Antisense oligonucleotides targeting Notch2 ameliorate the osteopenic phenotype in a mouse model of Hajdu-Cheney syndrome

Received for publication, October 11, 2019, and in revised form, January 24, 2020 Published, Papers in Press, January 28, 2020, DOI 10.1074/jbc.RA119.011440

© Ernesto Canalis†‡¶, Tamar R. Grossman†, Michele Carrer§, Lauren Schilling§, and Jungeun Yu‡¶
From the Departments of†Orthopaedic Surgery and §Medicine, and the ‡Ionis Pharmaceuticals, Inc., Carlsbad, California 92010

JBC ARTICLE

Notch receptors play critical roles in cell-fate decisions and in the regulation of skeletal development and bone remodeling. Gain–of–function NOTCH2 mutations can cause Hajdu-Cheney syndrome, an untreatable disease characterized by osteoporosis and fractures, craniofacial developmental abnormalities, and acro-osteolysis. We have previously created a mouse model harboring a point 6955C→T mutation in the Notch2 locus upstream of the PEST domain, and we termed this model Notch2tm1.1Ecan. Heterozygous Notch2tm1.1Ecan mutant mice exhibit severe cancellous and cortical bone osteopenia due to increased bone resorption. In this work, we demonstrate that the subcutaneous administration of Notch2 antisense oligonucleotides (ASO) down-regulates Notch2 and the Notch target genes Hes-related family basic helix–loop–helix transcription factor with YRPW motif 1 (Hey1), Hey2, and HeyL in skeletal tissue from Notch2tm1.1Ecan mice. Results of microcomputed tomography experiments indicated that the administration of Notch2 ASOs ameliorates the cancellous osteopenia of Notch2tm1.1Ecan mice, and bone histomorphometry analysis revealed decreased osteoclast numbers in Notch2 ASO-treated Notch2tm1.1Ecan mice. Notch2 ASOs decreased the induction of mRNA levels of TNF superfamily member 11 (Tnfsf11, encoding the osteoclastogenic protein RANKL) in cultured osteoblasts and osteocytes from Notch2tm1.1Ecan mice. Bone marrow-derived macrophage cultures from the Notch2tm1.1Ecan mice displayed enhanced osteoclastogenesis, which was suppressed by Notch2 ASOs. In conclusion, Notch2tm1.1Ecan mice exhibit cancellous bone osteopenia that can be ameliorated by systemic administration of Notch2 ASOs.

This work was supported by National Institutes of Health Grant DK045227 (to E. C.) from the NIDDK and Grant AR076747 (to E. C.) from the NIAMS, T. R. G. and M. C. are employed by Ionis Pharmaceuticals, Inc. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 To whom correspondence should be addressed: Depts. of Orthopaedic Surgery and Medicine, UConn Health, 263 Farmington Ave., Farmington, CT 06030-4037; Tel.: 860-679-7978; Fax: 860-679-1474; E-mail: canalis@uchc.edu.

Notch receptors are four single-pass transmembrane proteins that play a critical role in cell fate determination (1, 2). Notch1–3 and low levels of Notch4 transcripts are detected in bone cells, where they play a key role in osteoblast and osteoclast differentiation and function (3). Notch receptors are activated following interactions with ligands of the Jagged and Delta-like families, and JAGGED1 is the prevalent ligand expressed by skeletal cells (3, 4). Interactions of NOTCH with its ligands lead to the proteolytic cleavage of the NOTCH protein and to the release of the NOTCH intracellular domain (NICD)² (5, 6). The NICD is translocated into the nucleus where it forms a complex with recombination signal-binding protein for Ig of κ (RBPJκ) and mastermind (MAML) to induce the transcription of target genes, including those encoding Hairless Enhancer of Split (HES)-1, -5, and -7 and HES-related with YRPW motif (HEY)-1, -2, and -L (7–9).

Although activation of NOTCH1, -2, and -3 in the skeleton results in osteopenia, the mechanisms responsible for the bone loss are distinct (10–14). NOTCH2 has unique properties and impairs osteoblast maturation and induces osteoclastogenesis by acting directly on cells of the myeloid lineage and by inducing receptor activator of NF-κB ligand (RANKL) in cells of the osteoblast lineage (10, 12, 15).

Hajdu–Cheney syndrome (HCS) is a dominant inherited disease characterized by craniofacial developmental abnormalities, acro-osteolysis, generalized osteoporosis with fractures and neurological complications (16–18). HCS is associated with point mutations in, or short deletions of, exon 34 of NOTCH2 that lead to the creation of a stop codon upstream of the proline (P), glutamic acid (E), serine (S), and threonine (T) (PEST) domain (19–23). The PEST domain is required for the ubiquitination and degradation of NOTCH2. As a consequence, the mutations result in the translation of a stable truncated protein product and a gain–of–NOTCH2 function. Iliac crest bone biopsies obtained from subjects afflicted by HCS have demonstrated the presence of osteopenia, increased bone resorption, and trabecularization of cortical bone (24–26).

To gain an understanding of the HCS skeletal phenotype and the mechanisms involved, we introduced a Notch2 mutation

2 The abbreviations used are: NICD, NOTCH intracellular domain; ASO, antisense oligonucleotide; BMM, bone marrow macrophage; BV/TV, bone volume/tissue volume; CTX, carboxy-terminal collagen cross-link; Ctrl, control; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HCS, Hajdu-Cheney syndrome; HES, Hairless Enhancer of Split; M-CSF, macrophage–colony-stimulating factor; MAML, mastermind; μCT, microcomputed tomography; N2ICD, NOTCH2 intracellular domain; NRR, negative regulatory region; PEST, proline (P), glutamic acid (E), serine (S), and threonine (T); qRT, quantitative reverse transcription; RANKL, receptor activator of nuclear factor κB ligand; SMI, structure model index; TRAP, tartrate-resistant acid phosphatase.
(6955C→T) in the mouse genome to reproduce a mutation (6949C→T) found in a subject with HCS (10, 20, 22). The mutation creates a stop codon in exon 34 leading to the translation of a truncated NOTCH2 protein of 2318 amino acids (10). The mouse line, termed  Notch2<sup>tm1.1Ecan</sup>, exhibits NOTCH2 gain–of–function, and homozygous mice display craniofacial developmental abnormalities and newborn lethality. Heterozygous  Notch2<sup>tm1.1Ecan</sup> mice have cataract and cortical bone osteopenia due to enhanced bone resorption. This is secondary to an increase in the number of osteoclasts due to enhanced expression of RANKL by cells of the osteoblast lineage as well as due to direct effects of NOTCH2 on osteoclastogenesis (10, 15). The discovery of the mechanisms responsible for the bone loss provided clues to offer improved treatments to individuals with HCS, such as the use of the RANKL antibody denosumab (27). However, none of the available interventions offers the opportunity to correct the mechanisms responsible for the disease.

Approaches to down-regulate Notch signaling include the use of biochemical inhibitors of Notch activation, thapsigargin, antibodies to nicastrin, which forms part of the γ-secretase complex, or to Notch receptors or their ligands, and stapled peptides that prevent the assembly of a NICD/RBPJκ/MAML ternary complex (28–32). A limitation of these approaches is that either they are not specific inhibitors of Notch signaling or they prevent the indiscriminate activation of all Notch receptors, leading to a generalized Notch activation knockdown and side effects. Anti-Notch NRR antibodies have been effective at preventing the activation of specific Notch receptors (33–35). However, the pronounced down-regulation of Notch activation may result in gastrointestinal toxicity.

