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Spontaneous Mutation of *Dock7* Results in Lower Trabecular Bone Mass and Impaired Periosteal Expansion in Aged Female *Misty* Mice

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Abstract

Misty mice (*m/m*) have a loss of function mutation in *Dock7* gene, a guanine nucleotide exchange factor, resulting in low bone mineral density, uncoupled bone remodeling and reduced bone formation. *Dock7* has been identified as a modulator of osteoblast number and *in vitro* osteogenic differentiation in calvarial osteoblast culture. In addition, *m/m* exhibit reduced preformed brown adipose tissue innervation and temperature as well as compensatory increase in beige adipocyte markers. While the low bone mineral density phenotype is in part due to higher sympathetic nervous system (SNS) drive in young mice, it is unclear what effect aging would have in mice homozygous for the mutation in the *Dock7* gene. We hypothesized that age-related trabecular bone loss and periosteal envelope expansion would be altered in *m/m*. To test this hypothesis, we comprehensively characterized the skeletal phenotype of *m/m* at 16, 32, 52, and 78 wks of age. When compared to age-matched wild-type control mice (*+/+*), *m/m* had lower areal bone mineral density (aBMD) and areal bone mineral content (aBMC). Similarly, both femoral and vertebral BV/TV, Tb.N., and ConnD were decreased in *m/m* while there was also an increase in Tb.Sp. As low bone mineral density and decreased trabecular bone were already present at 16 wks of age in *m/m* and persisted throughout life, changes in age-related trabecular bone loss were not observed highlighting the role of *Dock7* in controlling trabecular bone acquisition or bone loss prior to 16 wks of age. Cortical thickness was also lower in the *m/m* across all ages. Periosteal and endosteal circumferences were higher in *m/m* compared to *+/+* at 16 wks. However, endosteal and periosteal

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expansion were attenuated in *m/m*, resulting in *m/m* having lower periosteal and endosteal circumferences by 78 wks of age compared to *+/+*, highlighting the critical role of *Dock7* in appositional bone expansion. Histomorphometry revealed that osteoblasts were nearly undetectable in *m/m* and marrow adipocytes were elevated 3.5 fold over *+/+* ($p=0.014$). Consistent with reduced bone formation, osteoblast gene expression of *Alp*, *Col1a1*, *Runx-2*, *Sp7*, and *Bglap* was significantly decreased in *m/m* whole bone. Furthermore, markers of osteoclasts were either unchanged or suppressed. Bone marrow stromal cell migration and motility were inhibited in culture and changes in senescence markers suggest that osteoblast function may also be inhibited with loss of *Dock7* expression in *m/m*. Finally, increased Oil Red O staining in *m/m* ear mesenchymal stem cells during adipogenesis highlights a potential shift of cells from the osteogenic to adipogenic lineages. In summary, loss of *Dock7* in the aging *m/m* resulted in an impairment of periosteal and endocortical envelope expansion, but did not alter age-related trabecular bone loss. These studies establish *Dock7* as a critical regulator of both cortical and trabecular bone mass, and demonstrate for the first time a novel role of *Dock7* in modulating compensatory changes in the periosteum with aging.

Keywords

Misty; aging; bone; *Dock7*; osteoporosis; periosteal

Introduction

Osteoporosis is characterized by enhanced skeletal fragility as a result of uncoupled bone remodeling, i.e. decreased bone formation, increased bone resorption, or both. Osteopenia predisposes to osteoporosis and affects approximately 54 million Americans [1]. Moreover, the two million osteoporosis-related fractures treated per year account for \$19 billion in health care costs [2]. Age is a major and independent risk factor for bone loss leading to osteoporosis: half of women and a quarter of men over the age of 50 will experience an osteoporosis related fracture in their lifetime [1]. Aging mouse models have some of the same characteristics of age-related bone loss in humans, including decreased bone formation, increased bone resorption, increased marrow adiposity, decreased trabecular and cortical bone, and expansion of the periosteal envelope with age [3, 4]. Furthermore, bone is compromised with estrogen deficiency as in ovariectomy and menopause [5, 6].

We previously demonstrated that *m/m* have very low bone mineral density, as well as reduced trabecular and cortical bone volume fraction [4]. The low bone mass phenotype in the *m/m* results from a mutation in the *Dock7* gene rendering the protein levels undetectable [4, 7–10]. DOCK7 is a guanine nucleotide exchange factor (GEF) and member of the Dock180 family of proteins [11, 12]. Dock7 interacts with Rac1, Cdc42, and RhoA catalyzing exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) [8, 9, 13–16]. Like other family members, DOCK7 contains a conserved dock homology region (DHR)-1 and DHR-2 domains. The DHR-1 domain interacts with phosphatidylinositol (3,5)-bisphosphate while DHR-2 domain contains the GTPase activity [12]. GTPase-dependent Dock7 activity has been implicated in ErbB2 and ErbB4 signaling [9, 17]. Further

work has revealed that Dock7 also has GTPase independent activity interacting with TACC3 to control kinetic nuclear migration in radial glial cells [18].

Dock7 has been linked to developmental changes in the nervous system including primary axonal elongation, Schwann cell migrations, and the aforementioned radial glial cell migration [8, 9, 17, 18]. Humans homozygous for mutations in the *Dock7* gene display signs of epilepsy, intellectual disability and cortical blindness [19]. Changes in bone metabolism have yet to be explored in this population.

