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Histone deacetylase 3 is required for maintenance of bone mass during aging

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Abstract

Histone deacetylase 3 (Hdac3) is a nuclear enzyme that removes acetyl groups from lysine residues in histones and other proteins to epigenetically regulate gene expression. Hdac3 interacts with bone-related transcription factors and co-factors such as Runx2 and Zfp521, and thus is poised to play a key role in the skeletal system. To understand the role of Hdac3 in osteoblasts and osteocytes, Hdac3 conditional knockout (CKO) mice were created with the Osteocalcin (OCN) promoter driving Cre expression. Hdac3 CKO_{OCN} mice were of normal size and weight, but progressively lost trabecular and cortical bone mass with age. The Hdac3 CKO_{OCN} mice exhibited reduced cortical bone mineralization and material properties and suffered frequent fractures. Bone resorption was lower, not higher, in the Hdac3 CKO_{OCN} mice, suggesting that primary defects in osteoblasts caused the reduced bone mass. Indeed, reductions in bone formation were observed. Osteoblasts and osteocytes from Hdac3 CKO_{OCN} mice showed increased DNA damage and reduced functional activity in vivo and in vitro. Thus, Hdac3 expression in osteoblasts and osteocytes is essential for bone maintenance during aging.

Keywords

Histone deacetylase; Osteocalcin-Cre; Osteoblast; Osteocyte; DNA damage

1. Introduction

Post-translational modifications of histones are important contributing factors in the epigenetic regulation of gene expression. Acetylated histones facilitate an open chromatin conformation that allows transcription factors, co-factors, and RNA polymerase II-complexes to access the DNA. In contrast, deacetylated histones encourage a condensed chromatin conformation and transcriptional repression. Histone acetyltransferases (HATs)

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and histone deacetylases (Hdacs) are counteracting enzymes that control histone acetylation levels. HATs and Hdacs also target lysine residues in many other proteins, such as transcription factors, altering their activity, cellular localization or stability [1], thus adding another layer of complexity to the mechanisms by which these enzymes regulate gene expression.

Hdacs regulate the activity of transcription factors important to bone development and maintenance and are crucial for skeletal development [2-7]. Germline deletion of Hdac8 causes craniofacial ossification defects [8], whereas germline deletion of Hdac4 or Hdac6 enhances endochondral ossification and bone mineral density [9, 10]. Hdacs also play important roles in the postnatal skeleton. The pan Hdac inhibitor, vorinostat, depletes the immature osteoblast population and causes trabecular bone loss [11, 12]. Another pan Hdac inhibitor, valproate, causes loss of both cortical and trabecular bone mass [13]. Biological responses to Hdac inhibition vary because Hdac expression changes during cellular differentiation. For example, Hdac1 and Hdac2 are abundantly expressed in osteoblast progenitors, but not in mature osteoblasts, whereas expression levels of Hdac4 and Hdac6 increase during osteoblastic differentiation [14]. Osteoblast progenitors appear to be particularly sensitive to Hdac inhibition, as stromal cells treated early in the time course of osteoblastic differentiation fail to form mature, matrix-producing osteoblasts, but cells treated with Hdac inhibitors at later culture stages are unaffected [11].

The role of Hdac3 in bone is of interest because it is strongly expressed in osteoblasts at all stages of differentiation [3, 14, 15] and it interacts with the master osteoblast transcription factor Runx2 [3, 4]. Germline deletion of Hdac3 causes embryonically lethality prior to skeleton formation [16], and thus its effects on bone must be assessed using conditional knockout models. Mice deficient in Hdac3 under the control of the Osterix (Osx) promoter (Hdac3 CKO_{OSX}) have a striking developmental phenotype of reduced body size, decreased bone length, and deficits in both cortical and trabecular bone mass [17]. This phenotype is explained, in part, by a deleterious impact on osteoblasts, as osteoblast numbers and bone formation rates were lower in Hdac3 CKO_{OSX} compared to wildtype mice [17]. However, Osterix (Sp7) is also expressed by chondrocytes, and indeed, Hdac3 CKO_{OSX} mice have a relative expansion of the hypertrophic zone in the growth plate cartilage, suggesting that aberrant chondrocyte hypertrophy contributes to the skeletal phenotype during development [17]. Recent studies confirmed that chondrocytes from Hdac3 CKO_{OSX} mice undergo accelerated hypertrophy but are impaired in their ability to produce a cartilaginous matrix [18], contributing to the growth plate defects in these mice. Thus, the phenotype of Hdac3 CKO_{OSX} mice is likely due to the combined effects of Hdac3 depletion in chondrocytes and osteoblasts.

To better understand the effects of Hdac3 in mature osteoblasts and osteocytes, independent of cartilage defects, we crossed Hdac3-floxed animals with mice containing the Osteocalcin (OCN)-Cre transgene [19]. Here we show that Hdac3 depletion in mature osteoblasts causes a progressive postnatal decrease in cortical and trabecular bone mass with age as compared to age-matched control mice. In vitro and in vivo studies attribute this osteopenic phenotype to increased DNA damage and an overall decline in osteoblast and osteocyte function, particularly in cortical bone.

2. Materials and Methods

2.1. Animal studies

Mice containing LoxP sites flanking exon 7 of Hdac3 (*Hdac3^{fl/fl}* mice) [16, 20] were crossed with transgenic mice expressing Cre recombinase under control of a segment (approximately 4 kb) of the human OCN promoter [19, 21] to create Hdac3^{fl/fl}, Cre+

conditional knockout mice (CKO; also referred to as Hdac3 CKO_{OCN} mice). All mice were maintained on a mixed CD1:C57Bl/6 background. The OCN-Cre mouse features an 88% excision index in osteoblasts and osteocytes [19, 22]. Mice were genotyped for Hdac3 alleles and the OCN-Cre transgene with PCRs using tail DNA as a template as previously reported [17, 19]. Because transgene and LoxP insertions may cause unpredictable and unknown consequences, Hdac3 CKO_{OCN} mice were compared to two different control groups when possible. These groups were: Hdac3^{fl/fl} mice that did not harbor the OCN-Cre transgene (hereafter referred to as Hdac3^{fl/fl}, Cre- control (Ctrl) mice), and mice expressing the OCN-Cre transgene but wildtype at the Hdac3 locus (hereafter referred to as Hdac3^{+/+}, Cre+ Ctrl mice). The latter group was not maintained past 12 wk of age because at the early ages the two control groups were largely indistinguishable from each other in skeletal phenotype. Data from the larger control group is shown in the figures. All animal research was conducted according to guidelines provided by the National Institutes of Health and the Institute of Laboratory Animal Resources, National Research Council. The Mayo Clinic Institutional Animal Care and Use Committee approved all animal studies. Animals were housed in an accredited facility under a 12-hour light/dark cycle and provided water and food (PicoLab Rodent Diet 20, LabDiet) ad libitum.