Antisense oligonucleotides (ASOs) are single-stranded synthetic nucleic acids that bind target mRNA by Watson–Crick pairing resulting in mRNA degradation by RNase H (36, 37). The administration of ASOs has emerged as a novel therapeutic approach to down-regulate WT and mutant transcripts, and it has been successful in the silencing of mutant genes in the central and peripheral nervous system, retina, and liver (38–45). ASOs have been used to down-regulate specific genes in the skeleton, although information about their possible use as a therapeutic intervention in genetic disorders of the skeleton is limited (46, 47).

The purpose of this work was to answer the question whether the phenotype of the  Notch2<sup>tm1.1Ecan</sup> mouse model could be ameliorated or reversed by down-regulating Notch2 expression with Notch2-specific ASOs. To this end, heterozygous  Notch2<sup>tm1.1Ecan</sup> and control littermate mice were treated with second generation phosphorothioate-modified ASOs targeting Notch2 and characterized by bone microarchitectural analysis. The direct effects of the Notch2 ASO on osteoblast, osteocyte, and osteoclast cultures from control and experimental mice also were tested.

**Results**

**Effect of Notch2 ASOs on Notch2 expression and signaling in vivo**

In initial experiments, we tested whether mouse Notch2 ASOs down-regulated Notch2 mRNA in vivo in tissues where Notch2 is expressed and is known to have a function (10, 48–52). The subcutaneous administration of ASOs targeting murine Notch2 to C57BL/6 WT mice at a dose of 50 mg/kg caused an ~40–50% down-regulation of Notch2 mRNA 40 h later in the spleen, kidney, and femur and an 80% reduction of Notch2 transcripts in the liver (Fig. 1). In a subsequent experiment, Notch2 ASOs, administered subcutaneously to WT C57BL/6 mice at 50 mg/kg, down-regulated Notch2 mRNA in femur by ~40% 48–96 h after the administration of the ASO.

There was evidence of enhanced Notch signaling in skeletal tissue from  Notch2<sup>tm1.1Ecan</sup> mice, and the Notch target genes Hey1, Hey2, and HeyL were induced in bone extracts from mutant mice in relationship to control littermates (Fig. 2). The subcutaneous administration of mouse Notch2 ASOs decreased the expression of Notch2 and Notch2<sup>G955C→T</sup> mutant mRNA. Notch2 ASOs also decreased the Notch target genes Hey1 and Hey2 in bone extracts from WT mice and Hey1, Hey2, and HeyL in extracts from Notch2<sup>tm1.1Ecan</sup> mice demonstrating a suppressive effect of Notch2 ASOs on Notch signaling in the skeleton. As a result, the mRNA levels of Hey1, Hey2, and HeyL in tibiae from Notch2<sup>tm1.1Ecan</sup> mice treated with Notch2 ASOs approached the levels found in tibiae from WT mice treated with control ASOs. A modest induction of Tnfsf11 (encoding RANKL,  p > 0.05) was observed in tibiae from Notch2<sup>tm1.1Ecan</sup> mice, and this was reduced by Notch2 ASOs.

**Effect of Notch2 ASOs on general characteristics, femoral microarchitecture, and histomorphometry of Notch2<sup>tm1.1Ecan</sup> mice**

Heterozygous  Notch2<sup>tm1.1Ecan</sup> mutant male mice were compared with WT sex-matched littermate mice in a C57BL/6 genetic background because the skeletal phenotype was similar in both sexes and the homozygous mutation of  Notch2<sup>tm1.1Ecan</sup> results in perinatal lethality. Confirming prior results,  Notch2<sup>tm1.1Ecan</sup> heterozygous mice had ~10% less weight than littermate controls, and their femoral length was slightly shorter than that of controls (Fig. 3) (10). Following the administration of mouse Notch2 ASOs, control and  Notch2<sup>tm1.1Ecan</sup> experimental mice appeared healthy, although a 6% decrease in weight was noted in WT mice treated with Notch2 ASOs when compared with control ASOs. Femoral length was not affected.

---

**Figure 1. Effect of control (open circles) or Notch2 ASOs (closed circles) administered subcutaneously at a dose of 50 mg/kg to C57BL/6 mice on Notch2 mRNA.** Panel A, Notch2 copy number corrected for Rpi38 was determined 40 h later in femur, spleen, kidney, and liver; panel B, Notch2 copy number was determined 6–46 h later in femur, and values are shown as relative expression corrected for Rpi38 and normalized to a control value of 1. Panel A, individual values are shown, and bars and ranges represent means ± S.D.; n = 3, except for 6-h data in panel B, where n = 2 of biological replicates. *p < 0.05.
Cortical osteopenia was not affected by Notch2 ASOs and Hajdu-Cheney syndrome. Transcript levels for Notch2, Notch26955C, Hey1, Hey2, HeyL, and Tnfsf11 were expressed as relative expression corrected for Rpl38 and normalized to a control value of 1. Individual values are shown, and bars and ranges represent means ± S.D.; n = 9–13 of biological replicates. *, significantly different between Notch2tm1.1Ecan mutant and WT mouse, p < 0.05; #, significantly different between Notch2 ASO and control ASO, p < 0.05.

Cancellous bone histomorphometric analysis revealed that osteoclast number was increased in Notch2tm1.1Ecan mutant mice; Notch2 ASOs did not change osteoclast number in WT mice, but significantly reduced the osteoclast number in Notch2tm1.1Ecan mutant mice so that the osteoclast number was not different between Notch2 ASOs and control littermate WT mice (Table 2). Confirming prior observations, osteoblast number was not different between control and Notch2tm1.1Ecan mutant mice. Accordingly, dynamic parameters of bone formation were not different between WT and mutant mice and were not affected by Notch2 ASOs. In accordance with the cellular phenotype of Notch2tm1.1Ecan mutant mice, fasting serum levels of carboxyl-terminal collagen cross-links (CTX) were increased from (means ± S.D.; n = 5–6) control 34.6 ± 9.7 ng/ml (p < 0.05) to 49.2 ± 8.9 ng/ml in Notch2tm1.1Ecan mice treated with control ASOs. Notch2 ASOs reduced the serum levels of CTX in both WT mice to 24.1 ± 9.7 ng/ml (p < 0.052) and Notch2tm1.1Ecan mice to 23.2 ± 3.9 ng/ml (p < 0.05) demonstrating a normalization of bone resorption in experimental mice.