The bone phenotype is more prominent in female *m/m* which have decreased bone formation, increased bone resorption and increased marrow adiposity. Furthermore, *m/m* have decreased innervation of brown adipose tissue and reduced interscapular temperature, suggesting a reduction of brown fat thermogenesis. In addition, thermogenic gene expression in response to cold was elevated in white fat, suggesting increased SNS signaling, which was in part responsible for bone loss in this model [4]. Studies have shown that the SNS signals to the osteoblast, via the β_2 -adrenergic receptor (β_2AR), decreasing bone formation and increasing osteoclastogenesis through an upregulation of *Rankl* expression [20–22]. Consistent with a phenotype that was partially dependent upon the SNS, treatment with the β -adrenergic receptor antagonist, propranolol, partially rescued the bone loss in the *m/m* [4].

We have previously shown that the *m/m* have low bone mass at 8 and 16 wks of age [4]. To determine whether the *m/m* altered characteristics of aging bone, we aged *+/+* and *m/m* to 78 wks of age and examined body composition, bone micro-architecture, and bone remodeling. Our current study demonstrated that female *m/m* have a persistent reduction in trabecular bone, a defect in cortical expansion both in the periosteal and endocortical envelopes, as well as increased marrow adiposity. Although the reduction in trabecular bone and increases in marrow adiposity recapitulate age-related bone loss, the absence of periosteal expansion in *m/m* suggests an important role for Dock7 in this particular characteristic of aging bone.

Methods

Mice

B6.D2(BKS)-*Dock7^m/J* (*Misty*, *m/m*) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), backcrossed to C57BL/6J (Jackson Laboratory), and bred to produce *m/m* and wild-type littermate control mice (*+/+*). Mice were housed in polycarbonate cages on sterilized paper bedding and maintained under 14:10 hour light:dark cycles in the barrier, AAALAC-accredited animal facility at Maine Medical Center Research Institute. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of Maine Medical Center, and followed the NIH guidelines for the Care and Use of Laboratory Animals. The experiments herein utilized only female *m/m* because female *m/m* have a more prominent low bone mass skeletal phenotype compared to male through 16 wks of age [4].

Dual-energy X-ray absorptiometry (DXA)

DXA for total and femoral areal bone mineral density (aBMD, g/cm²), areal bone mineral content (aBMC, g) and whole body composition exclusive of the head was performed using the PIXImus densitometer (GE-Lunar, Fairfield, CT, USA). The PIXImus was calibrated daily with a phantom provided by the manufacturer.

Micro-computed tomography (μ CT)

Micro-architecture of trabecular bone in the distal femoral metaphysis, L5 vertebral body, and cortical bone at the femoral mid-diaphysis was analyzed by a high resolution μ CT system (μ CT40, Scanco Medical AG, Bruttisellen, Switzerland). Scans were acquired using a 10 μ m³ isotropic voxel size, 70 kVP x-ray tube potential, 114 mA x-ray tube intensity, and 200 ms integration time. Trabecular bone in the distal femoral metaphysis was evaluated in a 1500 μ m (150 transverse slices) long region beginning 200 μ m superior to the distal growth plate and extending proximally. Trabecular bone in the L5 vertebral body was evaluated in a region beginning 100 μ m inferior to the cranial growth plate and extending distally to 100 μ m superior to the caudal growth plate. Trabecular bone in the femur and L5 vertebral body was segmented from soft-tissue using thresholds of 300 mgHA/cm³ and 386 mgHA/cm³, respectively. Measurements of trabecular regions included trabecular bone volume fraction (Tb.BV/TV, %), trabecular number (Tb.N., mm⁻¹), trabecular thickness (Tb.Th., μ m), trabecular separation (Tb.Sp., μ m), connectivity density (ConnD, 1/mm³), and structure model index (SMI). For cortical bone, soft tissue was segmented using a threshold of 696 mgHA/cm³ and then was evaluated in a 500 μ m (50 slice) long region at the femoral mid-diaphysis to measure total cross-sectional area (Tt.Ar, mm²), cortical bone area (Ct. Ar, mm²), medullary area (Ma.Ar, mm²), bone area fraction (Ct.Ar/Tt.Ar, %), cortical tissue mineral density (Ct.TMD, mgHA/cm³), cortical thickness (Ct.Th, mm), cortical porosity (%), and maximum, minimum, and polar moments of inertia ($I_{\max}$, $I_{\min}$, and pMOI, mm⁴). All scans were analyzed using manufacturer software (Scanco, version 4.05). Acquisition and analysis of μ CT data were performed in accordance with published guidelines [23].

Histomorphometry

Static and dynamic histomorphometry measurements were performed in *+/+* and *m/m* at 52 wks of age as previously described [4]. Mice were intraperitoneally injected with 20 mg/kg calcein and 40 mg/kg demeclocycline 9 days and 2 days, respectively, prior to sacrifice. Tibias were analyzed as described and standard nomenclature was used [24–26]. The calculations were performed using the Osteomeasure analysis software. Trabecular and cortex parameters were analyzed in 4 areas, total area was 1.3 mm width $\times$ 0.9 mm height (1 area was 0.65 mm width $\times$ 0.45 mm height). Mineral apposition rate (MAR) was calculated by the distance between 2 labels divided by number of days of interval. For the marrow adipocytes, the number and area of adipocyte ghosts were quantified in the same areas used to analyze trabecular structure and dynamic parameters. Osteomeasure (Osteometrics, Decatur, GA), was used to calculate an adipocyte number and volume and both values were divided by total area.