2.2. Dual-energy X-ray absorptiometry analyses and tissue collection

Bone mineral density (BMD) was assessed longitudinally via dual-energy X-ray absorptiometry (DXA) scanning (PIXImus, GE Healthcare) of live mice at 4, 12, and 24 wk of age. Three regions of interest were monitored: lower body (including the lumbar spine, pelvis, and hindquarters), lumbar vertebrae, and femoral midshaft. Body weights were recorded and serum was collected via cardiac puncture at sacrifice. To assess protein expression of Hdac3, long bones were removed from 5 to 6 wk old mice and dissected free of soft tissues. Epiphyses were removed from both femurs and one tibia from each mouse and marrow was flushed with saline; cortical bone segments were subsequently flash frozen in liquid nitrogen prior to protein isolation. For mRNA analyses, demarrowed cortical bone segments from one femur and one tibia were prepared from 2 wk old mice as described above and flash frozen in liquid nitrogen prior to mRNA extraction. To permit dynamic histomorphometry studies of bone remodeling, adult mice were administered subcutaneous injections of calcein (10 mg/kg) on days 4 and 1 prior to death as previously described [17]. Femurs and tibias were collected from mice at 4, 6, 12, and 24 wk of age for quantification of bone architecture, material properties, and remodeling indices. One femur from each of the 12 wk old mice was cleaned of soft tissue and stored at -20 deg C in saline-soaked gauze for preservation of mechanical properties. All remaining bones were fixed in 70% ethanol (for Fourier transform infrared (FT-IR) imaging) or 10% neutral buffered formalin (for static and dynamic histomorphometry) and stored in 70% ethanol prior to microCT analysis and histological preparation.

2.3. Tissue level gene expression

For mRNA analyses, demarrowed cortical bone explants were homogenized in TRIzol using a high-speed disperser (Ultra-Turrax T25, IKA). RNA was extracted and purified from the ground tissue with TRIzol reagent (Invitrogen) and was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Relative expression levels of mRNAs for genes of interest, including Hdac3, characteristic osteoblast and osteocyte genes, Rankl, Opg, and genes relating to proliferation (CyclinD1, Cdkn1a) (Table 1) were measured by real-time PCR (qPCR). Reactions were performed using 37.5 ng of cDNA per 15 μ l with Bio-Rad iQ SYBR Green Supermix and the Bio-Rad MyiQ Single Color Real-Time PCR Detection System. Transcript levels were normalized to the reference gene Gapdh. Gene expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method [23].

2.4 Cortical bone protein expression

Demarrowed cortical bone segments were pulverized with a mortar and pestle in liquid nitrogen, placed in RIPA buffer on ice, and sonicated to generate protein extracts. Lysates were cleared by centrifugation at 15,000 RPM for 15 minutes at 4 degrees C. Ten to forty micrograms of total protein from each sample were resolved by SDS-PAGE. Western blotting was performed with antibodies recognizing Hdac3 (1:5000, Abcam #ab63353), acetylated histone 3: K9 and K14 (1:10,000, Millipore #06-599), acetylated histone 4: K5, K8, K12, and K16 (1:10,000, Millipore 06-866), histone 3 (1:20,000, Millipore #05-928), and actin (1:5000 or 1:10,000, Santa Cruz, I-19 SC-1616). Relative expression of Hdac3 was quantified from band intensity with image analysis software (Image J).

2.5 Serum bone remodeling markers and serum calcium

Circulating levels of procollagen type 1 amino-terminal propeptide (PINP; bone formation marker) and tartrate-resistant acid phosphatase 5b (TRAcP5b; bone resorption marker) were measured with colorimetric assays (Rat/Mouse PINP EIA #AC-33F1, Immunodiagnostic Systems, Fountain Hills AZ; Mouse TRAP ELISA #SB-TR103, Immunodiagnostic Systems, Fountain Hills AZ). All samples were tested in duplicate within each assay. For quantification of total serum calcium, serum samples were diluted 1:40 in a solution of 1% lanthanum chloride and 0.1M HCl, and then analyzed via atomic absorption using a Perkin Elmer AAnalyst 800.

2.6. Bone structure and composition in the femur (micro-computed tomography and Fourier transform infrared imaging)

Bone architecture and mineralization were evaluated in femurs of male mice using ex vivo micro-computed tomography (microCT) and FT-IR imaging. For microCT studies, the central portion of the femoral diaphysis and secondary spongiosa in the distal femoral metaphysis of each bone were scanned in 70% ethanol on a μ CT35 scanner (Scanco Medical AG, Basserdorf, Switzerland). Cortical and trabecular bone scans were performed with 7 μ m voxel size using an energy setting of 70 kVp and an integration time of 300 ms. For the distal femoral metaphysis scans, a region of interest that was 0.7 mm long (100 slices) was analyzed in each mouse, located 0.9 mm proximal to the growth plate in 4 and 6 wk old animals and 0.5 mm proximal to the growth plate in 12- and 24 wk old animals. Trabecular bone volume fraction (Tb. BV/TV, %), trabecular number (Tb.N, mm^{-1}), trabecular thickness (Tb.Th, mm), and trabecular separation (Tb.Sp, mm), were computed using the manufacturer's software [24]. For the femoral diaphysis scans, a region of interest that was 0.7 mm long and centered about the exact midshaft of the bone was analyzed in each sample. Cortical bone area (Ct.Ar, mm^2) was calculated from cortical bone volume (Ct.BV, mm^3) as described previously [24]. Cortical bone thickness (Ct.Th, mm), and cortical bone material mineral density (Ct.Mat.Mn.Dn, mg hydroxyapatite (HA)/ cm^3) were computed with the manufacturer's software [24].

FT-IR spectroscopy was performed on 5- μ m thick undecalcified histology sections from 4 wk old mice using micro-attenuated total reflectance (ATR)-FT-IR spectroscopy (Nicolet 8700), coupled to a Continuum microscope (Thermo Electron Corp., Madison, WI). Four cortical bone locations on each section were chosen and spectra were collected. The microscope used an ATR slide on a germanium crystal and spectra were collected at a resolution of 4 cm^{-1} for 128 scans with a sampling area of 100 $\times$ 100 μ m. The mineral to matrix ratio was calculated by measuring the area of phosphate peak (900-1200 cm^{-1}) and normalizing it by the area under Amide I peak at 1590-1720 cm^{-1} . The carbonate to phosphate ratio was calculated by measuring the area under the carbonate peak at 850-890 cm^{-1} and normalizing it by the area of phosphate peak.

2.7. Bone material properties (nanoindentation)

Femurs were thawed and sectioned transversely with a diamond saw (Buehler Isomet, Lake Bluff, IL) to expose the femoral midshaft and subsequently potted in resin (Leco, St. Joseph, MI) at room temperature for nanoindentation testing. Embedded samples were metallographically polished to expose the bone material and to produce smooth surfaces needed for nanoindentation. Specimens were ground with silicon carbide abrasive papers of decreasing grit size (400, 600, 800, and 1200 grit) under deionized water, followed by micro-cloths (AUTOMET 250, Buehler) with silicon slurry of 0.05 μm grit. After polishing, specimens were ultrasonically cleaned to remove surface debris. Polished samples were tested in a longitudinal direction on a commercial nanoindentation machine (Hysitron TI 950, Minneapolis, MN) using a diamond Berkovich tip. A 2×2 point matrix of nanoindentation measurements was collected and averaged at each sampling site, and an average of 12 different sites around the circumference of each bone were tested. All nanoindentation tests were performed under load control, with a loading rate of 500 $\mu\text{N}/\text{second}$, and maximum load of 2 mN. To minimize the effects of viscoelasticity on property measurements, a 60 second hold period was conducted before the final unloading to diminish viscoelastic deformation to a negligible rate. Reduced modulus (E_r) and hardness (H) were computed for each sampling site using the Oliver-Pharr model [25-27] and were averaged for each bone.