Effect of Notch2 ASOs on Notch2 expression and signaling in osteoblast and osteocyte cell cultures

Mouse Notch2 ASOs added to the culture medium of osteoblast-enriched cells from WT C57BL/6 mice at 1–20 µM decreased Notch2 mRNA by ~40 to ~80% 72 h after ASO addition without evidence of cellular toxicity or changes in cell replication (Fig. 5). The effect of the Notch2 ASO was specific for Notch2 mRNA because, at a concentration as high as 20 µM, it did not decrease the expression of Notch1, -3, or -4 mRNA. The NOTCH2 intracellular domain (N2ICD), representative of NOTCH2 cleavage and signal activation, was increased in Notch2tm1.1Ecan osteoblasts, and the truncated form of Notch2, lacking the PEST domain (N2ICDΔPEST), was detected only in Notch2tm1.1Ecan cells. Therefore, the total levels of N2ICDs, intact and truncated, were ~2-fold greater in Notch2tm1.1Ecan cells than in control cells (Fig. 5). Notch2 ASOs decreased the total levels of N2ICD in WT and Notch2tm1.1Ecan cells demonstrating a suppression of NOTCH2 activation. Notch26955C→T transcripts were present in cells from Notch2tm1.1Ecan mutant mice but not in control cultures, and Hey1 and Hey2 transcripts were increased in Notch2tm1.1Ecan mutant mice. In contrast, Notch2tm1.1Ecan mice receiving Notch2 ASOs had a BV/TV that was 30% greater than in mutant mice receiving control ASOs. As a consequence, BV/TV in Notch2tm1.1Ecan mice was reduced by 28% when compared with control WT mice, whereas Notch2tm1.1Ecan mice treated with control ASOs exhibited a 45% reduction in BV/TV compared with WT littermate controls (Fig. 4). The partial restoration of BV/TV by Notch2 ASOs was associated with a significant increase in trabecular number. Notch2tm1.1Ecan mice presented with cortical osteopenia and cortical bone was thin, and bone area and cortical thickness were reduced (Table 1). The cortical osteopenia was not affected by Notch2 ASOs, so the cortical bone area and thickness in Notch2tm1.1Ecan mice treated with Notch2 ASOs were not different from values obtained in mutant mice treated with control ASOs.

Figure 3. Body weight and femoral length of 2-month-old male Notch2tm1.1Ecan mutant mice (closed circles) and sex-matched littermate controls (open circles) treated with Notch2 ASO or control ASO (Ctrl), both at 50 mg/kg subcutaneously once a week for 4 weeks prior to sacrifice. Transcript levels for Notch2, Notch26955C→T, Hey1, Hey2, HeyL, and Tnfsf11 are expressed as relative expression corrected for Rpl38 and normalized to a control value of 1. Individual values are shown, and bars and ranges represent means ± S.D.; n = 9–13 of biological replicates. *, significantly different between Notch2tm1.1Ecan mutant and WT mouse, p < 0.05; #, significantly different between Notch2 ASO and control ASO, p < 0.05.

Figure 4. Gene expression analysis of tibiae from 2-month-old male Notch2tm1.1Ecan mutant mice (closed circles) and sex-matched littermate controls (open circles) treated with Notch2 ASO or control ASO (Ctrl), both at 50 mg/kg subcutaneously once a week for 4 weeks prior to sacrifice. Transcript levels for Notch2, Notch26955C→T, Hey1, Hey2, HeyL, and Tnfsf11 are expressed as relative expression corrected for Rpl38 and normalized to a control value of 1. Individual values are shown, and bars and ranges represent means ± S.D.; n = 9–13 of biological replicates. *, significantly different between Notch2tm1.1Ecan mutant and WT mouse, p < 0.05; #, significantly different between Notch2 ASO and control ASO, p < 0.05.
osteoblasts confirming that Notch signaling was activated (Fig. 6). In accordance with prior observations, tumor necrosis factor superfamily member 11 (Tnfsf11), encoding RANKL, was induced, whereas Bglap, encoding osteocalcin, was not changed in Notch2tm1.1Ecan osteoblasts (10). Notch2 ASOs decreased Notch2 mRNA in WT and mutant cells and Notch2*6955C→T mRNA in osteoblasts from Notch2tm1.1Ecan mice. In addition, Notch2 ASOs decreased Hey1, Hey2, and Tnfsf11 mRNA in cells from Notch2tm1.1Ecan mice without an effect on Bglap expression (Fig. 6).

**Table 1**

Femoral cortical microarchitecture assessed by μCT of Notch2tm1.1Ecan male mice and littermate sex-matched controls treated with Notch2 ASOs

| Control ASO | Notch2 ASO |
|-------------|------------|
| Wildtype, n = 8 | Notch2tm1.1Ecan, n = 7 | Wildtype, n = 8 | Notch2tm1.1Ecan, n = 9 |
| Bone volume/tissue volume (%) | 91.0 ± 1.0 | 87.4 ± 1.8* | 90.7 ± 1.5 | 88.4 ± 0.9* |
| Porosity (%) | 9.0 ± 1.0 | 12.6 ± 1.8* | 9.3 ± 1.5 | 11.6 ± 0.9* |
| Cortical thickness (μm) | 162 ± 10 | 118 ± 17* | 161 ± 14 | 124 ± 9* |
| Total area (mm²) | 2.0 ± 0.2 | 1.8 ± 0.1* | 2.0 ± 0.2 | 1.9 ± 0.1 |
| Bone area (mm²) | 0.9 ± 0.1 | 0.7 ± 0.1* | 0.9 ± 0.1 | 0.7 ± 0.1* |
| Periosteal perimeter (mm) | 5.1 ± 0.2 | 4.8 ± 0.2* | 5.0 ± 0.3 | 4.8 ± 0.1 |
| Endocortical perimeter (mm) | 3.8 ± 0.2 | 3.8 ± 0.1 | 3.8 ± 0.2 | 3.9 ± 0.1 |
| Density of material (mg HA/ml) | 1126 ± 22 | 1093 ± 36* | 1114 ± 29 | 1095 ± 14 |

* Data are significantly different between Notch2tm1.1Ecan and wild type littermate mice, p < 0.05.
ASOs and Hajdu-Cheney

Table 2
Cancellous bone histological parameters of 2-month-old Notch2^{tm1.1Ecan} mice and littermate sex-matched controls treated with Notch2 ASOs

|                          | Control ASO | Notch2 ASO |
|--------------------------|-------------|------------|
|                          | Wildtype, n = 11 | Notch2^{tm1.1Ecan}, n = 10 | Wildtype, n = 13 | Notch2^{tm1.1Ecan}, n = 12 |
| Osteoblast surface/bone surface (%) | 12.6 ± 4.5 | 14.2 ± 5.7 | 9.5 ± 4.1 | 10.2 ± 4.4 |
| Osteoblasts/bone perimeter (1/mm) | 9.7 ± 3.4 | 11.1 ± 4.4 | 7.6 ± 3.3 | 8.3 ± 3.3 |
| Osteoclast surface/bone surface (%) | 6.9 ± 2.0 | 10.8 ± 2.4 | 6.4 ± 2.3 | 7.8 ± 2.7 |
| Osteoclasts/bone perimeter (1/mm) | 2.7 ± 0.6 | 4.2 ± 1.0 | 2.5 ± 0.9 | 3.1 ± 1.0 |
| Eroded surface/bone surface (%) | 3.0 ± 0.6 | 3.9 ± 2.0 | 2.6 ± 1.2 | 2.8 ± 1.2 |
| Mineral apposition rate (μm/day) | 1.2 ± 0.3 | 1.6 ± 0.7 | 1.3 ± 0.3 | 1.5 ± 0.4 |
| Mineralizing surface/bone surface (%) | 3.2 ± 1.1 | 2.1 ± 1.1 | 2.8 ± 1.5 | 2.0 ± 1.2 |
| Bone formation rate (μm^3/μm^3/day) | 0.03 ± 0.01 | 0.03 ± 0.02 | 0.04 ± 0.02 | 0.03 ± 0.02 |

*p* Data are significantly different between Notch2^{tm1.1Ecan} and wildtype controls, *p* < 0.05.

*p* Data are significantly different between Notch2 ASO and control ASO, *p* < 0.05.

