditioned medium from Hdac3-deficient osteoclasts was markedly reduced. Expression of alkaline phosphatase, type Ia1 collagen, and osteocalcin was also suppressed, but no change in Runx2 expression was observed. Our results demonstrate that Hdac3 controls bone modeling by suppressing osteoclast lineage cell responsiveness to RANKL and coupling to bone formation.

Skeletal element width (e.g. periosteal circumference) varies with sex, age, ethnicity, and geography (1–4), and can predict fracture risk independent of bone mass. Moreover, cortical bone strength is a function of the periosteal and endosteal radius, with slight changes in these radii imparting dramatic changes in bone strength (4). Periosteal bone apposition is thought to mainly contribute to bone modeling during growth, but bone modeling also relies on osteoclast activity (5). Although the overall process of bone width determination is defined, the molecular regulators of this characteristic during development are poorly understood. Quantitative trait loci and whole-genome association studies have shown that bone geometry has a genetic component and is controlled independently of other factors such as bone mineral density (6, 7).

RANKL (Odf, TRANCE, Opgl) and M-CSF (Csf1) are the two cytokines necessary and sufficient for osteoclastogenesis. RANKL is required for definitive osteoclastogenesis and exerts its actions through the tumor necrosis factor-type receptor RANK. RANK activation induces a number of signal transduction cascades, including NF-κB signaling. Activation of p65/RelA NF-κB in turn induces transcription of NFATc1, the master regulator of osteoclastogenesis. NFATc1 then acts in an autoamplification loop to induce further expression of p65 NF-κB and NFATc1 (8, 9).

Histone deacetylases (Hdac) catalyze the removal of acetyl groups from lysine side chains proteins such as histones and transcription factors. Hdac3 is broadly expressed and is predominantly localized to the nucleus. Hdac3-null mice exhibit early embryonic lethality (10), but conditional deletion within mesenchymal lineage cells demonstrates a central role for Hdac3 in skeletal development, including promotion of appositional growth and ossification (11, 12). In addition to its epigenetic function as a histone deacetylase, Hdac3 removes acetyl groups from lysine residues of other proteins including transcriptional factors. Hdac3 deacetylates the p65/RelA NF-κB subunit causing decreased NF-κB DNA-binding affinity, suppressed transcriptional activity, and enhanced association with the inhibitor κB complex (13, 14).

Peak bone mass and optimal bone geometry are achieved by bone modeling and are predictors of future osteoporosis and fracture risk (15–17). Our published data demonstrate that Hdac3 plays an essential role in development (11, 12), but the role of osteoclast-specific Hdac3 in bone modeling is unknown. To address this question, we generated conditional knockout mice by crossing Hdac3fl/fl mice with mice expressing Cre recombinase under the control of the Ctsk promoter (referred to as Hdac3 cKO Ctsk mice). Here we show that deletion of Hdac3 within Ctsk-expressing cells alters bone modeling by decreasing skeletal element width (e.g. periosteal circumference without a change in bone length). This coincides with increased numbers of periosteal osteoclasts. Trabecular bone mass is also diminished in Hdac3 cKO Ctsk mice. Mechanistically, Hdac3 deficiency increases RANKL responsiveness, NF-κB acetylation, and activity, resulting in enhanced osteoclast differentiation. Moreover, Hdac3-deficient osteoclasts have reduced...
expression of Sphk1, leading to decreased production of S1P. Likewise, conditioned medium generated from Hdac3-deficient osteoclasts does not support osteoblast-mediated matrix mineralization. Together these data suggest that Hdac3 deletion in Ctsk-expressing cells decreases skeletal element width by enhancing NF-κB transcriptional activity, osteoclast numbers, and bone resorption and that Sphk1 reduction facilitates this uncoupled bone loss.