2.8. Trabecular bone remodeling indices in the proximal tibia

Thin (5 μm) undecalcified trabecular bone sections were prepared from the proximal tibial metaphysis, distal femoral metaphysis, and femoral mid-diaphysis. Tibia sections were stained with VonKossa/MacNeal's tetrachrome to highlight osteoblast surfaces or tartrate resistant acid phosphatase (TRAP)/fast green to highlight osteoclast surfaces. Trabecular bone histomorphometric remodeling indices were measured in an area of tissue (1 mm^2) beginning 500 μm from growth plate in the metaphyseal spongiosa, and cortical bone indices for the femoral mid-diaphysis were quantified for the entire cross-section. Slides were digitized using a microscope and digital camera and analyzed using image analysis software at 200X or 400X magnification (OsteoMeasures, Osteometrics Inc., Decatur, GA). Trabecular bone osteoblast surface/bone surface (Ob.S/BS, %), trabecular bone osteoblast number (N.Ob/B.Ar or N.Ob/T.Ar, $\#/\text{mm}^2$), trabecular bone osteoclast surface/bone surface (Oc.S/BS, %), and trabecular bone osteoclast number (N.Oc/B.Ar or N.Oc/T.Ar, $\#/\text{mm}^2$) were quantified from the stained sections. Trabecular bone mineralizing surface (Tb. MS/BS, %), trabecular bone mineral apposition rate (Tb.MAR, $\mu\text{m}/\text{day}$), and trabecular bone formation rate (Tb. BFR/BV or BFR/TV, $\%/\text{day}$), were quantified from unstained sections. Mineralizing surface was also normalized to osteoblast surface (MS/Ob.S, %), to quantify formation on a per-cell basis [28]. Periosteal and endocortical mineralizing surface (Ps. MS/BS and Ec.MS/BS, %) and mineral apposition rate (Ps.MAR and Ec.MAR, $\mu\text{m}/\text{day}$) were quantified from the unstained femoral mid-diaphysis cross-sections [28].

2.9. Immunohistochemistry and DNA damage in the tibia

Tibias were decalcified for 5 days in 15% EDTA; complete decalcification was confirmed by x-ray. Decalcified bones were embedded in paraffin and sectioned at a thickness of 8 microns. Immunohistochemical staining was performed with antibodies directed to Hdac3 (Millipore 06-890, 1:50 dilution), or an IgG isotype control (Vector Laboratories I-1000). Chromogens were developed using a polyvalent secondary HRP detection kit (Abcam) followed by incubation in DAB (Sigma Aldrich). Sections were counterstained with fast green. DNA damage was assessed histologically with an in situ TUNEL detection kit (Roche #11 767 305 001, #11 767 291 910). TUNEL stained slides were mounted with DAPI-containing medium (Vectashield) and examined via fluorescence microscopy.

2.10. BMSC growth, mineralization and gene expression

Bone marrow was flushed from femurs and tibias of adult mice, and cells were immediately seeded into 6-well plates at 1×10^7 cells per well or in 12-well plates on glass coverslips at 4×10^6 cells per well in osteogenic culture medium (alpha-MEM, 20% FBS, 1% antibiotic / antimycotic (Invitrogen #15240-062), 1% non-essential amino acids, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 10 mM beta glycerol phosphate, and 10^{-8} M dexamethasone). Media were changed every three days after seeding, and bone marrow stromal cells (BMSC) were selected by their ability to adhere to the plate after the first three days in culture. BMSC were cultured for 21 days in osteogenic medium to promote calcified matrix production. Cells were fixed in 10% neutral buffered formalin and stained with 2% alizarin red to detect matrix calcification; staining was quantified with Bioquant Osteo [11]. For gene expression studies, RNA was harvested with TRIzol reagent (Invitrogen). RNA was reverse transcribed and expression levels of mRNAs associated with osteoblast maturation (Runx2, type I collagen, alkaline phosphatase, and Bglap) were quantified as described above and previously [11] (Table 1).

For ex vivo DNA damage studies, BMSC were grown on glass coverslips in osteogenic medium for six days, after which they were immunostained with an anti-phospho- γH2AX -Alexa Fluor® 488 conjugated antibody (Biolegend #613405, San Diego, CA; 1 $\mu\text{g}/\text{ml}$) as previously described [11]. DNA damage was quantified by assessing the percentage of cells demonstrating nuclear γH2AX staining using image analysis software (Bioquant Osteo, Nashville, TN) and a confocal microscope (Zeiss LSM 510) as described previously [11]. More than 150 cells were measured from each culture.

For TUNEL assays of DNA damage, BMSC were grown on glass coverslips in osteogenic medium for seven days. Cells were fixed with 4% paraformaldehyde, lysed with PBS containing 0.1% TritonX-100 and 0.1% sodium citrate, and stained with the TUNEL staining kit as described above (Roche). Fluorescent cells were quantified in 15 fields of view as the relative percentage of TUNEL positive nuclei using image analysis software (Bioquant Osteo).

2.10. Isolation and culture of immature mouse articular chondrocytes (IMACs)

Immature mouse articular chondrocytes (IMACs) were isolated as described by Gosset et al. [29]. Briefly, the femoral head and tibial plateau were dissected from 4 wk old mice. Cartilage pieces were digested twice in 3 mg/ml collagenase for one hour and then overnight in 0.5 mg/ml collagenase. The resulting suspension of freshly isolated IMACs was placed in micromass culture by plating 10 μl drops containing 2×10^5 cells in DMEM, 5% FBS supplemented with 25 $\mu\text{g}/\text{ml}$ ascorbic acid, 54 $\mu\text{g}/\text{ml}$ beta-glycerol phosphate and 1X ITS (Invitrogen, Carlsbad, CA) as previously published [30-32]. Culture medium was changed every three days for 21 days. Cells were fixed at various times and stained with either alcian blue (to highlight matrix proteoglycan content) or alizarin red (to highlight matrix calcification).

2.11. Statistics

Statistics were performed with JMP 9.0 statistical analysis software (SAS Institute Inc., Cary, NC). Data were compared between groups within each experiment with Student's t-tests (when only two groups were compared) or ANOVA (when three groups were compared) with Tukey-Kramer HSD post-hoc multiple comparisons test. A significance of $p < 0.05$ was used for all comparisons.