Figure 5. Effect of control (open circles) or Notch2 ASOs (closed circles) on Notch2 mRNA and NOTCH2 protein expression in calvarial osteoblast-enriched cells. Panel A, Notch2 mRNA levels were obtained 72 h after the addition of Notch2 (closed circles) or control ASO (open circles) at 1–20 μM to cells from WT C57BL/6 mice. Values are means ± S.D.; n = 3 technical replicates. Panel B, Notch1–4 mRNA levels were obtained 72 h after the addition of Notch2 (closed circles) or control ASO (open circles) at 20 μM to cells from WT C57BL/6 mice. Panels A and B, transcript levels are expressed as relative number following correction for Rpl38. Panels C and D, osteoblasts from WT (control) or Notch2^{tm1.1Ecan} mutant littermates were cultured with Notch2 or control ASOs at 20 μM for 72 h and analyzed for Notch2 mRNA expression (copy number corrected for Rpl38) (left) and for NOTCH2 by immunoblotting (right). The band intensity was quantified by Image Lab™ software (version 5.2.1), and the numerical ratio of NOTCH2/β-actin, N2ICD (including N2ICD^{PEST})/β-actin is shown below each blot with WT cells cultured with control ASO normalized to 1. Panels B and C, individual values are shown, and bars and ranges represent means ± S.D. of technical replicates; n = 3 for B and n = 4 for C. Panels A and B, *, significantly different between Notch2 ASO and control ASO, *p* < 0.05. Panel C, *, significantly different between Notch2^{tm1.1Ecan} and control ASO, *p* < 0.05; #, significantly different between Notch2 and control ASO, *p* < 0.05.

Effect of Notch2 ASOs on Notch2 expression and activity in BMM cultures and osteoclast formation

Notch2 ASOs were added to either BMM cultures at the initiation of the culture period or following the addition of RANKL for 2 days to determine their effect in cells of the myeloid lineage and in osteoclast precursors. Mouse Notch2 ASOs at 1 and 5 μM suppressed Notch2 mRNA levels in BMMs from WT C57BL/6 mice by 85–95% and in osteoclast precursors by 70–85% without evidence of cellular toxicity and without altering cell proliferation (Fig. 8). Confirming results in osteoblast cultures, the N2ICD was slightly increased in Notch2^{tm1.1Ecan} osteoclasts, and the truncated form of NOTCH2 lacking the PEST domain (N2ICD^{PEST}) was detected only in Notch2^{tm1.1Ecan} cells. Consequently, the total levels of N2ICD, intact and truncated, were ~2-fold greater in Notch2^{tm1.1Ecan} cells than in control cells (Fig. 8). Notch2 ASOs decreased the total levels of...
N2ICD in WT and Notch2tm1.1Ecan cells demonstrating a suppression of NOTCH2 activation.

There was a significant increase in osteoclast formation in BMMs from Notch2tm1.1Ecan mice cultured in the presence of M-CSF and RANKL (Fig. 9). The increased osteoclastogenesis was prevented by the addition of Notch2 ASOs to BMM cultures at 1/8262 M so that the osteoclastogenic potential of Notch2tm1.1Ecan cells cultured with Notch2 ASOs was no longer different from that of control cells. The decrease in osteoclastogenesis by Notch2 ASOs in Notch2tm1.1Ecan cells was associated with a concomitant decrease in Notch2 WT and Notch26955C3T mutant transcripts.

Discussion

Findings from this work confirm that a mouse model replicating a mutation found in HCS displays femoral cancellous and cortical bone osteopenia. The osteopenic phenotype is manifested early in life in mice of both sexes; and in this study, we elected to treat 1-month-old male mice with Notch2 ASOs in an attempt to ameliorate the osteopenic femoral phenotype of Notch2tm1.1Ecan mice (10). Because only male mice were treated, one needs to be cautious and not to extrapolate the results to female mice. Phenotypic alterations of experimental and control mice were assessed by CT, and analyses required the ex vivo exam of bone following the sacrifice of mice. Consequently, the same animal could not be analyzed before and after the administration of Notch2 ASOs. Another limitation of the work is the fact that all the analyses were performed in femoral bone because the osteopenia of Notch2tm1.1Ecan mice was established at this skeletal site (10). Although Notch2 ASOs down-regulated Notch2 WT and mutant transcripts in femoral bone, it was not determined whether the same effect occurs at other skeletal, possibly less vascularized, sites. The Notch2 ASO utilized is specific to Notch2 so that the results obtained should not be attributed to the down-regulation of other Notch receptors.

The phenotype of the Notch2tm1.1Ecan mutant mouse recapitulates aspects of HCS, including osteopenia, short limbs, and in the homozygous state of craniofacial abnormalities, including micrognathia and early-lethality (10, 15). However, neither Notch2tm1.1Ecan nor an alternate murine model of HCS manifest

3 E. Canalis, unpublished observations.
Figure 8. Effect of control (open circles) or Notch2 ASOs (closed circles) on Notch2 mRNA and NOTCH2 protein expression in the osteoclast lineage. Panel A, BMM (left panel) and osteoclast precursors (OCP) (right panel) from WT C57BL/6 mice were cultured. BMMs were cultured for 72 h in the presence of Notch2 or control ASOs at 1 or 5 μM (left panel) in the presence of M-CSF at 30 ng/ml or cultured in the presence of M-CSF at 30 ng/ml and then seeded in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml with Notch2 or control ASOs at 1 or 5 μM (right panel). Notch2 mRNA levels are expressed as copy number corrected for Rpl38. Individual values are shown, and bars and ranges represent means ± S.D. for three biological replicates. *, significantly different between Notch2 ASO and control ASO, p < 0.05. Panel B, BMMs from WT (control) or Notch26955C mutant littermates were cultured in M-CSF at 30 ng/ml and then seeded in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml with Notch2 or control ASOs at 1 μM for 48 h and analyzed for NOTCH2 by immunoblotting. The band intensity was quantified by Image LabTM software (version 5.2.1) and the numerical ratio of NOTCH2/β-actin. The band intensity was normalized to the β-actin signal. As shown below each blot with WT cells cultured with control ASO normalized to 1.

Figure 9. Osteoclast formation and gene expression analysis of BMM from Notch2tm1.1Ecan mutant (closed circles) and WT littermate controls (open circles) cultured for 72 h in the presence of M-CSF at 30 ng/ml and then seeded in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml and either Notch2 or control ASO (Ctrl) at 1 μM. Panel A, representative images of TRAP-stained multinucleated cells and data expressed as number of TRAP-positive multinucleated cells. The scale bars in the right corner represent 100 μm. Panel B, Notch2 and Notch3m559C−/− mRNA levels were obtained 96 h after the addition of Notch2 or control ASO (Ctrl) at 1 μM. mRNA levels are expressed as copy number corrected for Rpl38 copy number. Individual values are shown, and bars and ranges represent means ± S.D.; n = 4 of technical replicates of one representative out of three experiments. *, significantly different between Notch2tm1.1Ecan mutant and control cells; #, significantly different between Notch2 ASO and control ASO.

