The lateral meningocele syndrome mutation causes marked osteopenia in mice

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Lateral meningocele syndrome (LMS) is a rare genetic disorder characterized by neurological complications and osteoporosis. LMS is associated with mutations in exon 33 of NOTCH3 leading to a truncated protein lacking sequences for NOTCH3 degradation and presumably causing NOTCH3 gain of function. To create a mouse model reproducing human LMS-associated mutations, we utilized CRISPR/Cas9 to introduce a tandem termination codon at bases 6691–6696 (ACCAAG→TAATGA) and verified this mutation (Notch3tm1.1Ecan) by DNA sequencing of F1 mice. One-month-old male and female heterozygous Notch3tm1.1Ecan mice had cancellous and cortical bone osteopenia but exhibited no obvious neurological alterations, and histopathology of multiple organs revealed no abnormalities. Microcomputed tomography of these mutants revealed a 35–60% decrease in cancellous bone volume associated with a reduction in trabecular number and decreased connectivity. During maturation, cancellous and cortical bones were restored in female but not in male mice, which exhibited cancellous bone osteopenia at 4 months. Cancellous bone histomorphometry revealed increased osteoblast and osteocyte numbers and a modest increase in osteoclast surface and bone formation rate. Notch3tm1.1Ecan calvarial osteoblasts had increased proliferation and increased bone γ-carboxyglutamate protein (Bglap) and TNF superfamily member 11 (Tnfsf11) mRNA levels and lower Tnfsf11b levels. Tnfsf11 mRNA was increased in osteocyte-rich femora from Notch3tm1.1Ecan mice. Cultures of bone marrow–derived macrophages from Notch3tm1.1Ecan mice revealed increased osteoclast formation, particularly in cocultures with osteoblasts from Notch3tm1.1Ecan mice. In conclusion, the Notch3tm1.1Ecan mutation causes osteopenia despite an increase in osteoblast proliferation and function and is associated with enhanced Tnfsf11 expression in osteoblasts and osteocytes.

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3 The abbreviations used are: NICD, Notch intracellular domain; α-MEM, α-minimum essential medium; BMM, bone marrow–derived macrophage; FBS, fetal bovine serum; Hes, hairy and enhancer of split; Hey, HES-related with YRPW motif; LMS, lateral meningocele syndrome; M-CSF, macrophage colony-stimulating factor; μCt, microcomputed tomography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PTH, parathyroid hormone; qRT-PCR, quantitative RT-PCR; RANKL, receptor activator of NF-κB ligand; RBP/Jκ, recombination signal–binding protein for Ig of κ region; sg, single guide; TRAP, tartrate-resistant acid phosphatase; Tnfsf11, tumor necrosis factor superfamily member 11; Tnfsf11b, tumor necrosis factor receptor superfamily 11b; Bglap, γ-carboxyglutamate protein; sg, single guide; ANOVA, analysis of variance.

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etic disorder that presents with neuromuscular dysfunction, meningocles, and distinct facial features (28). The clinical features of LMS include developmental delay, decreased muscle mass, and cardiac valve defects. Skeletal abnormalities are frequent, including thickening of the calvarial vault, craniofacial defects, short stature, scoliosis, and bone loss (29, 30). Exome-wide sequencing of individuals affected by LMS demonstrated the presence of mutations in exon 33 of NOTCH3. These mutations create a stop codon so that the PEST domain is not translated (31). Because the PEST domain is necessary for the ubiquitination and degradation of NOTCH3, the NOTCH3 NICD is presumably stable, resulting in persistent signaling and gain of function.

Despite the skeletal manifestations reported in LMS, there is limited knowledge about the mechanisms that operate or the actions of NOTCH3 in the skeleton. The purpose of the present work was to provide an understanding of the skeletal manifestations of LMS and responsible mechanisms. For this purpose, a mouse (Notch3tm1.1Ecan) model reproducing the NOTCH3 mutation harbored by subjects affected by LMS was engineered. To create an LMS mutant mouse, we introduced a tandem termination codon, ACCAAG→TAATGA, into exon 33 of the Notch3 gene at bp 6691–6696 from the start of translation. In the present work, we report the skeletal phenotype and underlying mechanisms of Notch3tm1.1Ecan mice.

Results

Generation and general appearance of Notch3tm1.1Ecan mutant mice

The RNA-guided clustered regularly interspaced short palindromic repeat (CRISPR)-associated Cas9 nuclease system was used to target exon 33 of Notch3 and introduce a TAATGA tandem termination codon at positions 6691–6696 from the start of translation (32–34). This genetic intervention leads to the translation of the truncated NOTCH3 protein T2231X consisting of 2230 amino acids (versus 2318 in WT). Single guide (sg) RNA, designed to cleave between nucleotides 6691 and 6692 of Notch3, and a single-strand DNA containing the tandem stop codon TAATGA were coinjected into C57BL/6J one-cell embryos with Cas9 mRNA (Fig. 1). The proper insertion of the Notch3 6691–6696 ACCAAG→TAATGA mutation was confirmed by DNA sequencing of F1 mice (Fig. 1). Heterozygous mutant mice were compared with sex-matched WT littermates in a C57BL/6J genetic background. Breeding between heterozygous LMS mutant and WT mice yielded offspring composed of 55% WT and 45% heterozygous Notch3tm1.1Ecan mice. Notch3tm1.1Ecan mice appeared healthy, did not exhibit overt neuromuscular signs, and were fertile. Percent body fat, fasting serum insulin and glucose levels, and serum 17β-estradiol and parathyroid hormone (PTH) levels were not different between Notch3tm1.1Ecan and controls (Table 1). One-month-old heterozygous Notch3tm1.1Ecan mice did not appear different from controls and had a weight that was ~10% lower than controls. However, the difference in weight was less pronounced as the mice matured, and at 4 months of age Notch3tm1.1Ecan mice weighed ~5% less than control littermates. Femoral length was slightly shorter in Notch3tm1.1Ecan mice at 1 month but not at 4 months of age when femoral length was not different from controls (Fig. S1).