3. Results

3.1. Hdac3 CKO_{OCN} mice develop normally

Hdac3 was abundantly expressed in demarrowed cortical bone extracts from 5 to 6 wk-old Hdac3^{fl/fl}, Cre- control and Hdac3^{+/+}, Cre+ control groups, whereas Hdac3 expression levels were reduced by 40% to 90% in cortical bone of Hdac3^{fl/fl}, Cre+ CKO (Hdac3 CKO_{OCN}) mice (Figure 1A-C). Cells in the cortical bone of Hdac3 CKO_{OCN} mice had 4-fold higher levels of acetylated histone 3 (AcH3) as compared to Hdac3^{fl/fl}, Cre- control mice (Figure 1B, D). Acetylated histone 4 (AcH4) levels were also elevated, indicating that Hdac3 depletion had marked effects on chromatin structure. Immunohistochemistry confirmed that Hdac3 was reduced in both osteocytes and osteoblasts (Figure 1E-F). All Hdac3 CKO_{OCN} mice appeared grossly normal at birth and had normally patterned skeletons (Figure S1A-B). Femur length was comparable between Hdac3 CKO_{OCN} and control mice at all ages investigated (data not shown). No differences were observed in body weights of male Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- littermates at 3, 4, 6, and 12 wk of age (Figure S1C), whereas a small but significant decrease in body mass was observed in 24 wk old male Hdac3 CKO_{OCN} mice as compared to age-matched Hdac3^{fl/fl}, Cre- control mice. The body weights of female Hdac3 CKO_{OCN} were comparable to Hdac3^{fl/fl}, Cre- control mice at all ages investigated (Figure S1D).

3.2. Areal bone mineral density is reduced in Hdac3 CKO_{OCN} mice

DXA revealed a progressive decrease in BMD with age in both male and female Hdac3 CKO_{OCN} mice (Figure S2). No differences were observed in BMD between Hdac3^{fl/fl}, Cre- control and Hdac3 CKO_{OCN} mice at 4 wk of age, but by 12 wk of age, BMD of the lower body and femoral diaphysis were decreased (-6.6% lower body, -7.6% femur) in female Hdac3 CKO_{OCN} as compared to age- and sex-matched Hdac3^{fl/fl}, Cre- control mice (Figure S2). Similar results were observed at 24 wk of age, as BMD of both the lower body (-8.0%) and femoral diaphysis (-12.4%) were lower in the female Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- control mice. BMD also tended to be lower in male Hdac3 CKO_{OCN} mice relative to their respective controls, although not all differences achieved statistical significance (Figure S2). It is possible that the slight decrease in body mass in the aged males obscured trends in BMD, contributing to the lack of statistical significance.

3.3. Bone architecture, volumetric mineralization, and material properties are reduced in Ocn-Hdac3 CKO mice

For more accurate measurements of bone structure, trabecular and cortical bone architecture and volumetric mineralization were assessed by micro-computed tomography in male mice. No differences were observed in trabecular bone properties between Hdac3^{fl/fl}, Cre- control and Hdac3^{+/+}, Cre+ control mice through 12 wk of age (Table 2). Trabecular bone properties were comparable between Hdac3 CKO_{OCN} mice and both control groups at 4 wk of age (Table 2). However, by 6 wk of age, Hdac3 CKO_{OCN} mice displayed a 22% reduction in trabecular number and a 30% increase in trabecular separation as compared to age-matched Hdac3^{fl/fl}, Cre- control and Hdac3^{+/+}, Cre+ control mice. These differences persisted in 12 and 24 wk animals and increased in magnitude with age (Table 2); by 24 wk of age, Hdac3 CKO_{OCN} animals demonstrated a striking 50% decrease in trabecular bone volume fraction as compared to age-matched control mice (Table 2). Female Hdac3 CKO_{OCN} mice also showed prominent trabecular bone loss; bone volume fraction in female Hdac3 CKO_{OCN} mice was reduced by 85% as compared to Hdac3^{fl/fl}, Cre- control mice at 24 wk of age. (Figure 2A, Table S1). Taken together, these data demonstrate that Hdac3 CKO_{OCN} mice begin to lose trabecular bone mass between 4 and 6 weeks of age.

Few differences were observed in the morphology and mineralization of cortical bone at the femoral diaphysis between control groups and Hdac3 CKO_{OCN} mice at 4 wk and 6 wk of age. At 12 wk of age, however, Hdac3 CKO_{OCN} mice presented with lower cortical bone material mineral density as compared to age-matched Hdac3^{fl/fl}, Cre- (-3%) and Hdac3^{+/+}, Cre+ control (-5%) mice, and this difference persisted in the 24 wk old animals (Table 3). Cortical bone area was not different between groups at 4, 6, and 12 wk of age, but was lower (-16%) in the 24 wk old Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- control mice (Table 3). Cortical bone thickness was not significantly different between groups at any age investigated. Cortical bone properties were comparable between both control groups (Table 3). Female Hdac3 CKO_{OCN} mice also showed prominent cortical bone loss (Ct.Ar: -17%) and decreased cortical bone mineralization (-3%) in the femoral diaphysis as compared to Hdac3^{fl/fl}, Cre- controls at 24 wk of age (Table S2). Thus, cortical bone in adult Hdac3 CKO_{OCN} mice demonstrates altered geometry and reduced mineral density.

FT-IR analyses were performed on histological sections of cortical bone from 4 wk old mice to investigate bone composition. Surprisingly, despite the lack of change in cortical bone mineralization measured via microCT at this age, cortical bone from the Hdac3 CKO_{OCN} mice demonstrated a significantly lower (-38%) mineral to matrix ratio as compared to age-matched Hdac3^{fl/fl}, Cre- control mice (Figure 2B). Furthermore, the carbonate to phosphate ratio of Hdac3 CKO_{OCN} cortical bone was significantly higher (+31%) as compared to Hdac3^{fl/fl}, Cre- control cortical bone (Figure 2B). These data suggest that cortical bone composition is unfavorably altered by Hdac3 insufficiency in young mice.

Nanoindentation tests were performed on cortical bone samples from the femoral midshaft of 12 wk old mice to determine if the mineralization abnormalities had biological relevance. Hardness was 22% lower in Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- control mice (Figure 2C). Reduced modulus was 12% lower, although this difference did not achieve statistical significance (Figure 2C). Thus, loss of Hdac3 in mature osteoblasts and osteocytes compromises bone material properties.

3.4. Osteoblastic and osteocytic gene expression is reduced in Hdac3 CKO_{OCN} mice tissue

To gain insight into the early mechanisms responsible for the poor bone quality of the Hdac3 CKO_{OCN} mice, we analyzed cortical bone gene expression in 2 wk old animals. Expression levels of genes relating to osteoblast maturation (e.g., alkaline phosphatase (Alpl), Collagen 1a (Colla1), and Bglap) were lower in Hdac3 CKO_{OCN} mice as compared to Hdac3^{+/+}, Cre + controls. The reductions matched the decrease in Hdac3 expression in this heterogeneous cell population (Figure 3A). Osteocyte genes, including Sost and Mepe, were also reduced (Figure 3A) in the Hdac3 CKO_{OCN} mice, consistent with decreased expression of Hdac3 in this cell population seen via immunohistochemistry. No differences were observed in expression levels of representative genes relating to cell proliferation (CyclinD1 and Cdkn1a (p21)) (Figure 3A). Rankl and Opg expression were analyzed to determine if signals were favorable for an unbalanced increase in osteoclastogenesis that could explain the lower bone mass in the knockout mice. However, no differences were observed in expression of Rankl, Opg, or the ratio of Rankl to Opg expression in Hdac3 CKO_{OCN} mice as compared to Hdac3^{+/+}, Cre+ controls (Figure 3A-B).