Serum Protein Parameters

For PINP (AC-33F1) and CTx (AC-06F1), serum protein levels were measured using commercially available kits from IDS (Gaithersburg, MD) according to the manufacturer's instructions. The assay sensitivities were 0.7 and 2 ng/mL for PINP and CTx, respectively. The intra-assay variations were 6.3 and 6.9%, and the inter-assay variations were 8.5 and 12% respectively, for both assays. All measurements were performed in duplicate. For RANKL (MTR00), serum levels were measured using commercially available kit from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions. The assay sensitivity was less than 5 pg/mL. The intra- and inter-assay variations were 4.3 and 6.9 pg/mL respectively. All measurements were performed in duplicate.

Brown adipose tissue (BAT) and femurs histology

BAT was fixed in 10% neutral-buffered formalin immediately following sacrifice for 48 hours followed by storage in 70% ethanol. Samples were then paraffin embedded, sectioned, and stained with either an anti-uncoupling protein 1 (UCP-1; ab23841, Abcam, Cambridge, MA, USA) antibody at the Maine Medical Center Research Institute Histology Core or an anti-tyrosine hydroxylase (TH) antibody at the Michigan State University Histology Laboratory.

To view marrow adipocytes, femurs were fixed in 10% neutral-buffered formalin immediately following sacrifice for 48 hours and then decalcified in 14% EDTA, pH 7.3 until softened. Samples were then transferred to 70% ethanol before they were paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E).

RNA isolation and real-time PCR

Total RNA was isolated using a standard TRIzol extraction (15596018, ThermoFisher Scientific, Waltham, MA) method for tibias. cDNA was generated using the High Capacity cDNA Reverse Transcription kit (4368813, ThermoFisher Scientific, Waltham, MA) according to the manufacturer's instructions. Quantification of mRNA expression was carried out using an iQ SYBR Green Supermix and an iQTM5 multicolor Real-Time PCR detection system (1708882, Bio-Rad, Hercules, CA). *Hprt* was used as an internal standard control gene. Primers were designed and tested to be 95–100% efficient by PrimerDesign (South Hampton, UK) or designed using NCBI: PrimerBlast and ordered from Integrative DNA Technologies (Bethesda, MD). Sequences are listed in Table S1.

Bone marrow stromal cell (BMSC) culture

BMSCs were isolated from the femurs and tibiae of 6–8 wks old age-matched female *+/+* and *m/m* mice using previously established protocols [27]. Briefly, marrow was removed from the femurs and tibiae of 2–4 mice per genotype by centrifugation and resuspended in α MEM (12571, ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), and 1% penicillin/streptomycin (15140, ThermoFisher Scientific, Waltham, MA) using a 25 gauge syringe. Cells were passed through a 70 μ m strainer and plated at 10 million cells per well in a 6 well plate. Non-adherent cells were removed after 72 hrs and media was changed every 2–3 days of culture. BMSCs were grown to 80% confluence for transwell migration and adhesion assays.

Transwell Cell Migration Assay

Transwell cell migration assays were performed similarly to previously established protocols [28, 29]. Transwell inserts, 6.5 mm with 8 μ m pore size, (29442-120, Corning, Corning, NY) were coated with 10 μ g/mL collagen I (354236, Corning, Corning, NY) diluted in PBS overnight at 4°C. Collagen I solution was removed and inserts were blocked with BSA at 37°C, 5% CO₂ for 1 hr. BMSCs were lifted with 0.25% Trypsin and resuspended in α MEM with 0.5% BSA, 1 mM MgCl₂, and 0.2 mM MnCl₂. 100,000 BMSCs were applied to the top of each transwell insert and cells were incubated at 37°C, 5% CO₂. Following 1 or 2 hrs incubation, transwell inserts were removed and cells were stained with 0.1 mg/mL crystal violet, 5% methanol, in PBS for 10 minutes. Inserts were de-stained with 10% acetic acid in H₂O and cells on the underside of the insert were counted.

Cell Adhesion Assay

Cell adhesion assays were performed similarly to previously established protocols [28, 29]. Flat bottom 48 well non-tissue culture treated plates (CC7672-7548, USA Scientific, Ocala, FL) were coated with collagen I and blocked with BSA according to the protocol described for the transwell cell migration assay. BMSCs were lifted with 0.25% Trypsin and resuspended in α MEM with 0.5% BSA, 1 mM MgCl₂, and 0.2 mM MnCl₂. 50,000 BMSCs were added to each well and incubated at 37°C, 5% CO₂ for approximately 20 to 40 minutes or until the cells began to attach. Once cells attached, they were then washed with PBS, stained, and de-stained according to protocol used for the transwell migration assay. Cells adhered to the bottom of the wells were counted.

Ear mesenchymal stem cells (eMSC) culture

eMSCs were isolated from the ears of 3–10 wks old age-matched female *+/+* and *m/m* mice using previously established protocols [30, 31]. Briefly, the external ears were removed from 2–4 mice per genotype and digested in 2 mg/mL collagenase I (LS004196, Worthington Biochemical Corporation, Lakewood, NJ) in HBSS for 1 hr at 37°C while shaking. The digested tissue was filtered using a 70 μ m cell strainer. Red blood cells were lysed and the remaining cells were plated at 2 ears per well (6-well plate) in base media containing DMEM/F-12 (11330, ThermoFisher Scientific), 15% fetal bovine serum (97068-085, VWR Life Science Seradigm, Radnor, PA), and 100 μ g/mL primocin (ant-pm-1, InvivoGen, San Diego, CA). Cell culture media was refreshed every 2–3 days, and cells were passaged at 80% confluence.