Femoral microarchitecture of Notch3tm1.1Ecan mutant mice

Microcomputed tomography (μCT) of the distal femur demonstrated that 1-month-old male and female Notch3tm1.1Ecan mutant mice had a 35–60% decrease in cancellous bone volume (Table 2 and Figs. 2 and 3). The decrease in cancellous bone volume was associated with decreased trabecular number and connectivity and higher structure model index, indicating a predominance of rodlike trabeculae. Trabecular thickness was not affected. Cortical bone was thin and porous in 1-month-old Notch3tm1.1Ecan mutant mice of both sexes, although total bone area was not different from controls, indicating normal bone size (Table 2 and Figs. 2 and 3). The decrease in trabecular bone volume was sustained and observed in 4-month-old male, but not female, Notch3tm1.1Ecan mutant mice. Male mice had a significant decrease in cancellous bone volume of ~30% associated with decreased trabecular number and connectivity. However, cortical bone was not altered in Notch3tm1.1Ecan mice of either sex (Table 2).

Femoral histomorphometry of Notch3tm1.1Ecan mutant mice

In accordance with the μCT findings, cancellous bone histomorphometry of femora from Notch3tm1.1Ecan mice of both sexes revealed decreased bone volume/tissue volume and trabecular number (Table 3 and Fig. 3). There was an ~2-fold increase in the number of osteoblasts and in osteoblast surface/bone surface in Notch3tm1.1Ecan mutant mice of both sexes as well as a 13–33% increase in osteocyte cell density. The higher osteoblast number was associated with an increase in mineral apposition rate in male Notch3tm1.1Ecan mutant mice and a nonsignificant (1.8–2.1-fold) increase in bone formation rate. Osteoclast surface/bone surface was increased in male Notch3tm1.1Ecan mutant mice, but osteoclast number/bone perimeter was not affected significantly in either sex. At 4 months of age, the cellular phenotype in male mutant mice was sustained, and they exhibited decreased bone volume/tissue volume associated with a 60% increase in osteoblast number and elevated bone formation rate when compared with WT sex-matched littermate mice (Table 4). However, neither osteoclast surface nor osteoclast number was significantly affected in Notch3tm1.1Ecan mice. These results suggest that Notch3tm1.1Ecan mice have a higher number of osteoblasts, possibly as a manifestation of high bone remodeling, but the cells are not sufficiently active to maintain skeletal homeostasis.

Gene expression in Notch3tm1.1Ecan mutant mice

To search for mechanisms that could operate in Notch3tm1.1Ecan mutants, tibiae were analyzed for changes in gene expression. Quantitative RT-PCR (qRT-PCR) revealed expression of Notch36691-TAATGA transcripts and increased Hey1, Hey2, and HeyL mRNA levels in tibiae from mutant mice, demonstrating that Notch signaling was activated in bone (Fig. 4). In agreement with the increase in osteoclast surface in mutant mice, the expression of Tnfsf11 mRNA, encoding for receptor activator
of NF-κB ligand (RANKL), was increased in femora from Notch3tm1.1Ecan mutant mice. A modest increase in Tnfrsf11b mRNA, encoding for osteoprotegerin, was noted.

Calvarial osteoblasts

To pursue the consequences of the Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mutation in skeletal cells, calvarial osteoblasts were cultured. Osteoblasts from Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mice, but not from controls, expressed Notch3<sup>6691-1AAATGA</sup> transcripts. Hey1 was transiently increased in Notch3<sup>3<sub>sm1.1Ecan</sub></sup> osteoblasts, whereas HeyL was increased for a 3-week culture period, documenting sustained activation of Notch signaling (Fig. 5). Osteoblast cultures from Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mice expressed higher levels of Bglap (encoding for osteocalcin) and Tnfsf11 mRNAs and lower levels of Tnfrsf11b mRNA than control cultures. To estimate the rate of cellular proliferation, the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to its formazan salt was measured during the initial phases of the culture. This “MTT assay” measures mitochondrial activity of cells and

Table 1

|                        | Males Control | Notch3<sup>3<sub>sm1.1Ecan</sub></sup> | Females Control | Notch3<sup>3<sub>sm1.1Ecan</sub></sup> |
|------------------------|---------------|----------------------------------------|-----------------|----------------------------------------|
| Body fat (%)           | 17.9 ± 0.8    | 17.6 ± 0.6                             | 20.0 ± 0.4      | 21.5 ± 0.7                             |
| Glucose (mg/dl)        | 88 ± 2        | 84 ± 1                                 | 91 ± 2          | 86 ± 2                                 |
| Insulin (µU/ml)        | 114 ± 7       | 132 ± 6                                | 120 ± 36        | 110 ± 20                               |
| PTH (pg/ml)            | 426 ± 65      | 322 ± 107                              | 325 ± 89        | 294 ± 82                               |
| 17βE2 (pg/ml)          | 37 ± 4        | 42 ± 8                                 |                 |                                        |