3.5. Serum markers of bone remodeling and serum calcium levels are decreased in Ocn-Hdac3 CKO mice

To further explore the possibility that bone remodeling is altered in Hdac3 CKO_{OCN} mice, serum levels of TRAcP5b (a marker of bone resorption/osteoclast number), PINP (bone formation indicator), and calcium were measured. TRAcP5b levels were comparable between Hdac3^{+/+}, Cre+ control and Hdac3 CKO_{OCN} at 4 wk of age, but were 31% lower in

Hdac3 CKO_{OCN} as compared to Hdac3^{+/+}, Cre+ controls at 6 wk of age and 58% lower as compared to Hdac3^{fl/fl}, Cre- control mice 24 wk of age (Figures 4A-B). Serum P1NP (bone formation marker) levels followed a similar pattern, in that they were not different between Hdac3 CKO_{OCN} and Hdac3^{+/+}, Cre+ control mice at 4 wk of age but were 34% lower in Hdac3 CKO_{OCN} as compared to Hdac3^{+/+}, Cre+ control mice by 6 wk of age (Figure 4C). P1NP levels were not different between Hdac3 CKO_{OCN} and Hdac3^{fl/fl}, Cre- control mice at 24 wk of age (Figure 4D). The large age-related reduction in P1NP levels within control animals may contribute to this lack of difference between the control and Hdac3-CKO_{OCN} animals. However, another likely contributing factor is that aged Hdac3 CKO_{OCN} mice were prone to skeletal fractures (Figure S3), which can trigger bone formation associated with fracture healing and thus raise the P1NP levels in Hdac3 CKO_{OCN} mice. Indeed, subsequent skeletal examination indicated that all of the 24 month old Hdac3 CKO_{OCN} mice used for analysis of P1NP levels presented with discernible fractures, many of which were in various stages of remodeling (Figure S3), while no fractures were detected in their control littermates. Serum calcium levels were slightly lower in Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- control mice at 6 wk of age (-7.6%, $p = 0.173$) and similarly trended lower at 24 wk of age (-7.1%, $p = 0.073$) (Figure S4). Taken together, these data indicate that the reduced bone mass in the Hdac3 CKO_{OCN} mice is due to defects in bone formation, rather than to increases in bone resorption. These results are consistent with trends we observed in osteoblastic gene expression in the cortical bone of Hdac3 CKO_{OCN} mice.

3.6. Histological indices of bone remodeling

Histomorphometry was utilized to measure osteoblast and osteoclast numbers and activities. Histological indices of total osteoclast number (N.Oc/T.Ar) were 39% lower in Hdac3 CKO_{OCN} mice as compared to Hdac3^{+/+}, Cre+ controls, but other osteoclastic indices were not different between the groups (Table 4). Although there were significant reductions in trabecular number in 6 wk old Hdac3 CKO_{OCN} animals (Table 2), osteoblast numbers per bone surface or area (Ob.S/BS and N.Ob/B.Ar) were increased approximately 2-fold in 6 wk old Hdac3 CKO_{OCN} as compared to age-matched Hdac3^{+/+}, Cre+ control mice (Table 4). However, osteoblast number normalized to tissue area was not different between genotypes because of the decreased trabecular bone volume in the Hdac3 CKO_{OCN} mice. Closer examination revealed that osteoblasts occupying the bone surface in Hdac3 CKO_{OCN} animals had a flattened morphology, suggesting impaired function (Figure 5A). Consistent with this hypothesis, dynamic indices of trabecular bone formation (e.g., mineral apposition rate) were not different between Hdac3 CKO_{OCN} mice and Hdac3^{fl/fl}, Cre- controls despite higher osteoblast numbers in the Hdac3 CKO_{OCN} mice (Table 5), and mineralizing surface normalized to osteoblast surface (indicative of formation on a per-cell basis) was decreased in the Hdac3 CKO_{OCN} mice (Table 5). Periosteal and endocortical mineral apposition rates were not significantly different between the groups (Table 6). However, in the femoral diaphysis, periosteal and endocortical mineralizing surfaces were 20% and 30% lower, respectively, in Hdac3 CKO_{OCN} as compared to Hdac3^{fl/fl}, Cre- control mice, consistent with the reduced serum levels of P1NP that suggested lower bone formation. Together, these results indicate that osteoblasts are present in the Hdac3 CKO_{OCN} animals, but they are not functioning to full capacity. These results are consistent with changes in bone mass and matrix composition seen by DXA, microCT, and FT-IR.

3.7. Stromal cells from Hdac3 CKO_{OCN} mice are impaired in their ability to form matrix-producing osteoblasts and display signs of DNA damage

To understand the mechanisms responsible for the deleterious effects of Hdac3-insufficiency on osteoblast function in vivo, primary bone marrow stromal cells (BMSC) were isolated and cultured under osteogenic conditions. BMSC from the Hdac3 CKO_{OCN} mice cultured for 21 days showed a 50% to 70% reduction in the amount of mineralized matrix formed

relative to cells from age-matched Hdac3^{fl/fl}, Cre- control mice (Figure 6A). Microscopic examination showed both cellular and extracellular matrix deficiencies in the Hdac3 CKO_{OCN} cultures (Figure 6A). Hdac3 CKO_{OCN} BMSCs had reduced expression levels of Hdac3 and genes related to osteoblast maturation (Runx2, Col1a1, Alpl, and Bglap) as compared to cultures from Hdac3^{fl/fl}, Cre- control mice (Figure 6B). Osteoblasts from the Hdac3 CKO_{OCN} mice demonstrated 2- to 3-fold increases in DNA damage (evidenced by TUNEL and phosphorylated γ H2AX staining, respectively, (Figure 6C-D) relative to cells from Hdac3^{fl/fl}, Cre- control mice. Thus, Hdac3 insufficiency leads to impaired osteoblastic differentiation and induction of DNA damage in differentiating osteoblasts. Primary chondrocytes from the Hdac3 CKO_{OCN} mice were not impaired in their ability to produce a cartilaginous, calcified matrix, likely because chondrocyte expression of Hdac3 was not affected in the Hdac3CKO_{OCN} mice (Figure S5). These data demonstrate that Hdac3 is required for osteoblast survival and function.

3.8. Cortical osteocytes in Hdac3 CKO_{OCN} exhibit DNA damage in vivo

Because the in vitro data suggested that Hdac3 is required for osteoblast function and genome integrity and because cortical bone structure and hardness were reduced in Hdac3 CKO_{OCN} mice, TUNEL staining was performed on decalcified tibial sections to determine if cortical osteoblasts and osteocytes also had DNA damage. TUNEL positive cells were abundant in the cortical bone of Hdac3 CKO_{OCN} mice, but not in the cortical bone of Hdac3^{fl/fl}, Cre- control mice at both 6 and 12 wk of age (Figure 5B). No substantial differences were noted in the relative abundance of TUNEL positive cells in the growth plate or primary spongiosa. These data suggest that loss of Hdac3 promotes DNA damage in mature osseous cells.