Results

**Hdac3 conditional deletion alters bone modeling and reduces bone mass**

We first determined the specificity of the Ctsk-Cre diver developed by Chiu et al. (18). Genomic DNA was isolated from osteoclasts derived from Hdac3 cKO Ctsk bone marrow macrophages or their Cre− littersmates. Hdac3 floxed allele rearrangement was evident in Cre-expressing osteoclasts (Fig. 1A) but not within the ovary or testis (Fig. 1B). No reduction in Mendelian frequency of Hdac3 cKO Ctsk mice or other genotypes was noted. Immunohistochemical staining of sections from Hdac3 cKO Ctsk or WT littersmate tibiae showed no reduction in Hdac3 immunostaining by bone lining osteoblasts or bone-encased osteocytes within trabecular or cortical bone (Fig. 1C). In contrast, decreased levels within growth plate chondrocytes, particularly resting chondrocytes, was observed (Fig. 1C). Minimal reduction in Hdac3 levels were observed within the perichondrium or periosteum of Hdac3 cKO Ctsk mice (Fig. 1C). Detectable levels of Cre transcripts within the ovary and testis were not observed (data not shown). Reduction in Hdac3 expression by mature osteoclasts via qPCR was also noted (Fig. 1D).

Whereas no change in bone parameters was noted of 4-week-old animals, widths of the proximal femur and tibia of males were smaller at 12 weeks of age, indicating a bone-modeling defect that decreases acquisition of peak bone geometry (e.g. robustness) (Fig. 2A). Periosteal circumference and cortical thickness of the femoral midshaft were reduced as quantified by micro-CT (Fig. 2, B–D). No other changes in cortical bone were noted (Fig. 2D), and no changes were noted of 12-week-old female mice.

Histomorphometric analyses revealed more osteoclasts per periosteal bone surface with 59 and 47% increases at the medial and lateral periosteal cortical surfaces, respectively, within male cKO mice (Fig. 3). In addition, osteoclast perimeter per bone perimeter also increased (Fig. 3, E and F). Few osteoclasts were observed on the endocortical surface at this age, and no change in osteoclasts per endosteal bone surface was observed. These data further point to a defect in cortical bone modeling induced by Hdac3 deficiency.

We also evaluated trabecular bone parameters of Hdac3 cKO Ctsk mice. Decreased bone volume/total volume (53%), trabecular number (27%), and connective density (62%) of male mice was evident (Fig. 4, A–C). This corresponded with increased trabecular spacing (40%) and decreased bone mineral density (35%) (Fig. 4, E and F), but no change in trabecular thickness was noted (Fig. 4D). Female mice did not exhibit changes in trabecular bone at 12 weeks of age (Fig. 4).
Bone histomorphometric analyses were conducted and confirmed a 47% decrease in trabecular bone volume/total volume of Hdac3 cKO_Ctsk male mice (Fig. 5A). Increased osteoclasts per bone surface and osteoclast surface per bone surface reflected increases in osteoclast number and size by 37.5 and 52%, respectively (Fig. 5, B and C). Osteoblast number per bone surface was unaffected by Hdac3 deficiency (Fig. 5D). Serum markers of bone resorption (CTX) were elevated by 42%, but significant changes in the serum bone formation marker P1NP were not evident (Fig. 5, E and F). These data suggest an uncoupled increase in bone remodeling.

**Hdac3 deletion increases osteoclastogenesis**

To determine whether altered cortical geometry and reduced trabecular bone parameters of Hdac3 cKO_Ctsk mice corresponded with increased osteoclastogenesis, we performed ex vivo osteoclast differentiation assays. Bone marrow macrophages were collected from 6–8-week-old Hdac3^{fl/fl} mice. Osteoclasts were generated from bone marrow macrophages of male or female mice ex vivo with recombinant M-CSF and RANKL and infected with adenoviral GFP or AdCre on day 0 as previously published (19–21). Shown are data generated from male mice (Fig. 6). Hdac3 has a long t_{1/2}, so reductions in protein levels were not observed until day 2 (data not shown). On day 4, Hdac3 levels were successfully suppressed by AdCre transduction to 50% of WT levels (Fig. 6, B and C). A 4-fold increase in osteoclast numbers and a 2-fold increase in osteoclast area were obtained from bone marrow macrophage cultures of females or male AdCre-infected cells as compared with AdGFP controls (Fig. 6, A, D, and E). Hdac3^{fl/fl} AdGFP and AdCre-transduced osteoclasts derived from male or female mice were also seeded on dentin disks and cultured for 14 days. Toluidine blue staining of dentin disks cultured with AdCre-infected cells was increased by 60% (Fig. 6, F–H), suggesting that Hdac3 deficiency also increases bone resorption. These data correlate with our in vivo observations and support that Hdac3 suppresses osteoclast differentiation and bone resorption.