Figure 1. Domains of Notch3 and engineering of the Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mutant allele. A, domains of the Notch3 receptor depicting the 1) extracellular domain containing multiple epidermal growth factor (EGF)-like tandem repeats upstream of Lin12-Notch repeats (LNR); 2) heterodimerization domain (HD) that, in association with the Lin12-Notch repeats, forms the negative regulatory region; 3) transmembrane domain (TMD); and 4) NICD consisting of an RBPJκ-association module (RAM) linked to ankyrin (ANK) repeats and a nuclear localization sequence (NLS) upstream of a PEST domain. Under the NOTCH3 protein domains, the genomic structure of mutant exon 33 aligned with the corresponding protein structure is shown. Black bars represent exons (E) 29–32, the black box represents exon 33 containing the 6691–6696 ACCAAG→TAATGA mutation leading to a T2231X change at the amino acid level, and the white box represents the 3′-UTR. B, WT Notch3 exon 33 sequence with protospacer adjacent motif (PAM) for CRISPR/Cas9 targeting. C and D, genomic DNA from ear samples of F1 pups was used as a template for PCR, and products were sequenced by the Sanger method. Sequencing of the DNA fragment spanning the Notch3<sup>6691-1AAATGA</sup> mutation is shown in C. The 1:1 signal ratio for ACCAAG→TAATGA demonstrates heterozygosity for the mutation. The sequence of the mutant Notch3<sup>3<sub>sm1.1Ecan</sub></sup> allele is in D. E, sequence of donor oligonucleotide used to generate the Notch3<sup>6691-1AAATGA</sup> mutant, including a downstream c→g mutation to ensure the mutation was not a random event. * represents phosphorothioate linkage to protect from exonuclease degradation.

Table 1

Percent body fat and hormonal/metabolic parameters in 4-month-old Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mutant male and female mice and sex-matched control littersmates

Body fat was determined by total body densitometry, and glucose and hormone levels were measured in fasting serum samples from Notch3<sup>3<sub>sm1.1Ecan</sub></sup> mutant mice and sex-matched control littersmates. Values are means ± S.D.; n = 3–4. 17βE2, 17β-estradiol.
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As such is a proxy for cell proliferation. Osteoblasts from Notch3<sup>tm1.1Ecan</sup> mice proliferated at a rate 1.5 higher than that of cells from WT controls, an effect that is consistent with the increased number of osteoblasts in Notch3<sup>tm1.1Ecan</sup> mutants in vivo (Fig. 5). The results indicate enhanced osteoblast proliferation and differentiation in cells from Notch3<sup>tm1.1Ecan</sup> mutants as well as an increased ratio of RANKL to osteoprotegerin, providing a possible explanation for the findings in vivo.

Osteocyte-enriched cultures

Because a possible explanation for the Notch3<sup>tm1.1Ecan</sup> phenotype was an increase in osteoclastogenesis and because osteocytes play a major role in the control of bone resorption, we examined the expression of Tnfsf11 mRNA in enzymatically/EDTA-digested osteocyte-rich femora (14, 35). Osteocytes expressed the Notch3<sup>6691-TAATGA</sup> transcript and increased levels of Hey1, Hey2, and HeyL, indicating enhanced Notch signaling. Tnfsf11 mRNA was increased 1.6-fold in osteocytes from Notch3<sup>tm1.1Ecan</sup> mutant mice, whereas the expression of Tnfsf11b was not affected (Fig. 6).

In vitro osteoclast formation

To explore whether a direct effect on osteoclast differentiation was operational, bone marrow–derived macrophages (BMMs) were isolated from Notch3<sup>tm1.1Ecan</sup> mutants and littermate controls. BMMs were cultured in the presence of macrophage colony-stimulating factor (M-CSF) at 30 ng/ml for 3 days and subsequently in the presence of M-CSF (30 ng/ml) and RANKL at 10 ng/ml. The number of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells was increased in cultures from Notch3<sup>tm1.1Ecan</sup> mutants (Fig. 7). However, Notch3<sup>tm1.1Ecan</sup> mutant BMMs did not express Notch3<sup>6691-TAATGA</sup> transcripts, and neither mutant nor WT cells expressed detectable levels of Notch3 mRNA. The results indicate that even though Notch3<sup>tm1.1Ecan</sup> mutant cells exhibited enhanced osteoclast differentiation the cellular phenotype is likely secondary to events that occurred in vivo prior to cell isolation, such as exposure to increased levels of RANKL. To determine whether the osteoblast or osteoclast was responsible for the enhanced osteoclastogenesis, BMMs from control and Notch3<sup>tm1.1Ecan</sup> mice were cocultured with osteoblasts from the respective mouse lines. Osteoclast differentiation was increased in BMMs from Notch3<sup>tm1.1Ecan</sup> mutants whether they were cultured in the presence of WT or mutant osteoblasts. In addition, osteoblasts from Notch3<sup>tm1.1Ecan</sup> mice enhanced osteoclastogenesis in both WT and Notch3<sup>tm1.1Ecan</sup> mutant BMMs. The results demonstrate both a BMM- and an osteoblast-dependent effect as responsible for the increased osteoclast differentiation in Notch3<sup>tm1.1Ecan</sup> mutants.