4. Discussion

Hdac3 is essential for the proper development of many body systems. This was first emphasized by studies indicating that germline knockout of Hdac3 is embryonically lethal prior to E9.5 [16]. Subsequent reports described Hdac3's post-embryonic role in specific tissues, including the liver, skeletal and cardiac muscle, and in early skeletal development [17, 20, 33-35]. Our previous studies of Hdac3 in the skeletal system utilized a Cre recombinase expressed under the control of the Osterix promoter (Osx-Cre), which deleted Hdac3 in osteochondral progenitor cells [17]. These Hdac3 CKO_{OSX} mice had severe craniofacial and developmental abnormalities that were apparent within 1 week of birth. These skeletal deficiencies had both bone and cartilage components, consistent with the expression of Osx (Sp7) in both of these cell populations [36-38]. The goal of the current study was to better localize the effects of Hdac3-insufficiency to the osteoblast population, independent of effects in cartilage, to understand Hdac3's role in osseous development.

Hdac3 CKO_{OCN} mice had normal growth plates, and primary chondrocytes from the Hdac3-insufficient mice showed no impairment of their ability to produce matrix in vitro (Figure S5). This likely explains why the skeletons of Hdac3 CKO_{OCN} mice were normally patterned with bone lengths and body size comparable to control mice in the current study (Figure S1), whereas Hdac3 CKO_{OSX} mice were smaller than their control littermates [17]. Although the OCN-Cre transgene was reported to show activity in hypertrophic chondrocytes [39], Hdac3 is not expressed at this stage of chondrocyte maturation in wildtype mice (Figure S5) and thus all phenotypes in Hdac3 CKO_{OCN} are likely to emerge from functional deficits in osseous cells. Hdac3 insufficiency in committed osteoblasts caused DNA damage, ultimately impairing normal osteoblast function and bone formation. Unlike the Hdac3 CKO_{OSX} mice, Hdac3 CKO_{OCN} mice did not have a relative increase in marrow adiposity, possibly because cells were more firmly committed to the osteoblast lineage at the time of Hdac3 deletion.

Similarities did exist between the current study and our previous model, in that both Hdac3 CKO_{OSX} and Hdac3 CKO_{OCN} mice had low cortical and trabecular bone mass as compared to age-matched control mice, although the decreased bone mass was less severe in the Hdac3 CKO_{OCN} model and only became apparent as the animals aged. Bones of Hdac3 CKO_{OSX} mice fractured during tissue harvest [17], and comparably, in vivo fractures were observed in 12- and 24 wk old Hdac3 CKO_{OCN} mice (Figure S3). The observed fragility of the Hdac3 CKO_{OCN} mouse bones was likely due to both structural and compositional changes in the skeleton. Decreased cortical bone area in the aged Hdac3 CKO_{OCN} mice represents a geometrical change that negatively affects whole bone mechanical behavior [40]. At the material level, both microCT and FT-IR analyses revealed defects in cortical bone mineralization in the Hdac3 CKO_{OCN} mice as cortical bone tissue mineral density and mineral to matrix ratio were lower in the Hdac3-insufficient animals as compared to control littermates. A decreased mineral to matrix ratio and an increased carbonate to phosphate ratio (Figure 2B) are evidence of poor bone quality that increases fracture risk, as has been reported in dogs treated with calcitonin [41, 42]. Accordingly, decreased cortical bone mineralization in the Hdac3 CKO_{OCN} mice impacted tissue-level bone material properties, as seen by a reduction in cortical bone hardness via nanoindentation testing, as nanoindentation hardness of bone primarily reflects mineral content [25]. Thus, the increased prevalence of fractures in the aged Hdac3 CKO_{OCN} mice is consistent with the idea that whole bone mechanical strength is strongly related to alterations in bone mass, geometry, and matrix composition [40].

The low bone mass in the Hdac3 CKO_{OCN} mice was largely attributed to impaired bone formation, and not to increased bone resorption. Histological indices of total osteoclast number (N.Oc/T.Ar), serum TRAcP5b levels, and total serum calcium levels were lower, not higher, in the Hdac3-insufficient animals. It is worth noting that osteoclast numbers and serum TRAcP5b levels were similarly reduced in the Hdac3 CKO_{OCN} animals (N.Oc/T.Ar: -39%; serum TRAcP5b: -31%), consistent with the idea that serum TRAcP5b levels are indicative of absolute osteoclast number in the body [43]. These data, documenting the presence of fewer osteoclasts in the Hdac3 CKO_{OCN} mice, coupled with the lack of change in cortical bone expression levels of RANKL, OPG, or the ratio of RANKL/OPG, indicate that an osteoblast-mediated resorptive mechanism is not triggered by Hdac3 insufficiency, implying that changes in bone formation are responsible for the low bone mass phenotype of the Hdac3 CKO_{OCN} mice.

Impaired osteoblast function was best illustrated by lower serum PINP levels and lower cortical bone mineralizing surfaces in the Hdac3 CKO_{OCN} mice. Paradoxically, brightfield trabecular osteoblastic histological indices demonstrated that Hdac3 CKO_{OCN} mice had a greater number of osteoblasts occupying available trabecular bone surfaces as compared to control mice (Table 4). These parameters are deceiving, however, as the Hdac3 CKO_{OCN} mice did not have an increased amount of trabecular bone mineralizing surface. In fact, mineralizing surface normalized to osteoblast surface was lower in the Hdac3 CKO_{OCN} mice (Table 5), indicating that at least some of these osteoblasts were not functioning properly. Upon closer examination, many of the bone-lining osteoblasts in the Hdac3 CKO_{OCN} mice had a flattened morphology (Figure 5A), consistent with impaired function. Relative expression levels of characteristic osteoblast and osteocyte genes, including the genes encoding alkaline phosphatase (*Alpl*) and sclerostin (*Sost*), were decreased in cortical bone explants from Hdac3 CKO_{OCN} as compared to control mice (Figure 3), despite increases in histone acetylation. This phenotype may result from either increased expression of a transcriptional repressor or another cellular mechanism that delays differentiation and associated gene expression programs. In vitro studies with BMSC support the hypothesis that Hdac3 CKO_{OCN} cells differentiate inefficiently into mature osteoblasts, evidenced by the lack of matrix, increased DNA damage (as measured by P- γ H2AX and TUNEL

staining), and reduced expression of osteoblastic genes in the osteogenic BMSC cultures (Figure 6). Future studies will address whether Hdac3 insufficiency triggers other deleterious mechanisms (e.g., senescence) to impair osteoblastic differentiation. Consistent with our previous mouse model [17], trabecular bone mineral apposition rate was not different between control and Hdac3 CKO_{OCN} mice, which may indicate that the cells capable of surviving or progressing to the matrix production phase of osteoblastic differentiation functioned normally.

A relationship between Hdac3 and genome integrity was previously suggested, as mouse embryonic fibroblasts (MEFs) from *Hdac3*^{fl/-} animals infected with Adeno-Cre demonstrated genome instability shortly after Hdac3 deletion [16]. Subsequent analysis revealed that Hdac3-deficient MEFs suffered DNA damage that ultimately resulted in cell cycle arrest [16]. Genes relating to DNA damage, including the p53-inducible genes p21^{CIP1} and Phlda3, were also upregulated in Hdac3 CKO_{OSX} mice [17], and BMSC exposed to the pan-Hdac inhibitor vorinostat during osteoblastic differentiation demonstrated DNA damage and cell cycle arrest [11]. The current study confirms the importance of Hdac3 in bone cells, as Hdac3 CKO_{OCN} mice demonstrated DNA damage in differentiating osteoblasts and osteocytes in vivo and in vitro as compared to cells from control mice. Preventing DNA damage is a vital mechanism in the skeleton as it is an early marker of apoptosis, which is linked to key processes like targeted remodeling [44–47], the anabolic effects of intermittent parathyroid hormone therapy [48, 49], and glucocorticoid-induced osteonecrosis [50, 51]. Hdac3 is therefore positioned to be a key regulatory factor in the regulation of genome stability and bone mass. Understanding the roles of specific Hdac3 in regulating chromatin structure and DNA integrity could yield new strategies for modulating osteoblast behavior in skeletal disorders.