Adipogenic differentiation of eMSCs and Oil Red O staining

eMSCs were differentiated into adipocytes using previous established protocols [30, 31]. Briefly, cells were plated at 200,000 cells/well of a 6-well plate. At 100% confluence, adipogenesis was initiated with the addition of 62.5 mM IBMX (I7018), 10 mg/mL insulin (I6634), and 1 mM dexamethasone (D4902) to the base media. All adipogenesis reagents were purchased from Sigma-Aldrich (St. Louis, MO) except rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). At day 2 and 5 of adipogenic differentiation, cells were cultured in base media supplemented with 20 mM rosiglitazone (71740) and 2 mM insulin. Quantitative Oil Red O staining was used to assess lipid accumulation. Additionally,

cells were de-stained with isopropanol and the absorbance was measured at 490 nm with a standard curve using a MRX^{TC} Revelation by Dynex Technologies.

Proliferation of eMSC using BrdU incorporation

eMSCs were plated at 50,000 cells/well on glass cover slips in a 12 well plate in base media, incubating overnight at 37°C, 5% CO₂. The next day, media was changed and replaced with base media containing 30 µg/mL BrdU (B5002, Sigma-Aldrich, St. Louis, MO). Plates were incubated at 37°C, 5% CO₂ for 45 minutes. Cells were then washed and fixed with 100% EtOH and permeabilized with 0.1% Triton in PBS for 5 min. DNA was denatured by treating cells with 1N HCl for 30 min at 55°C. Cover slips were blocked with 0.1% Triton and 2% BSA in PBS and stained for immunofluorescence with an anti-BrdU-Alexa488 antibody 1:200 (FCMAB101A4, Millipore, Billerica, MA) and DAPI prolong antifade (P363966, ThermoFisher Scientific, Waltham MA) for total nuclei. To assess proliferation, cells were counted on 3 cover slips and 4 images/cover slip. The ratio of proliferative (BrdU and DAPI positive) and non-proliferative cells (BrdU negative, DAPI positive) per total cell number was calculated.

Statistical Analyses

All data are expressed as the mean $\pm$ standard error of the mean (SEM) unless otherwise noted. Results were analyzed for statistically differences using Student's *t*-test or ANOVA followed by Bonferroni's multiple comparison *post hoc* test where appropriate. All statistics were performed with Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

Results

m/m show loss of fat mass and low bone mineral density with age

To examine the effect of aging on the bone phenotype of female *m/m*, *m/m* and the *+/+* controls were aged to 78 wks and their body composition parameters were assessed by DXA analysis. The body weight of *m/m* was similar to *+/+* through 52 wks of age, but decreased significantly at 78 wks. This decrease in body weight observed between 52 and 78 wks of age was primarily due to loss of fat mass since the *+/+* cohort gained 60% fat mass and the *m/m* cohort lost 11% fat mass during this time. Lower fat-free mass in the *m/m* was previously reported, but remained constant with age [4]. Lower total and femoral aBMD and aBMC were observed at 78 wks of age in the *m/m* associated with a marked reduction in fat mass (Figure 1).

Very low trabecular bone mass is characteristic of *m/m*

In order to further elucidate the effect of aging on the bone phenotype of *m/m*, femurs and L5 vertebrae were collected at 16, 32, 52, and 78 wks of age and assessed by micro-computed tomography (μ CT). The *m/m* had reduced femur length and significantly lower trabecular bone volume fraction (BV/TV), trabecular number (Tb.N.), and connectivity density (Conn.D) at 16 through 52 wks in the distal femur. Consistent with this, trabecular separation (Tb.Sp.) increased significantly with age, but no difference in trabecular thickness (Tb.Th.) was observed (Figure 2, Table S2). No differences in Tb.BV/TV and Conn.D. were

observed between *m/m* and *+/+* at 78 wks of age due to the comprehensive loss of trabecular bone (Figure 2B, Table S2). Total relative trabecular bone loss from 16 to 78 wks was similar in *+/+* and *m/m* (80% vs 83% respectively), when compared to genotype-matched 16 wk controls. However, *+/+* did display a slightly higher rate of bone loss in femur when compared to *m/m* likely due to the higher starting Tb.BV/TV at 16 wks of age (Table S2, S4).

Similar to the microarchitecture of the distal femoral metaphysis, the microarchitecture of the L5 vertebrae showed a significant decrease in Tb.BV/TV, Tb.N., and Conn.D. with a significant increase in both Tb.Sp. and SMI score in *m/m* when compared *+/+*. This difference was present at 16 wks of age and persisted throughout aging resulting in significantly lower Tb.BV/TV and Tb.N. at 78 wks of age (Figure 3, Table S3). While relative vertebral Tb.BV/TV declined 36% from 16 to 78 wks in *m/m* and only 17% in the *+/+* when compared to the 16 wks old genotype matched control, no difference in the actual rate of trabecular bone loss (BV/TV) was observed in the vertebrae between genotypes (Table S4).