**Table 2**

Femoral microarchitecture assessed by μCT of 1- and 4-month-old Notch3<sup>tm1.1Ecan</sup> mutant male and female sex-matched mice and control littermates

μCT was performed in distal femora for trabecular bone and midshaft for cortical bone from 1- and 4-month-old male and female Notch3<sup>tm1.1Ecan</sup> mutant mice and sex-matched control littermates. Values are means ± S.D., *significantly different between control and Notch3<sup>tm1.1Ecan</sup>, p < 0.02 by unpaired t test.

| Males | 1 Month | 2 Month | 4 Month |
|-------|---------|---------|---------|
| Control | Notch3<sup>tm1.1Ecan</sup> | Control | Notch3<sup>tm1.1Ecan</sup> |
| Bone Volume/Total Volume (%) | 7.4 ± 1.3 | 4.9 ± 1.3* | 6.6 ± 2.4 | 6.3 ± 2.7* |
| Trabecular Separation (μm) | 191 ± 16 | 244 ± 31* | 217 ± 9 | 283 ± 23* |
| Trabecular Number (1/mm) | 53 ± 0.5 | 42 ± 0.5* | 46 ± 0.2 | 3.6 ± 0.3* |
| Trabecular Thickness (μm) | 28 ± 2 | 26 ± 3 | 38 ± 6 | 41 ± 7 |
| Connectivity Density (1/mm<sup>3</sup>) | 362 ± 124 | 381 ± 108* | 170 ± 51 | 106 ± 59* |
| Structure Model Index | 2.7 ± 0.1 | 3.0 ± 0.2* | 2.4 ± 0.3 | 2.6 ± 0.3 |
| Density of Material (mg HA/cm<sup>3</sup>) | 796 ± 60 | 781 ± 31 | 940 ± 87 | 969 ± 67 |

| Females | 1 Month | 2 Month | 4 Month |
|---------|---------|---------|---------|
| Control | Notch3<sup>tm1.1Ecan</sup> | Control | Notch3<sup>tm1.1Ecan</sup> |
| Bone Volume/Total Volume (%) | 8.4 ± 1.5 | 81 ± 1.6* | 891 ± 0.7 | 880 ± 1.5 |
| Porosity (%) | 15.6 ± 1.5 | 18.7 ± 1.6* | 10.9 ± 0.7 | 12.0 ± 1.5 |
| Cortical Thickness (μm) | 103 ± 10 | 87 ± 6* | 167 ± 8 | 158 ± 13 |
| Total Area (mm<sup>2</sup>) | 1.7 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.3 | 1.7 ± 0.1 |
| Bone Area (mm<sup>2</sup>) | 0.57 ± 0.05 | 0.51 ± 0.02* | 0.84 ± 0.06 | 0.81 ± 0.08 |
| Periosteal Perimeter (mm) | 4.7 ± 0.1 | 4.7 ± 0.1 | 4.7 ± 0.4 | 4.7 ± 0.2 |
| Endocortical Perimeter (mm) | 3.8 ± 0.1 | 3.9 ± 0.1* | 3.4 ± 0.4 | 3.4 ± 0.2 |
| Density of Material (mg HA/cm<sup>3</sup>) | 994 ± 17 | 974 ± 13* | 1226 ± 33 | 1226 ± 32 |

Figure 2. Femoral architecture assessed by μCT of distal femora from 1-month-old male (A) and female (B) Notch3<sup>tm1.1Ecan</sup> mutant (black bars) and control littermates (white bars) mice. Data are expressed as cancellous bone volume/total volume (BV/TV), trabecular number (Tb.N), connectivity density (Conn.D), and cortical bone thickness (Ct.Th). Values are expressed as means ± S.D. (error bars); number of observations for Notch3<sup>tm1.1Ecan</sup> and control males, n = 7 each, and Notch3<sup>tm1.1Ecan</sup> and control females, n = 4 each. *, significantly different between Notch3<sup>tm1.1Ecan</sup> mutant and WT controls, p < 0.05 by unpaired t test.
The present work indicates that mice harboring a Notch3 gain-of-function mutation exhibit bone loss that affects both the cancellous and cortical compartments. The osteopenia was detected in 1-month-old mice of both sexes, although in mature animals the osteopenic phenotype affected the cancellous bone of male, but not female, mutant mice. The abatement of the osteopenia in mature female mice might have been related to a decrease in bone remodeling, secondary to an effect of estrogens on the skeleton. Estrogens decrease the lifespan of osteoclasts by promoting their apoptosis and suppress the secretion of the cytokine IL-7 by osteoblasts with a consequent decrease in RANKL expression and osteoclastogenesis (36–38). The phenotype of the Notch3\textsuperscript{tm1.1Ecan} mutant mouse is congruent with the bone loss observed in humans with LMS. However, there is no information on sex- or age-related changes in bone mass in subjects affected by LMS because the number of cases reported is small (29, 31). Notch3\textsuperscript{tm1.1Ecan} mutant mice did not have obvious neurological manifestations reported in humans afflicted by the disease. Moreover, histopathology of brain, lungs, heart, liver, spleen, and kidneys did not reveal any obvious abnormalities in Notch3\textsuperscript{tm1.1Ecan} mice.4 A limitation of the present work is that relatively young mice were studied, and the phenotype might evolve as the mice age.

Stabilization of NOTCH3 protein is probably responsible for the increased Notch3 activity. The mutation in Notch3\textsuperscript{tm1.1Ecan} mice was introduced upstream of the PEST domain, and this domain is essential for the ubiquitination of NOTCH3 (39). Similarly, mutations in exon 34 upstream of the PEST domain of Notch1 or Notch2 result in Notch gain of function and enhanced Notch signaling (40–42). The mechanism in LMS is analogous to that found in Hajdu–Cheney syndrome where affected individuals harbor mutations in exon 34 of NOTCH2 (43–45). The inheritance of LMS is not established, and autosomal-dominant inheritance as well as de novo heterozygous truncating mutations in exon 33 of NOTCH3 has been reported (29).