N2ICDPEST is more stable than WT N2ICD because it is resistant to ubiquitin-mediated degradation, explaining the gain-of-NOTCH2 function and the induction of Notch target genes in Notch2tm1.1Ecan cells. Notch2tm1.1Ecan mice do not exhibit an increase in osteoblast number or a bone-forming response to the increase in bone resorption, indicating a possible negative regulation of osteoblastogenesis or osteoblast function by the Notch2 mutation. However, in this study, we confirm that osteoblast gene markers, such as Bglap (osteocalcin), are not affected in cells from Notch2tm1.1Ecan mice. The inactivation of Notch2 in cells of the osteoblastic lineage causes an increase in the osteogenic potential of these cells suggesting an inhibitory role of Notch signaling in osteoblastogenesis (54–56).

Although approaches to down-regulate Notch signaling are various, they are often not specific to this signaling pathway or to a specific Notch receptor. A recent alternative has been the use of antibodies to the negative regulatory region (NRR) of specific Notch receptors that prevent the exposure of the NRR to the γ-secretase complex and thus the activation of Notch (33–35). Recently, we demonstrated that anti-Notch2 NRR antibodies reverse the skeletal phenotype of Notch2tm1.1Ecan mice, and anti-Notch3 NRR antibodies reverse the skeletal phenotype of Notch3tm1.1Ecan mice, a model of lateral meningocele syndrome (34, 35). Although anti-Notch NRR antibodies are

acro-osteolysis (53). In this work, we confirm that Notch2 has unique actions on trabecular bone physiology and induces osteoblastogenesis by increasing the expression of RANKL by cells of the osteoblast lineage and by inducing the differentiation of cells of the myeloid lineage toward mature osteoclasts (15). Notch2 ASOs decreased both effects in vitro and decreased serum levels of CTX, a marker of bone resorption, so that CTX levels in Notch2tm1.1Ecan ASO-treated mice were not different from those of WT mice. These effects would explain the amelioration of the osteopenia observed in Notch2tm1.1Ecan mice.

Notch2 ASOs down-regulated Notch2 and Notch2p695SC→T transcripts and decreased the enhanced Notch signaling found in Notch2tm1.1Ecan cells as well as in bone extracts without an effect on basal levels of Notch activation. Only Notch2tm1.1Ecan mutant cells synthesized the truncated form of the N2ICD (N2ICDPEST) and the intact N2ICD. The summation of the intact and truncated forms of N2ICD resulted in an ~2-fold greater expression of N2ICD in Notch2tm1.1Ecan mutants than in control cells, and this was suppressed by Notch2 ASOs confirming the down-regulation of Notch2 signaling. The

ASOs and Hajdu-Cheney

Figure 9. Osteoclast formation and gene expression analysis of BMM from Notch2tm1.1Ecan mutant (closed circles) and WT littermate controls (open circles) cultured for 72 h in the presence of M-CSF at 30 ng/ml and then seeded in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml and either Notch2 or control ASO (Ctrl) at 1 μM. Panel A, representative images of TRAP-stained multinucleated cells and data expressed as number of TRAP-positive multinucleated cells. The scale bars in the right corner represent 100 μm. Panel B, Notch2 and Notch3m559C−/− mRNA levels were obtained 96 h after the addition of Notch2 or control ASO (Ctrl) at 1 μM. mRNA levels are expressed as copy number corrected for Rpl38 copy number. Individual values are shown, and bars and ranges represent means ± S.D.; n = 4 of technical replicates of one representative out of three experiments. *, significantly different between Notch2tm1.1Ecan mutant and control cells; #, significantly different between Notch2 ASO and control ASO.

N2ICDPEST is more stable than WT N2ICD because it is resistant to ubiquitin-mediated degradation, explaining the gain-of-NOTCH2 function and the induction of Notch target genes in Notch2tm1.1Ecan cells. Notch2tm1.1Ecan mice do not exhibit an increase in osteoblast number or a bone-forming response to the increase in bone resorption, indicating a possible negative regulation of osteoblastogenesis or osteoblast function by the Notch2 mutation. However, in this study, we confirm that osteoblast gene markers, such as Bglap (osteocalcin), are not affected in cells from Notch2tm1.1Ecan mice. The inactivation of Notch2 in cells of the osteoblastic lineage causes an increase in the osteogenic potential of these cells suggesting an inhibitory role of Notch signaling in osteoblastogenesis (54–56).

Although approaches to down-regulate Notch signaling are various, they are often not specific to this signaling pathway or to a specific Notch receptor. A recent alternative has been the use of antibodies to the negative regulatory region (NRR) of specific Notch receptors that prevent the exposure of the NRR to the γ-secretase complex and thus the activation of Notch (33–35). Recently, we demonstrated that anti-Notch2 NRR antibodies reverse the skeletal phenotype of Notch2tm1.1Ecan mice, and anti-Notch3 NRR antibodies reverse the skeletal phenotype of Notch3tm1.1Ecan mice, a model of lateral meningocele syndrome (34, 35). Although anti-Notch NRR antibodies are
specific, the significant down-regulation of the Notch receptor throughout the organism may lead to potential side effects, such as gastrointestinal toxicity. In this study, we demonstrate that down-regulation of Notch expression by specific Notch ASOs is a suitable alternative to decrease Notch activation in conditions of Notch gain–of–function. Although the effect of Notch2 ASOs was less pronounced than the one reported with anti-Notch2 NRR antibodies, Notch2 ASOs were effective at ameliorating the skeletal phenotype of Notch2 

_\textit{tm1.1Ecan}_ mice and appeared to be well-tolerated by this experimental model of HCS.

Although attempts have been made to transport ASOs to bone, complex delivery systems were necessary, and the technology has not been applied to the correction of gene mutations in the skeleton (57). In this study, we used a practical systemic approach to down-regulate Notch2 in skeletal and nonskeletal tissue. We demonstrate that a second generation phosphorothioate-modified murine Notch2 ASO down-regulated Notch2 in tissues where the gene is expressed and has a function, including bone. The decrease in Notch2 in a mouse model of Notch2 gain–of–function was associated with a concomitant decrease in Notch target gene expression in skeletal cells documenting a tempering effect on Notch activation. As a consequence, a recovery of bone mass was observed. Although this was not complete, a significant effect on BV/TV was achieved with amelioration of the Notch2 

_\textit{tm1.1Ecan}_ skeletal phenotype.

In conclusion, Notch2 ASOs down-regulate Notch2 expression and signal activation, decrease RANKL and osteoclastogenesis in a model of HCS, and consequently ameliorate its osteopoenic phenotype. The down-regulation of \textit{NOTCH2} may offer a potential therapeutic opportunity for subjects with HCS in the future.

**Experimental procedures**

**Notch2 antisense oligonucleotides**

ASOs targeting Notch2 mRNA were designed \textit{in silico} by scanning through the sequence of murine Notch2 pre-mRNA. The entire Notch2 pre-mRNA sequence was covered for potential 16-mer oligonucleotides complementary to the pre-mRNA. Sequence motifs that were intrinsically problematic because of unfavorable hybridization properties, such as polyG stretches, or potential toxicity due to immunogenic responses were avoided. Notch2 ASOs were tested for activity \textit{in vitro} for down-regulation of Notch2 mRNA in HEPA 1–6 cells at Ionis Pharmaceuticals (Carlsbad, CA), and 14 ASOs targeting Notch2 mRNA were screened for activity and toxicity \textit{in vivo} at the Korea Institute of Toxicology (Daejeon, Korea). To this end, 7-week-old BALB/c male mice were administered ASOs at a dose of 50 mg/kg once a week by subcutaneous injection for a total of 3.5 weeks (four doses). Body weights were measured weekly, and mice were euthanized 48 h after the last dose of ASO. Liver, kidney, and spleen were weighed, normalized to body weight, and compared with organs from control mice. Blood was obtained by cardiac puncture, and plasma was collected for the measurement of alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, and blood urea nitrogen. Total RNA was extracted from liver samples to determine Notch2 mRNA levels corrected for cyclophilin A expression. Based on the information obtained, ASOs found to down-regulate Notch2 liver mRNA by more than 75% compared with a control mismatched ASO without toxicity \textit{in vivo} were selected. Procedures were performed at and approved by the Animal Care and Use Committee of the Korea Institute of Toxicology. For this study, mouse Notch2 ASO Ionis 977472 of sequence GTTATATAATCTTCCA and control mismatched ASO Ionis 549144 of sequence GGCCAATACGCCGTCA were selected.