**Hdac3 deficiency increases NF-κB acetylation and RANKL responsiveness**

We and others have previously shown that Hdac3 deacetylates the p65/RelA subunit of the NF-κB transcriptional complex leading to decreased DNA-binding and transcriptional activity (12–14). We hypothesized that increased NF-κB transcriptional activity within Hdac3-deficient osteoclast lineage cells would enhance RANKL signaling. To test this, we first determined whether Hdac3 suppression increased NF-κB acetylation. Bone marrow macrophages were collected from 6–8-week-old male or female Hdac3^{fl/fl} mice, and osteoclasts were generated ex vivo with recombinant M-CSF and RANKL. The cultures were then transduced with AdGFP or AdCre on day 0 as previously published (19–21). The data from male mice are shown in Fig. 7. Hdac3 levels were successfully suppressed by AdCre transduction (Fig. 7, A and B). Increased acetylation of K310 NF-κB was observed in AdCre-infected cells (Fig. 7B). To support increased NF-κB transcriptional activity, we also demonstrated 4-fold increased activity of an NF-κB luciferase reporter activity by Hdac3-deficient osteoclasts (Fig. 7C). Two downstream transcriptional targets of RANKL-induced NF-κB activity are NFATc1 and the p65 NF-κB gene locus itself (8, 9); accordingly, NFATc1 and total NF-κB levels are enhanced by Hdac3 deficiency (Fig. 7B).

We next determined whether Hdac3 deficiency increased RANKL responsiveness by generating osteoclasts from bone marrow macrophages in the presence of 25 ng/ml M-CSF and

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Figure 2. Deletion of Hdac3 in Ctsk-Cre–expressing cells alters bone geometry. A–D, Hdac3 cKO_Ctsk mice and their control littermates were aged to 12 weeks. A, radiographs of the proximal femur and tibia of male mice. Scale bars, 2 cm. B, radiographs of the femoral midshaft of male mice. Dotted yellow lines denote the periosteal and endosteal surfaces. The scale bar in the right panel indicates 10 mm and that in the left panel represents 1 cm. C and D, microcomputed tomography was used to determine the periosteal circumference of male and female mice (C), as well as femoral cortical bone mass and geometry parameters of male mice (D) (n = 7/group). *, p < 0.05.
increasing RANKL concentrations (0, 15, 30, and 45 ng/ml) (Fig. 7, D–F). AdCre-infected cultures were hyper-responsive to RANKL and showed elevated osteoclast area at all concentrations above 0 ng/ml. A corresponding increase in M-CSF responsiveness was not observed. These data demonstrate that Hdac3 deficiency increases p65 NF-κB acetylation and RANKL responsiveness.

**Hdac3 facilitates the production of S1P and coupling to bone formation**

Deletion of Hdac3 within Ctsk-expressing cells decreased trabecular bone mass parameters (Figs. 1–4), suggesting that Hdac3 regulates coupling of bone resorption and bone formation. We surveyed a panel of osteoclast-derived factors implicated as coupling factors and found that suppression of Hdac3 led to diminished Sphk1 transcripts but not Sphk2 (Fig. 8, A and B). Likewise, Sphk1 protein levels and production of S1P was repressed by Hdac3 deficiency (Fig. 8C). Osteoclast-derived S1P acts in paracrine to promote bone mineralization; thus, we determined whether Hdac3 deletion affected mineralization of WT bone marrow stromal cell cultures. Conditioned medium (CM) from AdGFP or AdCre-infected Hdac3fl/fl osteoclasts was generated as previously described (22). Bone marrow stromal cells were harvested and cultured in AdGFP or AdCre CM and supplemented with 1 × 10^-7 M dexamethasone, 10 mM β-glycerol phosphate, and 50 μg/ml ascorbate and cultured for 28 days. Alizarin red staining was markedly reduced in AdCre CM bone marrow stromal cell cultures. Expression of alkaline phosphatase, type Iα1 collagen, and osteocalcin were also suppressed (Fig. 8, F–H), but no change in Runx2 expression was observed (Fig. 8G). These data indicate that conditioned medium derived from Hdac3-deficient osteoclasts does not support matrix production and mineralization as well as control osteoclast conditioned medium.