The osteopenia of Notch3\textsuperscript{tm1.1Ecan} mutants was associated with an increase in the number of osteoblasts secondary to enhanced cell replication. Although bone formation was increased, the enhanced osteoblast number and function were not sufficient to maintain skeletal homeostasis, so bone loss occurred. In vitro cultures of osteoblasts revealed increased Bglap expression in Notch3\textsuperscript{tm1.1Ecan} cells, indicating that osteoblast maturation was not impaired. There was a 45% increase in osteoclast surface/bone surface. This finding indicates that an increase in bone resorption contributed to the phenotype. This was secondary to an increased expression of RANKL and suppressed osteoprotegerin in cells of the osteoblast lineage. The capacity of osteoclast precursors to differentiate into mature osteoclasts in response to RANKL was enhanced in Notch3\textsuperscript{tm1.1Ecan} mutants, although cells of the myeloid lineage do not express either WT or mutant Notch3 transcripts. This suggests that the enhanced osteoclastogenesis in vitro reflected events occurring in vivo. Cocultures of BMMs with osteoblasts confirmed that the osteoblast of Notch3\textsuperscript{tm1.1Ecan} mutants contributed to the enhanced osteoclastogenesis, an effect congruent with the increased expression of RANKL by the osteoblast. There was a greater number of osteocytes in cancellous bone of Notch3\textsuperscript{tm1.1Ecan} mice, and this is probably secondary to the increased number of osteoblasts. Osteocytes are an important source of RANKL and likely contributed to the osteopenic phenotype observed (46–49).

The phenotype reported in Notch3\textsuperscript{tm1.1Ecan} mutant mice is different from that observed in Notch1 NICD–expressing mice or that reported in mice harboring a Notch2 gain-of-function mutation replicating Hajdu–Cheney syndrome (26). Notch1 activation in undifferentiated osteoblasts precludes their mat-

Figure 3. A, representative microcomputed tomography images of proximal trabecular bone and midshaft of femora showing cancellous bone osteopenia and decreased trabecular number and thinner cortical bone in Notch3\textsuperscript{tm1.1Ecan} mice. The complete data set is shown in Table 2. B, representative static cancellous bone histological sections stained with toluidine blue showing increased number of osteoblasts (arrows) (B, upper panels) and osteocytes (B, lower panels) in Notch3\textsuperscript{tm1.1Ecan} mice. C, calcein and demeclocycline labeling of cancellous bone showing a higher mineral apposition rate in Notch3\textsuperscript{tm1.1Ecan} mice compared with control. The complete data set is shown in Table 3. All representative images are from femora from 1-month-old male Notch3\textsuperscript{tm1.1Ecan} mutant and sex- and age-matched littermate WT controls. Scale bars, 50 μm.

Discussion

The present work indicates that mice harboring a Notch3 gain-of-function mutation exhibit bone loss that affects both the cancellous and cortical compartments. The osteopenia was detected in 1-month-old mice of both sexes, although in mature animals the osteopenic phenotype affected the cancellous bone of male, but not female, mutant mice. The abatement of the osteopenia in mature female mice might have been related to a decrease in bone remodeling, secondary to an effect of estrogens on the skeleton. Estrogens decrease the lifespan of osteoclasts by promoting their apoptosis and suppress the secretion of the cytokine IL-7 by osteoblasts with a consequent decrease in RANKL expression and osteoclastogenesis (36–38). The

4 E. Canalis, unpublished observations.
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Table 3
Cancellous bone histomorphometry of 1-month-old Notch3tm1.1Ecan mutant male and female mice and sex-matched control littermates

Histomorphometry was carried out on sagittal sections of distal femora from 1-month-old male and female Notch3tm1.1Ecan mutant mice and sex-matched control littermates. Values are means ± S.D.

| Static histomorphometry                | Males                             | Females                           |
|----------------------------------------|------------------------------------|-----------------------------------|
|                                        | Control (n = 6)                     | Notch3tm1.1Ecan (n = 6)           | Control (n = 7)                   | Notch3tm1.1Ecan (n = 7)               |
| Bone volume/tissue volume (%)          | 14.2 ± 3.1                         | 9.8 ± 2.6                         | 10.3 ± 1.5                       | 7.5 ± 2.3                             |
| Trabecular separation (μm)             | 226 ± 48                           | 307 ± 66                          | 268 ± 40                         | 427 ± 182                             |
| Trabecular number (1/mm)               | 3.9 ± 0.7                          | 3.0 ± 0.5                         | 3.4 ± 0.6                        | 2.4 ± 0.7                             |
| Trabecular thickness (μm)              | 36.0 ± 3.4                         | 32.1 ± 6.1                        | 30.4 ± 2.8                       | 31.0 ± 4.8                            |
| Osteoblast surface/bone surface (%)    | 10.5 ± 6.8                         | 25.4 ± 7.6                        | 12.1 ± 6.2                       | 21.6 ± 7.7                            |
| Osteoblasts/bone perimeter (1/mm)     | 10.3 ± 6.6                         | 25.4 ± 7.2                        | 11.9 ± 5.9                       | 20.3 ± 6.6                            |
| Osteoid surface/bone surface (%)      | 1.2 ± 1.4                          | 3.5 ± 2.4                         | 1.0 ± 0.9                        | 2.4 ± 1.6                             |
| Osteocytes (mm²)                       | 565 ± 46                           | 751 ± 151                         | 628 ± 29                         | 710 ± 84                              |
| Osteoclast surface/bone surface (%)   | 13.9 ± 5.9                         | 20.3 ± 4.2                        | 20.5 ± 3.3                       | 23.7 ± 4.1                            |
| Osteoclasts/bone perimeter (1/mm)     | 6.6 ± 1.4                          | 8.0 ± 1.7                         | 7.9 ± 1.1                        | 8.7 ± 1.9                             |
| Eroded surface/bone surface (%)       | 8.3 ± 5.8                          | 6.1 ± 2.3                         | 6.2 ± 2.4                        | 7.5 ± 2.2                             |