**Notch2 

_\textit{tm1.1Ecan}_ mutant mice**

A mouse model of HCS, termed Notch2 

_\textit{tm1.1Ecan}_ , harboring a 6955C→T substitution in exon 34 of Notch2 was previously reported and validated (10). Notch2 

_\textit{tm1.1Ecan}_ mice were backcrossed into a C57BL/6J background for eight or more generations, and genotyping was conducted in tail DNA extracts by PCR using forward primer Nch2Lox gtF 5’-CCCTTCTCTCCT-GTGCCTAG-3’ and reverse primer Nch2Lox gtR 5’-CTCA-GAGCCAAAGCCTCAG-3’. In this study, 1-month-old mice heterozygous for the Notch2 

_\textit{tm1.1Ecan}_ allele and control mice were obtained by crossing heterozygous mutants with WT mice to assess the impact of Notch2 ASOs on the Notch2 

_\textit{tm1.1Ecan}_ skeletal phenotype. One-month-old male Notch2 

_\textit{tm1.1Ecan}_ heterozygous mutant and control sex-matched littermate mice were treated with Notch2 ASO (Ionis 977472) or control ASO (Ionis 549144) that was suspended in PBS and administered subcutaneously at a dose of 50 mg/kg once a week for 4 consecutive weeks. Mice were euthanized at 2 months of age. Studies were approved by the Institutional Animal Care and Use Committee of UConn Health.

**μCT**

Bone microarchitecture of femurs from experimental and control mice was determined using a μCT (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland), which was calibrated periodically using a phantom provided by the manufacturer (58, 59). Femurs were scanned in 70% ethanol at high resolution, energy level of 55 kilovoltage peaks, intensity of 145 μA, and integration time of 200 ms. A total of 100 slices at midshaft and 160 slices at the distal metaphysis were acquired at an isotropic voxel size of 216 μm³, with a slice thickness of 6 μm, and then chosen for analysis. Trabecular bone volume fraction and microarchitecture were evaluated starting ~1.0-mm proximal from the femoral condyles. Contours were manually drawn a few voxels away from the endocortical boundary every 10 slices to define the region of interest for analysis. The remaining slice contours were iterated automatically. Trabecular regions were assessed for total volume, bone volume, bone volume fraction (bone volume/total volume), trabecular thickness, trabecular number, trabecular separation, connectivity density, and SMI, using a Gaussian filter (σ = 0.8), and a threshold of 240 per mil eq to 355.5 mg/cm³ hydroxyapatite (58, 59). For analysis of femoral 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 perimeter, endostele perimeter, and material density
were performed using a Gaussian filter (\(\sigma = 0.8,\) support = 1), and a threshold of 400 per mil eq to 704.7 mg/cm\(^3\) hydroxyapatite.

### Bone histomorphometric analysis

Static cancellous bone histomorphometry was carried out on experimental and control mice. The 5-μm longitudinal sections of undecalcified femurs embedded in methyl methacrylate were cut on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI) and stained with 0.1% toluidine blue. Static and dynamic parameters of bone formation and resorption were measured in a defined area between 360 and 2160 μm from the growth plate, using an OsteoMeasure morphometry system (OsteoMetrics, Atlanta, GA). Stained sections were used to measure osteoblast and osteoclast number and eroded surface. Mineralizing surface per bone surface and the mineral apposition rate were measured on unstained sections visualized under UV light and a triple diaminodio-2-phenylindole/fluorescein/Texas Red set long-pass filter, and bone formation rate was calculated. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (60, 61).

### Osteoblast-enriched cell cultures

The parietal bones of 3–5-day-old control and Notch2\(^{tm1.1Ecan}\) mutant mice were exposed to Liberase TL 1.2 units/ml (Sigma) for 20 min at 37 °C, and cells were extracted in five consecutive reactions (62). Cells from the last three digestions were pooled and seeded at a density of 10 × 10\(^4\) cells/cm\(^2\), as described previously (63). Osteoblast-enriched cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with nonessential amino acids (both from Life Technologies, Inc.) and 10% heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) in a humidified 5% CO\(_2\) incubator at 37 °C. Confluent osteoblast-enriched cells were exposed to DMEM supplemented with 10% heat-inactivated FBS, 100 μg/ml ascorbic acid, and 5 mM β-glycerophosphate (both from Sigma) in the presence of Notch2 ASO or control ASO at various doses as indicated in figure legends.

### Osteocyte-enriched cultures

Femurs from 6- to 7-week-old WT or Notch2\(^{tm1.1Ecan}\) mice were collected after sacrifice; the surrounding tissues were dissected; the proximal epiphysis was excised; and the bone marrow was removed by centrifugation. The distal epiphysis was removed, and to release the endosteal and periosteal cellular layers, the femoral fragments were sequentially exposed for 20-min periods to type II collagenase pretreated with 17 μg/ml N\(^\text{a-}\)tosyl-L-lysine chloromethyl ketone hydrochloride and 5 mM EDTA (Life Technologies, Inc.) at 37 °C, as described previously (4, 64). Osteocyte-enriched bone fragments were obtained and cultured individually in DMEM supplemented with nonessential amino acids, 100 μg/ml ascorbic acid, and heat-inactivated 10% FBS for 72 h in a humidified 5% CO\(_2\) incubator at 37 °C in the presence of control or Notch2 ASOs, as indicated in figure legends (4, 65).

### Bone marrow-derived macrophage (BMM) cultures and osteoclast formation

To obtain BMMs, bone marrow cells were isolated from long bones by flushing the marrow with a 26-gauge needle. Red blood cells were lysed in lysis buffer containing 150 mM NH\(_4\)Cl, 10 mM KHCO\(_3\), and 0.1 mM EDTA (pH 7.4). The cell suspension was centrifuged, and the pellet was suspended in α-minimum essential medium (Life Technologies, Inc.) containing 10% heat-inactivated FBS and recombinant human macrophage colony-stimulating factor (M-CSF) at 30 ng/ml. M-CSF was purified as indicated in the figure legends. TRAP-positive cells. RANKL cDNA and expression vector were obtained from D. Fremont (St. Louis, MO), and M-CSF was purified as described (66). Cells were seeded at a density of 3 × 10\(^5\) cells/cm\(^2\) on uncoated Petri dishes and cultured for 3 days.