***m/m* exhibit reduced cortical expansion**

Cortical thickness (Ct.Th.) was significantly reduced in the femoral mid-shaft of *m/m* from 16 through 78 wks of age when compared to *+/+* (Figure 4A). While periosteal (Ps.) and endocortical (Ec.) circumferences were higher in *m/m* at 16 wks of age, expansion of both surfaces was minimal with age (Figure 4, Table S5, S6). By 78 wks of age, *+/+* exhibited a 16% increase in periosteal circumference and 23% increase in endosteal circumference over the 16 wks old *+/+*. However, *m/m* showed only a 5% and 7% increase in periosteal and endosteal circumferences, respectively, demonstrating a statistical reduction in cortical expansion (Figure 4B, C, Table S5). The lack of expansion on both the Ps. and Ec. surfaces resulted in no net change in cortical thickness by 78 wks of age. These data strongly suggest that *m/m* display defects in cortical expansion which could impact skeletal strength.

Osteoblast number is reduced and marrow adiposity is increased in the *m/m*

In order to determine how age affected bone cell activities in *m/m*, we performed static and dynamic histomorphometry on the proximal tibia in mice at 52 wks of age. Histomorphometric analysis was performed at 52 wks rather than 78 wks of age because little trabecular bone was evident on μ CT by 78 wks of age. Histomorphometric analysis showed BV/TV was reduced in *m/m*, similar to analysis of the microarchitecture by μ CT (Table 1). Analysis of the cellular parameters revealed that osteoblasts number (N.Ob/B.Pm) was undetectable in *m/m* while no change in osteoclast number or surface was detected. Statistical changes in Ps. and Ec. mineral apposition rates (MAR) were not observed; although both MAR values showed a decreasing trend (Table 1). No change in either serum PINP and CTx levels were detected at either 16 or 52 wks between *+/+* and *m/m* (Figure 5). Furthermore, serum RANKL levels also remained unchanged providing further evidence and rationale for the lack of alternation in osteoclast numbers with age (Figure 5).

In terms of adipocytes, marrow adipocyte number (N.Ad/T.Ar) and marrow adipocyte volume (Ad.V/T.Ar) were increased over 3-fold in *m/m* compared to *+/+* (Table 1).

Representative images for dynamic labeling and marrow adipocyte quantification are shown in Figure S1. Furthermore, H&E staining of the *m/m* femurs showed a dramatic accumulation of adipocytes in the marrow space when compared to *+/+* indicating that *m/m* mutation leads to enhanced in age-related marrow adiposity (Figure 6).

Reduced osteoblast gene expression and dysfunctional osteoblast motility point to a role for osteoblasts in the sustained low bone mass phenotype associated with aging in *m/m*

In order to gain some insight into the mechanism by which bone mass is reduced in *m/m*, expression of osteoblast, osteoclast, and inflammatory genes were analyzed in whole bone isolated from *+/+* and *m/m* at 16 and 52 wks of age. Not surprisingly, osteoblast gene expression was reduced in both 16 and 52 wk old *m/m* consistent with reduced osteoblast number observed by this study and previous studies in younger *m/m* (Figure 7A) [4]. *Trap5b* and *NfκB* expression were also significantly reduced at 52 wks (Figure 7B). Both *Rankl* and *Opg* expression were lower in *m/m* at 52 wks, with *Opg* expression changes more pronounced than *Rankl*. Despite this, serum RANKL levels remained unaffected (Figure 5), and no change in osteoclast number per unit of bone surface was observed by histomorphometry (Table 1). Local inflammation was assessed with *Tnf* and *Il6* expression and no changes were present in whole bone suggesting that the low bone mass phenotype was not a result of an age-associated inflammatory phenotype in the bone environment (Figure 7C). Taken together, these data suggest changes in osteoblast number are responsible, in part, for the reduced peak bone mass and reduced cortical expansion with aging in *m/m*.

A reduction in osteogenic differentiation has previously been established in *m/m* highlighting the potential role of *Dock7* in controlling bone formation directly in the osteoblast. We further investigated the cell autonomous role of *Dock7* in bone marrow stromal cells (BMSCs). To determine if osteoblast number in *m/m* were reduced by increased senescence in bone cells, expression of senescence markers *p16* and *p21* were analyzed in whole bone. While no change in senescence markers was present in whole bone at 16 wks of age, a reduction in *p21* expression was observed in *m/m* compared to *+/+* at 52 wks of age (Figure 8A). In contrast, BMSCs from 8 wks old *m/m* demonstrated a modest increase in *p16* indicating that loss of *Dock7* in *m/m* may increase senescence specifically in the BMSC population (Figure 8B). However, the heterogeneity of cells in the whole bone RNA may be masking *p16* changes observed in BMSCs.

As osteoblast numbers are reduced, we investigated osteoblast function, specifically migration and adhesion of BMSCs. Cell migration onto a collagen I matrix was decreased in BMSCs isolated from *m/m* compared to control *+/+* in a transwell assay (Figure 9A). Conversely, cell adhesion on to a collagen I matrix was increased and may provide some rationale for the decreased migration (Figure 9B). In combination with previous data, these results suggest a reduction of osteogenic differentiation as well as provide additional evidence that the low bone mass in *m/m* may not only be influenced by reduced osteoblast numbers, but also by dysfunction in the osteoblasts themselves.