**Dynamic histomorphometry**

| Mineral apposition rate (μm/day)       | 2.3 ± 0.3                          | 4.0 ± 0.8                         | 2.9 ± 0.9                        | 3.9 ± 1.2                             |
| Mineralizing surface/bone surface (%) | 2.9 ± 0.7                          | 3.8 ± 2.5                         | 4.1 ± 2.0                        | 6.0 ± 3.5                             |
| Bone formation rate (μm³/μm²/day)     | 0.07 ± 0.02                        | 0.15 ± 0.09                       | 0.12 ± 0.06                     | 0.21 ± 0.09                           |

* Significantly different between control and Notch3tm1.1Ecan, p < 0.05 by unpaired t test.

Although the goal was to replicate LMS, a limitation of the work is the use of a global mutation, so systemic or indirect consequences of the mutant Notch3tm1.1Ecan in bone cannot be excluded. However, no obvious neurological alterations were observed in mutant mice, and histopathology of multiple organs revealed no abnormalities. Other limitations of the work relate to the use of the CRISPR/Cas9 approach to create the Notch3tm1.1Ecan mouse model because it could have resulted in off-target effects. However, most of the off-target effects reported with CRISPR/Cas9 technology have used human-induced pluripotent or embryonic stem cells transfected with Cas9 and sgRNA expression vectors, generating Cas9 and sgRNA in significant amounts for a prolonged period of time and therefore increasing the possibility of off-target effects. A limited amount of Cas9 mRNA and sgRNA was injected into one-cell embryos to create the Notch3tm1.1Ecan mutants described, decreasing the likelihood of off-target effects. Moreover, the sgRNA selected scored high by inverse likelihood of off-target binding, and we did not detect a change in phenotype following three to four rounds of matings of Notch3tm1.1Ecan heterozygous mice with WT mice (53). This suggests that off-
target effects, which should segregate out, are not responsible for the phenotype.

Notch3 is associated with a variety of pathological conditions in addition to LMS. Notch3 promotes tumor cell proliferation and has been implicated in the bone-invasive potential of carcinoma of the breast and multiple myeloma and in the pathogenesis of mandibular torus (27, 54–57). Mutations in the notch3 gene are associated with a variety of pathological conditions, including LMS, osteopenia, and vascular disorders (58–60).

Experimental procedures

Lateral meningocele mutant mice (Notch3<sup>tm1.1Ecan</sup>)

RNA-guided CRISPR-associated Cas9 nuclease technology was used to create a mutation mimicking that found in a subject afflicted by LMS at the Center for Mouse Genome Modification of UConn Health (31–34). The subject harbored a single bp insertion at c.6692_93insC (from the start of translation) of NOTCH3, predicting the premature termination of a protein product devoid of the PEST domain. To engineer the Notch3<sup>tm1.1Ecan</sup> global mutant allele, the corresponding human NOTCH3 mutation was introduced into the mouse genome (6691–6696 ACCAAG→TAATGA), leading to a T2231X change at the amino acid level and creating a truncated protein of 2230 amino acids (versus 2318 in wildtype) devoid of the PEST domain. The databases http://zifit.partners.org/ZiFiT<sup>5</sup> (80, 81) and http://crispr.mit.edu<sup>5</sup> were utilized to evaluate potential sgRNAs, and Notch3 sgRNA 5’-AACC CGCAGUAGCCCACCA-3’ was selected because of its high score and limited probabilities of off-target effects and designed to cleave between nucleotides 6691 and 6692 of exon 33 of Notch3 adjacent to a protoscaler adjacent motif (Fig. 1). A synthetic DNA fragment containing a T7 promoter driving sequences coding for Notch3 sgRNA was used as a template to synthesize the sgRNA. A single-strand DNA of 146 nucleotides containing the tandem stop codon TAATGA and 70 nucleotides of Notch3 homologous 5’- and 3’-arms (Fig. 1) were co injected into C57BL/6J one-cell embryos with Notch3 sgRNA and Cas9 mRNA. Injected embryos were transferred into CD1 pseudo-pregnant foster females, and progeny were screened by PCR. The proper insertion of the Notch3 6691–6696 ACCAAG→TAATGA mutation was confirmed by sequencing of genomic DNA obtained from ear punches from F1 mice (Fig. 1) (GENEWIZ, South Plainfield, NJ). Heterozygous F1 mice were crossed with C57BL/6J WT mice to establish the mutant mouse line. Notch3<sup>tm1.1Ecan</sup> heterozygous mutant mice were crossed with WT mice to generate Notch3<sup>tm1.1Ecan</sup> heterozygous and sex-matched littermate controls in a C57BL/6J genetic background for study. Genotypes were determined by PCR analysis of tail DNA using forward primer 5’-GTGCTCAGCTTTGGTCTG-3’ and reverse primer 5’-GCCAGAAGCGCCTACATTA-3’ for Notch3<sup>tm1.1Ecan</sup> or 5’-CCGAGAAGCGGCGGCTTGG-3’ for the WT allele (Integrated DNA Technologies, Coralville, IA). Studies were approved by the Institutional Animal Care and Use Committee of UConn Health.