For osteoclast formation, cells were collected following treatment with 0.05% trypsin/EDTA and seeded at a density of 4.7 × 10\(^8\) cells/cm\(^2\) on tissue culture plates in the presence of M-CSF at 30 ng/ml and murine RANKL at 10 ng/ml until the formation of multinucleated tartrate-resistant acid phosphatase (TRAP)-positive cells. RANKL cDNA and expression vector were obtained from M. Glogau (Toronto, Ontario, Canada), and GSH S-transferase–tagged RANKL was expressed and purified as described (67). TRAP enzyme histochemistry was conducted using a commercial kit (Sigma), in accordance with manufacturer’s instructions. TRAP-positive cells containing more than three nuclei were considered osteoclasts. Cultures were carried out in the presence of Notch2 or control ASO at various doses as indicated in figure legends.

### Cell proliferation assay

Cell replication was determined using the Cell Counting Kit-8. In this kit, the tetrazolium salt WST-8 (2- (2-methoxy-4-nitrophenyl)-3- (4-nitrophenyl)-5- (2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) produces a formazan dye, measured at an absorbance of 450 nm, upon reduction by cellular dehydrogenases. The assay quantifies viable cells and was used in accordance with the manufacturer’s instructions (Dojindo Molecular Technologies, Rockville, MD).

### Quantitative reverse transcription (qRT)-PCR

Total RNA was extracted from either cultured cells or tibiae following the removal of the bone marrow by centrifugation, and mRNA levels were determined by qRT-PCR (68, 69). For this purpose, equal amounts of RNA were reverse-transcribed using the iScript RT-PCR kit (Bio-Rad), according to the manufacturer’s instructions, and were amplified in the presence of specific primers (Table 3, all primers from Integrated DNA Technologies (IDT), Coralville, IA), and iQ 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 \(Bglap\) (from J. Lian, Burlington, VT), \(Hey1\) and \(Hey2\) (both from T. Iso, Gunma, Japan), \(HeyL\) (from D. Srivastava, San Francisco, CA), \(Notch2\) (from Thermo Fisher Scientific), \(Notch1\) (from J. S. Nye, Cambridge, MA), \(Notch4\) (from Y. Shirayoshi, Tottori, Japan), or \(Tnfsf11\) (from Source BioScience, Nottingham, UK) (70–75). \(Notch3\) copy number was estimated by comparison with a serial dilution of an ~100-base pair synthetic DNA template (IDT) cloned into pcDNA3.1 (Thermo Fisher Scientific).
by isothermal single reaction assembly using commercially available reagents (New England Biolabs, Ipswich, MA) (76).

To measure levels of the Notch2<sup>6955C→T</sup> mutant transcript, total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase in accordance with the manufacturer’s instructions (Life Technologies, Inc.) in the presence of reverse primers for Notch2 and of reverse primers for ribosomal protein L38 (Rpl38) (Table 3). Notch2 cDNA was amplified by PCR in the presence of specific primers, a 6-carboxyfluorescein-labeled DNA probe of sequence 5′-CATTGCTAGGCAGC-3′ covalently bound to a 3′-minor groove binder quencher (Life Technologies, Inc.) and SsoAdvanced Universal Probes Supermix (Bio-Rad) at 60°C for 45 cycles (10, 77). Notch2<sup>6955C→T</sup> transcript copy number was estimated by comparison with a serial dilution of a synthetic DNA fragment (IDT) containing 200 bp surrounding the 6955C→T mutation in the Notch2 locus, and it was cloned into pcDNA3.1(+) (Life Technologies, Inc.) by isothermal single-reaction assembly using commercially-available reagents (New England Biolabs, Ipswich, MA) (76).

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 corrected for Rpl38 copy number, as estimated by comparison with a serial dilution of Rpl38 cDNA (from ATCC) (78). In selected experiments, control data were normalized to one following correction for Notch2 transcript copy number, as estimated by comparison with a serial dilution of Notch2<sup>6955C</sup> cDNA.

**Immunoblotting**

Pre-osteoclasts or osteoblasts from control or Notch2<sup>ΔEca</sup> mice were extracted in buffer containing 25 mm Tris-HCl (pH 7.5), 150 mm NaCl, 5% glycerol, 1 mm EDTA, 0.5% Triton X-100, 1 mm sodium orthovanadate, 10 mm NaF, 1 mm phenylmethylsulfonyl fluoride, and protease inhibitor mixture (all from Sigma). Quantified total cell lysates (35 μg of total protein) were separated by SDS-PAGE in 8% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore, Billerica, MA). The blots were probed with anti-NOTCH2 (C651.6DbHN) antibodies (Developmental Studies Hybridoma Bank (DSHB C651.6DbHN, University of Iowa, Iowa City)) and β-actin (3700) antibodies (Cell Signaling Technology, Danvers, MA) and exposed to anti-rabbit IgG and anti-rat IgG conjugated to horseradish peroxidase (Sigma) and incubated with a chemiluminescence detection reagent (Bio-Rad). Chemiluminescence was detected by ChemiDoc™ XRS+ molecular imager (Bio-Rad) with Image Lab™ software (version 5.2.1), and the amount of protein in individual bands was quantified (15).

**Serum carboxy-terminal collagen cross-links assay**

Serum samples from control and experimental mice were obtained after an overnight fast. CTX levels were measured using an ELISA kit according to manufacturer’s instructions (Immunodiagnostic Systems, Gaithersburg, MD).

**Statistics**

Data are expressed as means ± S.D. Statistical differences were determined by analysis of variance with Holm-Sidak’s post hoc analysis for pairwise or multiple comparisons.

**Author contributions**—E. C. conceptualization; E. C. and J. Y. formal analysis; E. C. supervision; E. C. funding acquisition; E. C. writing—original draft; E. C., T. R. G., M. C., L. S., and J. Y. writing-review and editing; T. R. G. and M. C. resources; L. S. and J. Y. investigation.

**Acknowledgments**—We thank D. Srivastava for Hey1 cDNA; D. Fremont for M-CSF cDNA; M. Glogauer for RANKL cDNA; T. Iso for Hey1 and Hey2 cDNA; J. Lian for Bglap cDNA; J. S. Nye for Notch1 cDNA; Y. Shirayoshi for Notch4 cDNA; T. Eller for technical assistance; and Mary Yurczak for secretarial support.

**References**

1. Siebel, C., and Lendahl, U. (2017) Notch signaling in development, tissue homeostasis, and disease. Physiol. Rev. 97, 1235–1294 CrossRef Medline