**Discussion**

We previously described roles for Hdac3 within mesenchymal skeletal lineage cells (11, 12, 23, 24), but the role of Hdac3 in bone-resorbing osteoclasts has not been explored. In this report, we show that deletion of Hdac3 in Ctsk-expressing cells diminishes periosteal bone diameter and limits acquisition of trabecular bone mass. These defects in bone modeling are accompanied by enhanced osteoclast numbers in vivo and increased levels of the serum resorption marker CTX1.
attribute enhanced osteoclast differentiation to elevated NF-κB acetylation, resulting in heightened RANKL responsiveness. Furthermore, our results suggest that decreased bone mass may result from suppressed Sphk1/S1P production leading to diminished bone coupling. The in vivo phenotypes are specific to 12-week-old male mice, because female littermates did not exhibit changes, suggesting that additional physiological factors affect osteoclast activity and bone phenotypes.

Previous studies demonstrated that Hdac3 promotes long bone appositional growth and maintains bone mass (11, 12, 23, 24). In our study, we observed a defect in bone modeling resulting in decreased periosteal circumference, without a corresponding deficit in long bone lengthening in 12-week-old male mice. Decreased trabecular bone mass accompanied this observation. We attribute the decreased bone width and trabecular bone to enhanced osteoclast activity, but these effects could also be due to Hdac3 deletion in other Ctsk-Cre–expressing cells.

Varying degrees of Ctsk expression, Cre detection, and Cre activity have been reported of the two distinct Ctsk-Cre drivers within different cell types. This includes mesenchymal lineage cells such as perichondrial cells (25, 26), hypertrophic chondrocytes (18), osteoblasts (26, 27), within the groove of Ranvier (26, 28), and potentially by cells within the ovaries and testes (26, 29). We observed decreased Hdac3 levels within osteoclasts and growth plate chondrocytes, but minimal reduction within perichondrial or periosteal cells (Fig. 1C). The Ctsk-Cre driver developed by Nakamura et al. (26) is a targeted insertion of Cre into the Ctsk allele, whereas Chiu et al. (18) generated a Ctsk-Cre mouse via random insertion into the genome. In this study, we employed the Ctsk-Cre driver developed by Chiu et al. (18). Given the limitations of the Ctsk driver, future studies will be aimed at assessing the function of Hdac3 within periosteal and perichondrial stem cells and pericyte-associated stem cells (30, 31).

The effects of Hdac3 deficiency within mesenchymal lineage cells result in skeletal phenotypes in both males and females (11, 12, 23, 24), but in our study we found that only males had diminished bone width and trabecular bone mass. Hdac3 can repress the transcriptional activity of the androgen and estrogen receptors in other cell types, so the effects on bone geometry and acquisition may be via these mechanisms (32, 33). Ex vivo osteoclast numbers were equally enhanced by Hdac3 deficiency in male and female cells, further supporting an endocrine-mediated mechanism. Future studies will be performed utilizing gonadectomy models to understand these sex-specific phenotypes.

A prior study by Pham et al. (34) used an siRNA-mediated approach to suppress Hdac3 levels in progenitor cells, resulting in decreased osteoclastogenesis. Our contrasting results suggest that the functions of Hdac3 with progenitor cells and lineage-committed cells vary. Moreover, we used a genetic approach to specifically target Hdac3, whereas siRNA-mediated knockdown could result in off-target effects. Likewise, our data may be the result of Hdac3 deletion within other cell types, so further study is warranted.

To maintain bone mass, bone resorption must equal bone formation; thus, at sites of bone remodeling, coupling to bone formation must occur. This coupling is thought to be diminished in conditions of decreased bone mass. Several osteoclast-derived factors have been identified as putative bone formation coupling factors, including semaphorins, ephrins, interleukin-6 family cytokines, Wnt ligands, and S1P (35). We assessed a panel of coupling factors, including Bmp6, Cardiotrophin,
Cthrc1, Ephrin B2, Semaphorin D, Sphk1/2, and Wnt10b. Of these, only Sphk1 was altered by Hdac3 suppression. Sphingosine is a lipid produced through ceramide metabolism; the S1P bioactive form is made via sphingosine kinase (Sphk) 1/2-mediated phosphorylation of sphingosine. S1P is generated by a wide variety of cell types, but its concentration is highest in serum. S1P has both intrinsic and extrinsic functions, the latter of which depends on export through the Spns transporters (36). S1P interacts with the G-protein coupled receptor leading to downstream anabolic signaling. In bone, production of S1P by osteoclasts leads to enhanced osteoblast proliferation, migration, and mineralization (22, 37, 38). S1P also affects osteoclasts directly, acting to promote osteoclast precursor migration to bone surfaces. When administered in vivo, S1P agonists limit osteoclast mediated bone resorption induced by ovariectomy in mice and bone resorption associated with multiple sclerosis in humans (39–42). Our data show that Hdac3 deficiency limits S1P production and suggest that bone coupling is diminished.