Serum glucose and hormone assays

17β-Estradiol, murine PTH, and insulin were measured by ELISA assays in serum from Notch3<sup>tm1.1Ecan</sup> and control mice following an overnight fast using commercially available kits (17β-estradiol ELISA kit from Abcam, Cambridge, MA; murine insulin ELISA kit from Thermo Fisher Scientific, Waltham, MA; and mouse PTH(1–84) ELISA kit from Quidel Corp., San Diego, CA). Serum glucose was measured using an EnzyChrom glucose assay kit from BioAssay Systems ( Hayward, CA).

Densitometry and body composition

Total body fat (grams) was measured on anesthetized mice using a PIXImus small animal DEXA system (GE Medical Systems/Lunar, Madison, WI) (62).

Microcomputed tomography

Femoral microarchitecture was determined using a μCT instrument (Scanco μCT 40, Scanco Medical AG, Bassersdorf, Switzerland) and a microcomputed tomography system (Scanco CT40, Scanco Medical AG, Bassersdorf, Switzerland). Bone volumes and densities were analyzed using software (CTAn, Scanco Medical AG, Bassersdorf, Switzerland).

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Switzerland), which was calibrated periodically using a phantom provided by the manufacturer (63, 64). Femora were scanned in 70% ethanol at high resolution, energy level of 55 peak kilovoltage (kVp), intensity of 145 mA, and integration time of 200 ms as reported previously (26, 50). A total of 100 slices at midshaft and 160 slices at the distal metaphysis were acquired at anisotropic voxel size of 216 μm³ and a slice thickness of 6 μm and chosen for analysis. Cancellous bone volume fraction (bone volume/total volume) and microarchitecture were evaluated starting 1.0 mm proximal from the femoral condyles. Contours were manually drawn every 10 slices, a few voxels away from the endocortical boundary, to define the region of interest for analysis, whereas the remaining slice contours were iterated automatically. Total volume, bone volume, bone volume fraction, trabecular thickness, trabecular number, connectivity density, structure model index, and material density were measured in trabecular regions using a Gaussian filter (σ = 0.8) and user-defined thresholds (63, 64). For analysis of cortical bone, contours were iterated across 100 slices along the cortical shell of the femoral midshaft, excluding the marrow cavity. Analysis of bone volume/total volume, porosity, cortical thickness, total cross-sectional and cortical bone area, periosteal and endosteal perimeter, and material density were conducted using a Gaussian filter (σ = 0.8, support = 1) with operator-defined thresholds.

**Bone histomorphometry**

Bone histomorphometry was carried out in 1- and 4-month-old mice injected with 20 mg/kg calcein and 50 mg/kg demeclocycline at a 2- and 7-day interval, respectively, and sacrificed 2 days after demeclocycline administration as reported previously (26, 50). Femora were dissected, fixed in 70% ethanol, and embedded in methyl methacrylate. For cancellous bone analysis, bones were sectioned at a thickness of 5 μm along the sagittal plane on a Microm microtome (Richards-Allan Scientific, Kalamazoo, MI) and stained with 0.1% toluidine blue. Static and dynamic parameters of bone morphometry were measured in a defined area between 0.35 and 2.16 mm from the growth plate at a magnification of 100× using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA). Stained sections were used to measure trabecular bone area; trabecular number and thickness; osteoblast, osteocyte, and osteoclast number; and osteoid and eroded surface. Mineralizing surface per bone surface and mineral apposition rate were measured on unstained sections.
sections visualized under UV light and a triple diaminido-2-phenylindole/fluorescein/Texas Red set long-pass filter, and bone formation rate was calculated. Terminology and units used for cancellous and cortical bone histomorphometry are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (65, 66).

### Osteoblast-enriched cell cultures and cell viability assay

Parietal bones from 3–5-day-old Notch3<sup>gmi1.1Ecan</sup> mice and littermate controls were exposed to 1.2 units/ml Liberase TL (Sigma-Aldrich) for 20 min at 37 °C, and cells were extracted in five consecutive reactions (67). Cells from the last three digestions were pooled and seeded at a density of 10,000 cells/cm<sup>2</sup> as described previously (50, 68). Osteoblast-enriched cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with nonessential amino acids (both from Thermo Fisher Scientific), 20 mM HEPES, 100 μg/ml ascorbic acid (both from Sigma-Aldrich), and 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) in a humidified 5% CO<sub>2</sub> incubator at 37 °C as described (50). M-CSF cDNA, encoding RANKL, and expression vector were obtained from M. Glogauer (Toronto, Canada), and glutathione S-transferase–tagged RANKL was expressed and purified as described (72). To determine the contribution of osteoblast-derived factors to osteoclast formation, calvarial osteoblast-enriched cells from Notch3<sup>gmi1.1Ecan</sup> mutant and WT control littermates were seeded at a density of 15,700 cells/cm<sup>2</sup> in α-MEM in the presence of BMMs from either genotype at a density of 47,000 cells/cm<sup>2</sup> and cultured 1,25-dihydroxyvitamin D<sub>3</sub> at 10 nM. Cultures were carried out until the formation of multinucleated TRAP-positive cells was achieved. TRAP enzyme histochemistry was conducted using a commercial kit (Sigma-Aldrich), in accordance with the manufacturer’s instructions, as reported previously (50). TRAP-positive cells containing three or more nuclei were considered osteoclasts.