Potential role for adipose tissue and the SNS in regulating bone metabolism

Previous studies have indicated that *m/m* have decreased innervation of BAT and reduced interscapular temperature and UCP-1 staining in BAT indicating BAT dysfunction. These data would suggest a potential upregulation of SNS activity in order to compensate for reduced BAT activity. As increased SNS activity is one marker of aging and has been linked to bone loss, we investigated BAT innervation in the aged *m/m*. At 52 wks of age, no changes in innervation of *m/m* BAT were present as measured by tyrosine hydroxylase (TH) staining (Figure 10A). Furthermore, no changes in UCP-1 protein levels were observed in *m/m* BAT at 52 wks (Figure 10B). These data suggest that BAT dysfunction may play a larger role in modulating bone metabolism in *m/m* prior to 16 wks of age, a time when BAT is thought to be most active.

Finally, the role of marrow adipose tissue on bone metabolism is not completely understood. We have demonstrated that *m/m* has increased marrow adipose tissue at 52 wks, but it is unclear if this is a direct result of loss of *Dock7* in adipocyte precursors. Ear mesenchymal stem cells (eMSCs) were used as a robust primary cell culture model to study adipogenic differentiation with the capacity to turn into adipocytes or osteoblast-like cells [30, 31]. eMSCs isolated from *m/m* demonstrated increased Oil Red O staining in adipogenic differentiation media. Proliferation was also decreased in *m/m* eMSCs. In combination with the decreased osteogenic differentiation previously observed and the increased marrow adipogenesis in *m/m*, these data suggest that loss of *Dock7* in *m/m* may divert progenitors from the osteoblastic lineage toward the adipogenic lineage (Figure 10C).

Discussion

In this study, we found that the *m/m* mouse model, with a spontaneous mutation in the *Dock7* gene, exhibits a low bone mineral density phenotype that is maintained throughout skeletal aging [4]. For example, by 52 weeks of age, there is almost a complete absence of femoral trabeculae. Cortical expansion was also significantly compromised on both periosteal and endocortical surfaces in *m/m* leading to a marked reduction in appositional growth. Furthermore, there was a striking increase in marrow adiposity in the *m/m* and a marked decrease in osteoblast number suggesting a possible shift in lineage toward adipogenesis. This tenet is further reinforced by previous studies from our group showing that calvarial osteoblasts from neonatal *m/m* mice differentiated at a much slower rate than *+/+* [4].

Trabecular bone is integral for the mechanical integrity of bone in humans [32]. Analysis of bone mineral density in previous studies shows that *m/m* have statistically lower aBMD and aBMC as early as 4 wks of age by DXA analysis and decreased trabecular number as early as 8 wks of age measured by μ CT analysis [4]. These studies revealed that peak trabecular and cortical bone acquisition is compromised with loss of *Dock7* in these mice. Aging of these mice now highlights that the low bone mineral density and low trabecular bone phenotype persist through the 78 weeks without any sign of compensation. The almost complete absence of trabecular bone seen in *m/m* starting at 52 wks age is due to fewer trabeculae, greater trabecular separation, and reduced connectivity density. However, the rate of trabecular bone loss in *m/m* remains unchanged in vertebrae and even slightly lower in

m/m femur compared to *+/+*. As there is no acceleration of trabecular bone loss with age, these studies suggest that low trabecular bone mass associated with loss of *Dock7* in *m/m* primarily occurs during bone acquisition or with bone loss prior to 16 wks of age.

In contrast to trabecular bone, cortical bone provides much of the structural support for bone and expansion of the cortex. Appositional growth primarily involves mineralization on the periosteal surface by osteoblasts and resorption on the endosteal surface by osteoclasts [33, 34]. Interestingly, younger *m/m* showed greater periosteal and endosteal circumferences when compared to *+/+* resulting in a larger bone medullary area at an early age. This undoubtedly resulted from the lower trabecular bone volume in *m/m*. On the other hand, *m/m* mice had minimal expansion of periosteal and endocortical envelopes with age. These data may reflect defects in either bone formation on the periosteal or endocortical surface and/or reduced resorption on the endosteal surface. In respect to the former, osteoblast number was negligible in the aged *m/m* mice by histomorphometry at 52 wks with no significant change in osteoclast number. These data suggest there is an important and persistent cell autonomous defect from absence of *Dock7* in the endosteal osteoblast and possibly in the periosteal cells as well. Relative to changes in resorption, the number of osteoclasts did not differ between *m/m* and *+/+* mice at 52 wks. However, it is worth noting that *Rankl*, *Trap5*, and *Nfkb* mRNA were significantly reduced in the aged but not the young *m/m* mice when compared to age-matched *+/+*, suggesting there may well be a modest reduction in endosteal resorption with age in the *m/m* vs. *+/+*. However, the reduction in *Trap5* and *Nfkb* gene expression, with no changes in osteoclast number per unit bone surface, may in part be due to the reduced trabecular bone surface present in the *m/m*. Furthermore, the absence of increased osteoclastogenesis in the presence of suppressed *Opg* suggests a functional uncoupling of osteoblast-dependent osteoclast recruitment.

During human skeletal aging, resorption on the endosteal surface is compensated for through enhanced periosteal apposition to preserve bone strength [35–38]. Indeed, with estrogen deprivation after menopause, the marrow cavity expands and there is an increase in the periosteal circumference. However, some investigators believe that periosteal expansion is hampered in postmenopausal women with osteoporosis [33]. In the *m/m*, there is minimal periosteal expansion with age compared to *+/+*. The absence of age-related periosteal apposition may compromise skeletal integrity in the mutant mice. But it should be noted that the perimeter of the endosteal surface minimally expanded during aging in *m/m*. Hence it is conceivable that the reduction of periosteal expansion relates to a defect on the endocortical surface, either in the osteoblast, or possibly in the osteoclast signaling to the osteoblast. Either way, this model provides a unique opportunity for understanding age-related signaling between bone compartments.