In conclusion, our data confirm that Hdac3 expression in osteoblasts is essential for proper bone formation. Loss of Hdac3 in committed osteoblasts and osteocytes impaired tissue-specific gene expression, caused DNA damage, decreased bone formation, and caused low bone mass. These data demonstrate that Hdac3 is required for osteoblast and osteocyte function.

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Research Highlights

- Deleting Hdac3 in mature osteoblasts with Osteocalcin-Cre (Hdac3 CKO_{OCN}) caused progressive cortical and trabecular bone loss with age.
- Cortical bone mineralization and material properties were compromised in Hdac3 CKO_{OCN} as compared to control mice.
- Bone loss in Hdac3 CKO_{OCN} mice was attributed to decreased bone formation and osteoblast/osteocyte function.
- Hdac3 deletion caused DNA damage in osteoblasts and osteocytes in vitro and in vivo.
- Chondrocyte activity and growth plate development were not affected in Hdac3 CKO_{OCN} mice

Figure 1. Hdac3 expression is reduced in Hdac3 CKO_{OCN} mice

(A) Western blots of demarrowed cortical bone protein extracts from 6 wk old Hdac3^{fl/fl}, Cre- control, Hdac3^{+/+}, Cre+ control, and Hdac3 CKO_{OCN} mice. Relative intensity of Hdac3 normalized to Actin appears beneath each lane. (B) Western blots of demarrowed cortical bone protein extracts from 5 wk old Hdac3^{fl/fl}, Cre- control and Hdac3 CKO_{OCN} mice. (C-D) Relative intensity of Hdac3 normalized to Actin (C) and AcH3 normalized to H3 (D) for bands in (B) are plotted; means $\pm$ SEM are presented. (E-F) Immunohistochemistry of Hdac3 expression in osteocyte nuclei (arrows) (E) and osteoblasts (arrows) (F) within cortical bone of 6 wk old Hdac3^{fl/fl}, Cre- control or Hdac3 CKO_{OCN} mice. AcH3: acetylated histone 3, AcH4: acetylated histone 4, Ctrl: control.

Figure 2. Bone architecture and bone quality are reduced in Hdac3 CKO_{OCN} mice

(A) Representative 3D reconstructions of trabecular bone architecture in the distal femoral metaphysis from Hdac3^{fl/fl}, Cre- control and Hdac3 CKO_{OCN} mice. (B) Cortical bone extracellular matrix composition was analyzed via FT-IR in histological sections from 4 wk old mice. (C) Cortical bone material properties in 12 wk old mice were measured by nanoindentation. Hardness was significantly reduced in the Hdac3 CKO_{OCN} mice, but reduced modulus (Er) was not different between Hdac3 CKO_{OCN} and Hdac3^{fl/fl}, Cre-control mice ($p = 0.318$). Means + SEM are presented * $p < 0.05$ vs. Hdac3^{fl/fl}, Cre- control. Sample numbers are stated on each bar.

Figure 3. Osteoblast and osteocyte gene expression is decreased in demarrowed cortical bone from 2 wk old Hdac3 CKO_{OCN} mice

(A) Transcript levels for the specified genes were normalized to the reference gene Gapdh according to the $2^{-(\Delta\Delta CT)}$ method. Data are expressed as fold change in gene expression relative to Hdac3^{+/+}, Cre+ control mice. (B) The ratio of Rankl to Opg expression was not different between Hdac3^{+/+}, Cre+ control and Hdac3 CKO_{OCN} mice. Means + SEM of n = 4 Hdac3^{+/+}, Cre+ control, 3 Hdac3 CKO_{OCN} mice are presented. *p 0.05, **p < 0.07.

Figure 4. Serum markers of bone remodeling are decreased in Hdac3 CKO_{OCN} mice
 Serum was collected from male mice at sacrifice. (A-B) Serum levels of TRAcP5b (a marker of bone resorption) in control and Hdac3 CKO_{OCN} mice at 4, 6 and 24 wk of age. (C-D) Serum levels of P1NP (a marker of bone formation) in the same animals. Means + SEM are presented. *p = 0.05 vs. age-matched controls. **p < 0.1.

Figure 5. Osteoblast morphology and TUNEL staining suggest impaired osteoblast differentiation and genome integrity in Hdac3 CKO_{OCN} mice

(A) Trabecular bone from 6 wk old Hdac3^{+/+}, Cre+ control or Hdac3 CKO_{OCN} mice stained by VonKossa/MacNeals tetrachrome. Osteoblasts (arrows) in the Hdac3 CKO_{OCN} mice frequently had a more flattened morphology as compared to osteoblasts in control mice. (B) TUNEL staining of growth plate and cortical bones from Hdac3^{+/+}, Cre+ control and Hdac3 CKO_{OCN} mice (white arrows indicate TUNEL positive osteocytes). Green = TUNEL, blue = DAPI.

Figure 6. Osteogenic culture of bone marrow stromal cells (BMSC) recapitulates osteoblastic defects in Hdac3 CKO mice

(A) Cells isolated from 4 wk, 12 wk, and 24 wk old mice were grown in osteogenic medium for 21 days. Representative alizarin red stained wells from Hdac3^{fl/fl}, Cre- control and Hdac3 CKO^{OCN} mouse cultures from all three ages of mice are presented. (B) mRNA expression in the Hdac3^{fl/fl}, Cre- control and Hdac3 CKO^{OCN} cultures from 4 wk and 24 wk old mice after 21 days of osteogenic differentiation. Transcript levels for the specified genes were normalized to the reference gene Gapdh, and all samples were analyzed in triplicate. Data are expressed as fold change in gene expression relative to Hdac3^{fl/fl}, Cre- control cultures. (C) BMSC seeded onto glass coverslips were cultured in osteogenic media for 6 days, after which they were stained with antibodies for phosphorylated γH2AX, an indicator of DNA damage. Data plotted are the number of γH2AX positive nuclei versus the total number of DAPI positive nuclei. More than 150 cells were measured from each culture. (D) BMSC seeded onto glass coverslips were cultured in osteogenic media for 7 days, after which they were TUNEL stained for another quantification of DNA damage, measured as TUNEL positive nuclei fraction (#TUNEL⁺ nuclei / # DAPI-stained nuclei). TUNEL staining was quantified in 15 fields of view from each culture. Means + SEM are presented. *p < 0.05 vs. WT cultures.

Table 1

Primer sequences used in PCR reactions.