Together our data demonstrate that Hdac3 deficiency results in bone modeling defects and limits trabecular bone mass acquisition. Reduced bone width was observed without a change in bone length, pointing either to a defect in osteoclast or to chondrocyte activity during bone modeling. In this study, we focused on the osteoclast functions of Hdac3. Enhanced osteoclast numbers are due to increased NF-κB acetylation and transcriptional activity resulting in increased RANKL responsiveness and diminished coupling to bone formation.

Materials and methods

**Generation of Hdac3 conditional knockout mice**

Hdac3<sup>fl/fl</sup> (10) mice were crossed with mice expressing Cre-recombinase under the control of the Ctsk promoter to delete Hdac3 within Ctsk-expressing cells (18). The mice were genotyped for Cre or the Hdac3 floxed allele as previously described (43). Conditional knockout animals from these crossings are referred to as Hdac3 cKO<sub>Ctsk</sub> mice and are on the C57Bl/6 background. The animals were housed in an accredited facility under a 12-h light/dark cycle and provided water and food ad libitum. All animal research was conducted according to guidelines provided by the National Institute of Health and the Institute of Laboratory Animal Resources, National Research

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**Figure 5. Osteoclast numbers are suppressed within trabecular bone by Hdac3 deficiency.** A–F, Hdac3 cKO<sub>Ctsk</sub> mice and their control littermates were aged to 12 weeks. Sections obtained from control or Hdac3 cKO<sub>Ctsk</sub> tibias were TRAP and Fast Green–stained. A–C, BV/TV (A), osteoclast per bone surface (B), and osteoclast surface per bone surface (C) were evaluated. *, *p* < 0.05. D, sections were also Masson’s trichrome–stained, and the number of osteoblasts per bone surface was determined. *, *p* < 0.05. E and F, serum was collected from 12-week-old mice, and CTX-1 (E) and P1NP ELISAs (F) were performed. *, *p* < 0.05. Oc., osteoclast; Pm., perimeter; B., bone.
Council. The Mayo Clinic Institutional Animal Care and Use Committee approved all animal studies.

Radiographs and microcomputed tomography

Radiographs of the right hind limb of 12-week-old mice were collected using a Faxitron X-ray imaging cabinet (Faxitron Bioptics, Tucson, AZ, USA). Femurs from 12-week-old male and female Hdac3 cKO Ctsk mice and their control littermates were isolated and fixed in 10% neutral buffered formalin for 24 h. Femurs were then stored in 70% ethanol prior to scanning at 70 kV, 221 ms with a 10.5-μm voxel size using a Scanco Viva40 micro-CT. For cortical bone analyses, a region of interest was defined at 10% of total femur length beginning at the femoral midpoint; defining the outer cortical shell and running a mid-shaft analysis with 260-threshold air filling correction analyzed samples. For trabecular measurements, a region of interest was defined at 10% of total femur length starting immediately proximal to the distal growth plate; the samples were analyzed using a 220-threshold air filling correction. Study staff were blinded to sample identities during analyses.
Hdac3 controls osteoclast activities

Figure 8. Osteoclast-specific Hdac3 deficiency decreases Sphk1 expression and mineral deposition. Bone marrow macrophages were collected from Hdac3<sup>fl/fl</sup> mice, cultured with RANKL and M-CSF, and infected with either AdGFP or AdCre. Shown are data from averaged data from males, but studies were performed in females and males of each genotype in three independent experiments. A and B, after 4 days in culture, Sphk1 (A) and Sphk2 (B) levels were assessed by qPCR. *, p < 0.05 Western blotting was performed. The average change (n = 3) from AdGFP is shown to the right of each band. **, p < 0.05. C, depiction of experiment performed in E–H. D, bone marrow stromal cells were cultured in AdGFP or AdCre conditioned medium supplemented with dexamethasone, βGP, and ascorbate for 21 days. E–I, Alizarin red staining was performed (E). Expression of alkaline phosphatase (F), type 1a1 collagen (G), Osteocalcin (H), and Runx2 (I) were evaluated by qPCR. F–I, average C<i>α</i> levels are shown above each bar. *, p < 0.05, mOcl, mouse osteoclast-like cells; Ob, osteoblast.