Rac1 activity is important for normal bone development as well as adhesion, proliferation, and differentiation of osteoblasts in addition to controlling osteoclast number [39, 40]. Therefore, loss of *Dock7*, a modulator of Rac1 and Cdc42 [8, 9, 14, 41], may in fact be altering osteoblast and osteoclast differentiation and function. Previous studies have already suggested a cell autonomous role for *Dock7* directly in controlling osteoblast differentiation and with an overall reduction of differentiation of calvarial osteoblasts *in vitro* [4]. Therefore, it follows that absence of *Dock7* may affect the differentiation and proliferation

of periosteal osteoblasts, as well as endocortical osteoclasts, which could result in the reduction of cortical expansion.

The SNS has emerged as a major regulator of bone metabolism with activation of the β 2AR on osteoblasts as a primary SNS target leading to an increase in *Rankl* expression and increased osteoclastogenesis/bone resorption causing lower bone mass [20, 22, 42]. Increases in SNS activity with aging have been linked to age-related bone loss. Although, we did not find an increase in resorption at 52 wks dynamically or by bone turnover markers/gene expression in our study, it remains unclear whether higher SNS tone is responsible for the non-cell autonomous effects on bone with age in *m/m* [43]. In fact, *Dock7* has been studied for its role in brain and nervous system [8, 9, 14, 18, 19, 44, 45], but it remains unclear if any of these alterations in the nervous system may affect bone development or remodeling. Propranolol treatment, a β 2AR antagonist, has been shown to partially rescue *m/m* bone phenotype in young mice, suggesting that the SNS may indeed be contributing to bone loss in the *m/m* [4]. However, we found no change in innervation of BAT tissue or UCP-1 protein levels in BAT in aged mice, suggesting that the role of the SNS in bone metabolism in *m/m* may be limited to the time of bone development and early age-related trabecular bone loss.

While the *m/m* clearly demonstrated low trabecular bone mass and reduced periosteal expansion, there are some limitations with this global *Dock7* deletion model. The skeletal phenotype of *m/m* is likely due to both a cell autonomous role for *Dock7* in bone lineage cells as well as non-cell autonomous effects of *Dock7* in other organ systems such as the SNS [4]. We currently cannot delineate which mechanism is dominant in controlling age-related bone loss in the aged *m/m*. However, we have developed a conditional deletion of *Dock7* using CRISPR/Cas9 technology and are currently working toward selectively deleting *Dock7* in osteoblasts, osteoclasts, and nervous system [46]. Additionally, we cannot be sure without mechanical testing whether the changes in periosteal apposition translate into a weaker bone. However, we did observe a substantial decrease in femoral moment of inertia at 78 wks of age, indicating *m/m* femur bones would likely have reduced load to fracture.

In conclusion, we found that the aged *m/m* mouse model had many of the same characteristics of the aging osteoporotic skeleton in humans. These hallmarks include low bone mineral density, reduced trabecular bone mass, decreased osteoblast number, and increased marrow adiposity. While trabecular bone loss was not accelerated with aging, absence of *Dock7* resulted in the novel findings of impaired cortical expansion in the *m/m*, suggesting a critical role for *Dock7* in appositional bone growth and possibly the function of periosteal osteoblasts. Since the *m/m* model displayed a low bone mass phenotype that is maintained during the aging process, this spontaneous mutant may become a useful model for further aging studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Loss of *Dock7* in the *Misty* mice results in low bone mineral density. During the aging process, *Misty* mice show a similar rate of trabecular bone loss and reduced cortical expansion. Reduced cortical expansion may result from decreased osteoblast number and defects in osteoblast function. These data highlight the *Misty* mice as a model to study apposition bone growth and communication between the bone compartments.

Figure 1. *m/m* show severe age-related bone loss

Female *m/m* (open squares) and *+/+* (closed circles) were weighed and screened using Lunar PIXImus Densitometer for body composition including (A) body mass, (B) fat-free mass, (C) fat mass, (D) total aBMD, (E) total aBMC, (F) femoral aBMD, and (G) femoral aBMC. Points represent mean $\pm$ SEM of $n=13-15$ per group. 2-way ANOVA: $P<0.0001$ for genotype in all measurements except fat mass (not significant). *Post hoc*: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to *+/+*.

Figure 2. +/+ and *m/m* have similar trabecular bone loss with age

(A) Representative μ CT images of distal femur trabecular bone, and (B) femoral microarchitecture examined in +/+ and *m/m* from 16 to 78 wks of age. Trabecular microarchitecture was measured by μ CT and femur length, trabecular bone volume/total volume (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp.), connectivity density (Conn.D.), and structural modeling index (SMI) were calculated. Closed circles (+/+) and open squares (*m/m*) represent mean $\pm$ SEM of $n=9-10$ per group. 2-way ANOVA: $P<0.0001$ for genotype in all measurements except Tb.Th. and SMI (not significant). *Post hoc*: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to +/+.