Gene name	ID	Forward primer sequence	Reverse primer sequence
Alkaline phosphatase/Alpl	NM_007431	CACAGATTCCCAAAGCACCT	GGGATGGAGGAGAGAAGGTC
Ccnd1	NM_007631	AGAAGGAGATTGTGCCATCCA	CTCACAGACCTCCAGCATCCA
Cdkn1a Cdkn1(p21)	NM_007669	CAAGAGGCCCACTACTTCCT	ACACCAGAGTGCAAGACAGC
Col1a1	NM_007742	GCTTCACCTACAGCACCCCTTGT	TGACTGTCTTGCCCCAAGTTC
Gapdh	NM_008084	GGGAAGCCCATCACCATCTT	GCCTCACCCCATTGATGTT
Hdac3	NM_010411	CCCGCATCGAGAATCAGAAC	TCAAAGATTGTCTGGCGGATCT
Mepe	NM_053172	TGCTGCCCTCCTCAGAAATATC	GTTCGGCCCCAGTCACTAGA
Opg/Tnfrsf11b	NM_008764	CCAAGAGCCCAGTGTTTCTT	CCAAGCCAGCCATTGTTAAT
Osteocalcin/Bglap	NM_007541	CCTGAGTCTGACAAAGCCTTCA	GCCGGAGTCTGTTCACCTT
Osterix/Sp7	NM_130458	GGAGGTTTCACTCCATTCCA	TAGAAGGAGCAGGGGACAGA
Phex	NM_011077	AAGCTGGACCAAGCAACACT	AAGGGCATCCCGATAAGATT
Rankl/Tnfsf11	NM_011613	GCTGGGACCTGCAAATAAGT	TTGCACAGAAAACATTACACCTG
Runx2	NM_009820	GGCACAGACAGAAGCTTGATGA	GAATGCGCCCTAAATCACTGA
Sost	NM_024449	ACTTGTGCACGCTGCCTTCT	TGACCTCTGTGGCATCATTCC

Table 2

Trabecular bone architecture in male mice

Age	Genotype	n	BV/TV	Tb.N, 1/mm	Tb.Th, mm	Tb.Sp, mm
4 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.081 (0.019)	4.89 (0.68)	0.029 (0.001)	0.209 (0.031)
	Hdac3 ^{+/+} , Cre+ control	4	0.101 (0.029)	4.99 (0.88)	0.032 (0.003)	0.209 (0.031)
	Hdac3 CKO _{OCN}	7	0.094 (0.030)	4.75 (0.61)	0.033 (0.004)	0.185 (0.075)
6 wk	Hdac3 ^{fl/fl} , Cre- control	4	0.163 (0.067)	6.25 (0.75)*	0.036 (0.005)	0.160 (0.019)*
	Hdac3 ^{+/+} , Cre+ control	5	0.132 (0.015)	6.18 (0.62)*	0.032 (0.001)*	0.161 (0.018)*
	Hdac3 CKO _{OCN}	12	0.111 (0.027)	4.80 (0.44)	0.038 (0.004)	0.208 (0.016)
12 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.120 (0.023)	5.15 (0.53)*	0.038 (0.004)	0.192 (0.020)*
	Hdac3 ^{+/+} , Cre+ control	3	0.138 (0.049)	5.45 (0.43)*	0.038 (0.006)	0.180 (0.015)*
	Hdac3 CKO _{OCN}	9	0.104 (0.046)	4.18 (0.39)	0.045 (0.009)	0.241 (0.023)
24 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.101 (0.025)*	4.11 (0.19)*	0.043 (0.004)*	0.240 (0.011)*
	Hdac3 CKO _{OCN}	7	0.052 (0.013)	3.63 (0.35)	0.038 (0.005)	0.277 (0.027)

Means and (standard deviations) are shown.

* p 0.05 vs. Hdac3 CKO_{OCN}

Table 3

Cortical bone architecture and mineralization in male mice

Age	Genotype	n	Ct.Ar, mm ²	Ct.Th, mm	Ct.Mat.M.Dn, mgHA/ccm
4 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.421 (0.035)	0.099 (0.008)	1054 (12)
	Hdac3 ^{+/+} , Cre+ control	3	0.485 (0.061)	0.112 (0.012)	1122 (18)*
	Hdac3 CKO _{OCN}	7	0.446 (0.042)	0.111 (0.008)	1066 (19)
6 wk	Hdac3 ^{fl/fl} , Cre- control	4	0.624 (0.118)	0.137 (0.014)	1077 (6)
	Hdac3 ^{+/+} , Cre+ control	5	0.566 (0.043)	0.122 (0.006)	1091 (24)*
	Hdac3 CKO _{OCN}	11	0.605 (0.086)	0.138 (0.013)	1063 (15)
12 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.545 (0.080)	0.173 (0.016)	1198 (17)*
	Hdac3 ^{+/+} , Cre+ control	3	0.556 (0.022)	0.171 (0.008)	1182 (10)*
	Hdac3 CKO _{OCN}	9	0.521 (0.080)	0.171 (0.016)	1143 (18)
24 wk	Hdac3 ^{fl/fl} , Cre- control	5	0.858 (0.057)*	0.193 (0.007)	1260 (9)*
	Hdac3 CKO _{OCN}	7	0.721 (0.098)	0.177 (0.026)	1226 (21)

Means and (standard deviations) are shown.

* p 0.05 vs. Hdac3 CKO_{OCN}

Table 4

Static trabecular bone histological remodeling indices

Genotype	n	Ob.S/BS, %	N.Ob/B.Ar, #/mm ²	N.Ob/T.Ar, #/mm ²	n	Oc.S/BS, %	N.Oc/B.Ar, #/mm ²	N.Oc/T.Ar, #/mm ²
Hdac3 ^{+/+} , Cre+ control	7	14.5 (6.1) *	605 (233) *	83.8 (35.1)	6	19.7 (7.4)	486 (221)	73.5 (24.3) *
Hdac3 CKO _{OCN}	8	22.5 (6.1)	1129 (415)	82.0 (27.2)	7	16.6 (5.2)	572 (278)	44.7 (15.8)

Means and (standard deviations) are shown.

* p 0.05 vs. Hdac3 CKOOCN

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Table 5

Dynamic trabecular bone histological remodeling indices

Genotype	n	MS/BS, %	MS/Ob.S, %	MAR, μm/day	BFR/BV, %/yr	BFR/TV, %/yr
Hdac3 ^{+/+} , Cre+ control	4	37.5 (6.1)	3.32 (1.20) *	2.41 (0.21)	5.66 (0.42)	0.58 (0.23)
Hdac3 CKO _{OCN}	5	38.7 (3.3)	1.88 (0.52)	2.46 (0.62)	5.18 (0.70)	0.43 (0.15)

Means and (standard deviations) are shown.

* p 0.05 vs. Hdac3 CKOOCN

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Table 6

Cortical bone histological remodeling indices

Genotype	n	Ps.MS/BS, %	Ps.MAR, μm/day	Ec.MS/BS, %	Ec.MAR, μm/day
Hdac3 ^{+/+} , Cre+ control	3	74.8 (3.2) *	3.88 (0.19)	38.2 (4.0) **	1.56 (0.25)
Hdac3 CKO _{OCN}	3	50.9 (2.8)	4.91 (0.39)	30.6 (6.4)	2.44 (0.50)

Means and (standard deviations) are shown.

** p < 0.1 vs. Hdac3 CKOOCN;

* p 0.05 vs. Hdac3 CKOOCN