Histology and static bone histomorphometry

Following micro-CT analyses, femurs from 12-week-old mice were fixed in 10% neutral buffered formalin and then decalcified in 15% EDTA for 14 days. Tibiae from 12-week-old animals were paraffin-embedded, and longitudinal 5-μm sections were collected using the posterior cruciate ligament as a landmark for section depth. The sections were then TRAP/Fast Green–stained (Sigma–Aldrich, catalog no. 387A-1KT) or Masson’s trichrome stained (Sigma–Aldrich, catalog no. HT15-1KT). For periosteal osteoclast numbers present on the medial and lateral cortical bone surface, the region between the femoral midshaft to the onset of the distal growth plate was evaluated. Standardized histomorphometry was performed using OsteoMeasure software (44). Study staff were blinded to sample identities during analyses.

Immunohistochemical staining

Tibiae from 12-week-old male Hdac3 cKO<sub>C<sup>±</sup></sub>csk and their control littermates were fixed in 10% neutral buffered formalin and decalcified in 15% EDTA, and paraffin-embedded. Immunohistochemical staining was performed on 5-μm sections with antibodies directed to Hdac3 (Millipore, catalog no. 06-890, 1:50) or an irrelevant IgG control. Chromogens were developed using a polyclonal secondary horseradish peroxidase detection kit (AbCam) followed by incubation in 3,3'-diaminobenzidine (Sigma–Aldrich).

Ex vivo osteoclastogenesis, luciferase reporter, and dentin disk resorption assays

Bone marrow macrophages were collected from 4–6-week-old Hdac3<sup>fl/fl</sup> male or female mice as previously described (45). The cells were cultured in phenol red–free α-MEM overnight in the presence of 35 ng/ml rM-CSF (catalog no. 410-ML, R&D Systems, Minneapolis, MN, USA). Nonadherent cells were collected and cultured with 60 ng/ml rRANKL (catalog no. 315-11, Preprotech, Rocky Hill, NJ, USA) and 35 ng/ml M-CSF. For dose-response assays using Hdac3<sup>fl/fl</sup> cells, the cells were exposed to increasing RANKL concentrations and infected with adenoviral GFP or AdCre at a multiplicity of infection of 300 as previously described (19–21). For reporter assays, the cells were infected with an NF-κB reporter adenovirus (Vector Biolabs, catalog no. 1740) at a multiplicity of infection of 100. For bone resorption assays, nonadherent cells were seeded onto dentin disks (catalog no. NC1309388, Fisher Scientific, Waltham, MA, USA) cultured with 60 ng/ml RANKL and 35 ng/ml M-CSF. The cells were fed every 3–4 days with phenol red–free α-MEM supplemented with 35 ng/ml M-CSF and 60 ng/ml RANKL. On day 14, the cells were lysed with 1% domestic bleach, and dentin disks were stained with 1% toluidine blue. Shown are the averaged data from males, but studies were performed in females and males of each genotype in three independent experiments.

RNA extraction and semiquantitative PCR

Total RNA was extracted from primary osteoclasts using TRIzol (Invitrogen) and chloroform, and 2 μg was reverse-transcribed using the SuperScript III first-strand synthesis system (Invitrogen). The resulting cDNAs were used to assay gene expression via real-time PCR using the following gene-specific primers: alkaline phosphatase (5'-ACTGGTACTCAGACAA-CGAGAT-3'), 5'-ACGTCAATGCTCCTGATTTAG-3'), Col1a1 (5'-GCTTCACCTACGACCCCTTTG-3', 5'-TGA-
CTGTCTTGCCCCCAAG TTC-3', Ctsk (5'-TCCGA AAAAG AGCCTAGC GAA-3', 5'-AGAGAT TTTATCCACCTG CATG-3'), Hdac3 (5'-CCC GACATCGAAT CAGAAC-3', 5'-TCAAGAGTGTCTG CGGATT-3'), Osteocalcin (5'-CCGTTGAGCTG C-3', 5'-GCCGGA GTCGTGTC ACTACCTT-3'), Sphk1 (5'-CTGATGCG TGGTTGTGATA CT-3', 5'-TGGATGTA GTAGCAGGTCTT-3'), Sphk2 (5'-CTTCAACAA CAGGC CCTA-3', 5'-GCCACGTTGGT AGTTGAT-3'), RANK (5'-TAAAA GTCTGTGATCGGCA GGAG-3', 5'-CCGTATC CTGTGAGCTG C-3'), Runx2 (5'-GGCACAGACA GCTGTGATC-3', 5'-GAATGCGCCTAA ATCAG-3'), and Tubulin (5'-CTGCTCATC AGACAGAATC AGAAC-3', 5'-GGATTATAGGG CCTCCACCACAG-3'). Fold changes in gene expression for each sample were calculated using the 2-ΔΔCq method relative to control after normalization of gene-specific Cq values to Tubulin Cq values (43). Shown are data from averaged from three males per group, but studies were performed in females and males of each genotype in three independent experiments.