Figure 3. +/+ and m/m have similar trabecular bone loss in vertebrae with age

(A) Representative images of L5 vertebrae, and (B) vertebral microarchitecture examined in +/+ and m/m from 16 to 78 wks of age. Trabecular microarchitecture was measured by μ CT and bone volume/total volume (BV/TV), trabecular number (Tb.N.), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp.), connectivity density (Conn.D.), and structural modeling index (SMI) were calculated. Closed circles (+/+) and open squares (m/m) represent mean $\pm$ SEM of n=8–10 per group. 2-way ANOVA: $P < 0.0001$ for genotype in all measurements except Tb.Th. (not significant). *Post hoc*: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to +/+.

Figure 4. *m/m* have decreased cortical expansion

(A) Cortical thickness, (B) periosteal (Ps) circumference, and (C) endosteal (Ec) circumference were measured by μ CT. Closed circles (+/+) and open squares (*m/m*) represent mean $\pm$ SEM of $n=9-10$ per group. 2-way ANOVA: $P<0.0001$ for genotype in all measurements. *Post hoc*: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to +/+.

Figure 5. Serum markers of bone formation and bone resorption were unchanged in m/m
P1NP, CTx, and RANKL protein levels were measured by enzyme immunoassay in $+/+$ and m/m at 16 and 52 wks of age (n=10). 2-way ANOVA: All measures had non-significant P values for genotype. *Post hoc*: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to $+/+$.

Figure 6. *m/m* have extreme age-related increase in marrow adiposity

Femurs were isolated from 16, 32, 52, and 78 wks, decalcified, paraffin embedded, and stained with H & E. Images show accumulation of white marrow adipocyte ghosts in the distal femur epiphysis.

Figure 7. Decreased osteoblast gene expression persists in the aged *m/m*

Gene expression of whole tibia was analyzed by qRT-PCR for expression of (A) osteoblast genes, (B) osteoclast genes, and (C) inflammatory cytokines. $n=8-10$ per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to *+/+*.

Figure 8. Gene expression of senescence markers is modestly altered in m/m

Gene expression of *p16* and *p21* senescence markers was analyzed by qRT-PCR in (A) whole tibia of 16 and 52 wks old mice and (B) BMSCs from $+/+$ and m/m mice. Values represent mean $\pm$ SEM of $n=6-10$ per group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to $+/+$.

Figure 9. BMSCs motility is impaired in BMSCs on a Collagen I matrix

(A) BMSC migration was assessed in a transwell assay on membranes coated with collagen I. (B) Adhesion of BMSCs was analyzed in collagen I coated wells. The number of cells on the membrane (A) or well (B) were quantified. Closed circles (+/+) and open squares (m/m) represent mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to +/+.

Figure 10. Adipogenic differentiation is increased in *m/m*

(A) Tyrosine hydroxylase (TH) positive sympathetic nerve fibers were quantified in histological BAT sections from 52 wks old *+/+* and *m/m*. Representative images of staining are shown on right. Black arrows mark examples of TH positive nerve fibers. (B) UCP-1 protein expression was detected in histological BAT sections from 52 wks old *+/+* and *m/m* with an anti-UCP-1 antibody. (C) Adipogenic differentiation was analyzed in eMSC by Oil Red O staining after 7 days in adipogenic media. Proliferation was measured in undifferentiated eMSCs with BrdU staining.

Table 1
***m/m* have decreased bone volume and increased marrow adipocytes**

Trabecular and cortical bone were assessed by static and dynamic histomorphometry in the tibia at 52 wks of age. Trabecular bone was analyzed 200 μ m below the growth plate and cortical bone was analyzed 5 mm below the tibia-fibula junction.

Parameters	+/-	n	m/m	n	p value (t test)
BV/TV (%)	0.78 $\pm$ 0.96	9	0.12 $\pm$ 0.28	10	0.05
Ad.V/T.Ar (%)	5.12 $\pm$ 3.59	9	19.6 $\pm$ 15.5 [*]	10	0.01
N.Ad/T.Ar (/mm ²)	67.7 $\pm$ 51.4	9	234 $\pm$ 169 [*]	10	0.01
Oc.S/B.Pm (%)	9.94 $\pm$ 6.55	8 ^a	10.5 $\pm$ 12.1	4 ^a	0.92
N.Oc/B.Pm (/mm)	3.45 $\pm$ 2.63	8 ^a	4.20 $\pm$ 4.86	4 ^a	0.73
Ob.S/B.Pm (%)	0.87 $\pm$ 1.79	8 ^a	undetected	4 ^a	N.A.
N.Ob/B.Pm (/mm)	0.96 $\pm$ 2.04	8 ^a	undetected	4 ^a	N.A.
Ps.MAR (μ m/day)	0.63 $\pm$ 0.31	8 ^a	0.49 $\pm$ 0.16	3 ^a	0.46
Ec.MAR (μ m/day)	1.21 $\pm$ 0.43	9	0.99 $\pm$ 0.22	9 ^a	0.18

BV/TV = bone volume/total volume; Ad.V/T.Ar = adipocyte volume/total area; N.Ad/T.Ar = number of adipocytes/total area; Oc.S/B.Pm = osteoclast surface/bone perimeter; N.Oc/B.Pm = number of osteoclast/bone perimeter; Ob.S/B.Pm = osteoblast surface/bone perimeter; N.Ob/B.Pm = number of osteoblast/bone perimeter; Ps.MAR = periosteal mineral apposition rate; Ec.MAR = endocortical mineral apposition rate.

^{*} p<0.05 by Student's *t*-test compared to +/-.

^a Sample numbers were reduced as not all sections displayed trabecular bone or labels.