### Quantitative reverse transcription–polymerase chain reaction

Total RNA was extracted from cells with the RNeasy kit (Qiagen, Valencia, CA) and from homogenized tibiae or osteocyte-enriched femoral fragments, following the removal of the bone marrow by centrifugation, with the micro RNeasy kit (Qiagen), in accordance with the manufacturer’s instructions, as reported previously (26, 50, 73, 74). The integrity of the RNA from tibiae and osteocyte-rich fragments was assessed by microfluidic electrophoresis on an Experion instrument (Bio-Rad), and only RNA with a quality indicator number equal to or higher than 7.0 was used for subsequent analysis. Equal amounts of RNA were reverse transcribed using the iScript RT-PCR kit (Bio-Rad) and amplified in the presence of specific primers (Inte-
grated DNA Technologies) (Table 5) with iQ SYBR Green Supermix or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) at 60 °C for 35 cycles. Transcript copy number was estimated by comparison with a serial dilution of cDNA for Bglap (encoding for osteocalcin; from J. Lian, University of Vermont, Burlington, VT), Hey1 and Hey2 (both from T. Iso, Figure 7. BMMs harvested from long bones of Notch3tm1.Ecan mutants (black bars) and WT littermate controls (white bars) cultured for 72 h in the presence of M-CSF at 30 ng/ml. In A, cells were seeded on culture dishes in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml and assessed for the appearance of osteoclasts defined as TRAP-positive multinucleated cells. In B, BMMs from control and Notch3tm1.Ecan mutant mice were seeded on culture dishes in the presence of osteoblasts (OB) from control or Notch3tm1.Ecan mutant mice with 1,25-dihydroxyvitamin D3 at 10 nM and assessed for the appearance of TRAP-positive multinucleated cells. Representative images of TRAP-stained multinucleated cells are shown in the left panels. Data are expressed as total number of TRAP-positive multinucleated cells/well. Values are means ± S.D. (error bars); n = 4 technical replicates. *, significantly different between Notch3tm1.Ecan and WT control BMMs, p < 0.05 in A by unpaired t test and in B by ANOVA with Holm–Sidak post hoc analysis. #, significantly different between Notch3tm1.Ecan and WT osteoblasts by ANOVA.

Table 5

| Gene     | Strand | Sequence (5'–3')                      | GenBank accession no. |
|----------|--------|---------------------------------------|-----------------------|
| Bglap    | Forward| 5'–GACTCCGGCGCTACTTTGGGTAAG-3'        | NM_001037939          |
|          | Reverse| 5'–CCCCAGCAAAACTTCTCCCTTA-3'          |                      |
| Hes1     | Forward| 5'–ACCAAGACGGCCTCTGACGAGAAAGT-3'      | NM_008235             |
|          | Reverse| 5'–ATCTTCTCCCTTCCCTCTT-3'             |                      |
| Hey1     | Forward| 5'–ATCTCAAAAACTACGGATCCAGAC-3'        | NM_010423             |
|          | Reverse| 5'–GTTGTTGCCGAGAGAGAGG-3'             |                      |
| Hey2     | Forward| 5'–AGCGAGACAAATTACCTCGGAC-3'          | NM_013904             |
|          | Reverse| 5'–AGCTTTGCAGAGCAACCTCTTTC-3'         |                      |
| HeyL     | Forward| 5'–CGATACCTCTTCTGTGATCCAGAC-3'        | NM_013905             |
|          | Reverse| 5'–AGCTTTGAGAGCAACCTCTTTC-3'          |                      |
| Notch3   | Forward| 5'–CCGATTCTCCTGCTGATCC-3'             | NM_008716             |
|          | Reverse| 5'–TGAAACAAGGCGCTTCGAC-3'             |                      |
| Notch3 (5'-TAATGCA) | Forward| 5'–AACCAGCTTCTTCCTACT-3'               | Not applicable        |
|          | Reverse| 5'–CTGAGAATGCCAGCTGGCA-3'              |                      |
| Rpl38    | Forward| 5'–AGAACACAGAATATATAGGAATTTCAAAGTT-3' | NM_001048057; NM_001048058; NM_023372 |
|          | Reverse| 5'–CTGCGTTCAGCTCTGTGTTGCTCC-3'        |                      |
| Tnfrsf11b| Forward| 5'–TGGAGAAAGGAATGGACACGACAC-3'        | NM_008764             |
|          | Reverse| 5'–CCCTCCTACACAGGGTGA-3'              |                      |
| Tnfsf11  | Forward| 5'–ATTAGAAATCTCAGGAGAAATAGGACAC-3'    | NM_011613             |
|          | Reverse| 5'–CCCTGAAAGGCTTTCTACCC-3'            |                      |

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Gunma University, Gunma, Japan, *HeyL* (from D. Srivastava, Gladstone Institute of Cardiovascular Disease, San Francisco, CA), *Tnfsf11* (from Source BioScience, Nottingham, UK), or *Tnfsf11b* (from American Type Tissue Culture Collection (ATCC), Manassas, VA) (75–77). *Notch3*tm1.1Ecan* transcript copy number was estimated by comparison with a serial dilution of a synthetic DNA fragment (Integrated DNA Technologies) containing ~90 bp surrounding the *Notch3* 6691–6696 ACCAAG→TAATGA mutation and cloned into pcDNA3.1(−) (Life Technologies) by isothermal single-reaction assembly using commercially available reagents (New England Biolabs, Ipswich, MA) (78). Amplification reactions were conducted in a CFX96 qRT-PCR detection system (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step. Data are expressed as copy number or relative transcript expression, both corrected for *Rpl38* (from ATCC) (79).

**Statistics**

Data are expressed as means ± S.D. All data except for osteoblast and BMM cultures represent biological replicates. qRT-PCR values were derived from two technical replicates of biological replicates as indicated in the text and tables. Statistical differences were determined by unpaired Student’s *t* test or analysis of variance (ANOVA) with Holm–Šidák post hoc analysis for pairwise or multiple comparisons.

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