Western blotting

The cell lysates were collected in a buffered SDS solution (0.1% glycerol, 0.01% SDS, 0.1 m Tris, pH 6.8) on ice. Total protein concentrations were obtained with the Bio-Rad DC assay (Bio-Rad). Proteins (20 µg) were then resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blotting was performed with antibodies (1:2000 dilution) for acetyl-K310 NF-kB (Cell Signaling Technology, catalog no. 3045), p65 NF-kB (Cell Signaling Technology, catalog no. 8242), Hdac3 (AbCam catalog no. ab7030), NFATc1 (AbCam, catalog no. ab175134), S1P (AbCam, catalog no. ab140592), Sphk1 (AbCam, catalog no. ab71700), Histone 3 (Millipore, catalog no. 05-928), actin (Sigma–Aldrich, catalog no. A2228), and tubulin (Developmental Studies Hybridoma Bank, E7) and corresponding secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology). Antibody binding was detected with the Supersignal West Femto chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA). Shown are data from averaged from three males, but studies were performed in females (n = 3 per group) or males (n = 3 per group) of each genotype in three independent replicate experiments, each containing 12 pooled replicate wells per group.

TRAP staining

The cells were fixed in 10% neutral buffered formalin for 10 min and then washed three times with PBS. The fixed cells were TRAP-stained using an acid phosphatase, leukocyte (TRAP) kit (catalog no. 387A-1KT, Sigma–Aldrich). Each experiment was repeated at least three times (n = 3 mice per group) with replicate experiments performed, and the data from a representative experiment is shown.

Bone marrow stromal cell mineralization assays

Bone marrow stromal cells were flushed from the marrow cavity of 6–8-week-old male or female WT mice as previously described (46). Shown are data from averaged data from males, but studies were performed in females and males of each genotype in three independent experiments. The cells were cultured in conditioned medium derived from AdCre- or AdGFP-infected Hdac3fl/fl osteoclasts that was diluted 1:1 in fresh α-MEM and supplemented with 20% fetal bovine serum, 50 µg/ml ascorbate, 10 mM β-glycerol phosphate, and 1 x 10^7 M dexamethasone. The cultures were fed every 3–4 days with respective conditioned medium plus osteogenic supplements. RNA was extracted, or cells were fixed and stained with Alizarin red on day 21. Three replicate experiments were performed.

Imaging and quantification

For osteoclastogenesis experiments, three images were collected using a 10× objective per cover glass to generate technical replicates. Three cover glasses were used per experiment. The number and area of each image was quantified using ImageJ software. The average osteoclast area and the average number of osteoclasts per field was determined. A logarithmic curve fit was applied to describe osteoclast number and area data resulting from increasing RANKL concentrations. Each experiment was repeated independently three times (n = 3 mice per group). For Western blotting quantification, The Images were digitally collected and inverted, and the average grayscale value were determined using ImageJ software. The levels were normalized to Actin, and the ratio of normalized grayscale level of AdCre to AdGFP was determined.

Statistics

The data obtained are the means ± S.D. The p values were determined with the Student’s t test when only one experimental comparison was made. For assessment of significance with greater than two conditions, a one-way analysis of variance was performed, p < 0.05 was considered statistically significant.

Data availability

All data are contained within the article.

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Abbreviations—The abbreviations used are: S1P, sphingosine-1-phosphate; qPCR, quantitative PCR; CT, computed tomography; M-CSF, macrophage colony-stimulating factor; CM, conditioned medium; α-MEM, α-minimum essential medium.; CTX, C-terminal telopeptide; βGP, β-glycerophosphate; TRAP, Tartrate-resistant acid phosphatase; Ob, Osteoblast; Sphk, Sphingosine kinase.

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