### Table 3

Primer sets used for qRT-PCR determinations

| Gene  | Strand        | Sequence                          | GenBank<sup>TM</sup> accession no. |
|-------|---------------|-----------------------------------|-----------------------------------|
| Bglap | Forward       | 5′-GACTCCGGGCCTAATCTTGGTAA-3′      | NM_001037939                      |
|       | Reverse       | 5′-CCGAGCACAACCTTCCCTCTTA-3′      |                                   |
| Hey1  | Forward       | 5′-ATCTCAACAAACTACGGATCCCGAC-3′   | NM_010423                         |
|       | Reverse       | 5′-GTTGAGGATGATGTGCTGAAAGG-3′     |                                   |
| Hey2  | Forward       | 5′-ACGCGGAACAAATTTACCTGGGGAC-3′   | NM_013904                         |
|       | Reverse       | 5′-GGTATGTGCCGTCAGTTCGAC-3′       |                                   |
| HeyL  | Forward       | 5′-CAGTAGCTTCTGTAATGGCAAC-3′      | NM_013905                         |
|       | Reverse       | 5′-AGCTTGGAGGAGCCCTTGTTTC-3′      |                                   |
| Notch1| Forward       | 5′-GTCGCCACCATGACACTACCAAGTC-3′   | NM_008714                         |
|       | Reverse       | 5′-GGGGTTGTTCACCGAAGTGTA-3′       |                                   |
| Notch2| Forward       | 5′-TGAAGTTGAGATGAGTAATCCCAAGCC-3′ | NM_010928                         |
|       | Reverse       | 5′-GAAGCTCCGTGCTGACCCCAG-3′       |                                   |
| Notch3| Forward       | 5′-CCGATTCCTCCTGCTGTCTCC-3′       | NM_008716                         |
|       | Reverse       | 5′-TGAAACGAGGGCCTCTGAC-3′         |                                   |
| Notch4| Forward       | 5′-CCAGGCAAGACAGACTGGGTGAC-3′     | NM_010929                         |
|       | Reverse       | 5′-GCAAGCAGCAATCAAGGTGT-3′        |                                   |
| Rpl38 | Forward       | 5′-AGAAACAGGTAATTTGGAAGTCCAGT-3′  | NM_00104857; NM_00104858; NM_023372|
|       | Reverse       | 5′-CTGCTCTAGACTCTGCTCTT-3′        |                                   |
| Tfnsf11| Forward      | 5′-TATAGAATCCGAGCAGCCTCATGAC-3′   | NM_011613                         |
|       | Reverse       | 5′-CCCTGAAAGGCTGTGTTCACTCC-3′     |                                   |
2. Zanotti, S., and Canalis, E. (2016) Notch signaling and the skeleton. *J. Biol. Chem.* 291, 8198–8205
3. Canalis, E. (2018) Notch in skeletal physiology and disease. *Osteoporos. Int.* 29, 2611–2621
4. Schroeter, E. H., Kisslinger, J. A., and Kopan, R. (1998) Notch-1 signaling requires ligand-induced proteolytic release of intracellular domain. *Nature* 393, 382–386
5. Koval, R. A. (2008) More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene* 27, 5099–5109
6. Kaneda, T., Siz, P., Song, L., Aster, J. C., and Blacklow, S. C. (2006) Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell* 124, 973–983
7. Wilson, J. J., and Koval, R. A. (2006) Crystal structure of the CSL–Notch–mastermind ternary complex bound to DNA. *Cell* 124, 985–996
8. Canalis, E., Schilling, L., Yee, S. P., Lee, S. K., and Zanotti, S. (2016) Hajdu-Cheney mouse mutants exhibit osteopenia, increased osteoclastogenesis and bone resorption. *J. Biol. Chem.* 291, 1538–1551
9. Bai, S., Kopan, R., Zou, W., Hilton, M. J., Ong, C. T., Long, F., Ross, F. P., and Teitelbaum, S. L. (2008) NOTCH1 regulates osteoclastogenesis directly in osteoclast precursors and indirectly via osteoblast lineage cells. *J. Biol. Chem.* 283, 6509–6518
10. Fukushima, H., Nakao, A., Okamoto, F., Shin, M., Kajiya, H., Sakano, S., Bigas, A., Jimi, E., and Okabe, K. (2008) The association of Notch2 and NF-kB accelerates RANKL-induced osteoclastogenesis. *Mol. Cell. Biol.* 28, 6402–6412
11. Canalis, E., Yu, J., Schilling, L., Yee, S. P., and Zanotti, S. (2018) The lateral meningocele syndrome mutation causes marked osteopenia in mice. *J. Biol. Chem.* 293, 14165–14177
12. Canalis, E., Parker, K., Feng, J. Q., and Zanotti, S. (2013) Osteoblast lineage-specific effects of Notch activation in the skeleton. *Endocrinology* 154, 623–634
13. Yu, J., and Canalis, E. (2019) The Hajdu-Cheney mutation sensitizes mice to the osteoclastic actions of tumor necrosis factor α. *J. Biol. Chem.* 294, 14203–14214
14. Cheney, W. D. (1965) Acro-osteolysis. *Am. J. Roentgenol. Radium. Ther. Nucl. Med.* 94, 595–607
15. Hajdu, N., and Kaunitz, R. (1948) Cranio-skeletal dysplasia. *Br. J. Radiol.* 21, 42–48
16. Canalis, E. (2018) Clinical and experimental aspects of notch receptor signaling: Hajdu-Cheney syndrome and related disorders. *Metabolism* 80, 48–56
17. Gray, M. J., Kim, C. A., Bertola, D. R., Arantes, P. R., Stewart, H., Simpson, M. A., Irving, M. D., and Robertson, S. P. (2012) Serpine1 fibula polycystic kidney syndrome is part of the phenotypic spectrum of Hajdu-Cheney syndrome. *Eur. J. Hum. Genet.* 20, 122–124
18. Isidor, B., Lindenbaum, P., Pichon, O., Bézieau, S., Dina, C., Jacquemont, S., Martin-Coignard, D., Thauvin-Robinet, C., Le Merrer, M., Mandel, M. E. (2011) Mutations in NOTCH2 in families with Hajdu-Cheney syndrome. *Hum. Mutat.* 32, 1114–1117
19. Simpson, M. A., Irving, M. D., Asilimaz, E., Gray, M. J., Dafou, D., Elmslie, F. V., Mansour, S., Holder, S. E., Brain, C. E., Burton, B. K., Kim, K. H., Pauli, R. M., Aftimos, S., Stewart, H., Kim, C. A., et al. (2011) Mutations in NOTCH2 cause Hajdu-Cheney syndrome, a disorder of severe and progressive bone loss. *Nat. Genet.* 43, 303–305
20. Zhao, W., Petit, E., Gafni, R. I., Collins, M. T., Robey, P. G., Seton, M., Miller, K. K., and Mannstadt, M. (2013) Mutations in NOTCH2 in patients with Hajdu-Cheney syndrome. *Osteoporos. Int.* 24, 2275–2281
21. Uddell, I., Schumacher, H. R., Jr, Kaplan, F., and Fallon, M. D. (1986) Idiopathic familial acro-osteolysis: histomorphometric study of bone and literature review of the Hajdu-Cheney syndrome. *Arthritis Rheum.* 29, 1032–1038
22. Blumenauer, B. T., Cranney, A. B., and Goldstein, R. (2002) Acro-osteolysis and osteoporosis as manifestations of the Hajdu-Cheney syndrome. *Clin. Exp. Rheumatol.* 20, 574–575
23. Andami, G., Rossini, M., Gatti, D., Orsolini, G., Idolazzi, L., Viapiana, O., Scarpa, A., and Canalis, E. (2016) Hajdu-Cheney syndrome: report of a novel NOTCH2 mutation and treatment with denosumab. *Bone* 92, 150–156
24. Byrom, S. W. (2011) The cautionary tale of side effects of chronic Notch1 inhibition. *J. Clin. Invest.* 121, 508–509
25. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craesselaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. I., Goate, A., and Kanap, R. (1999) A presenilin-1-dependent γ-secretase-like protease mediates release of Notch intracellular domain. *Nature* 398, 518–522
26. Duggan, S. P., and McCarthy, J. V. (2016) Beyond γ-secretase activity: the multifunctional nature of presenilins in cell signalling pathways. *Cell Signal.* 28, 1–11
27. Ilagan, M. X., and Kopan, R. (2013) Selective blockade of transport via SERCA inhibition: the answer for oncogenic forms of Notch? *Cancer Cell* 23, 267–269
28. Moellerling, R. E., Corneo, M., Davis, T. N., Del Bianco, C., Aster, J. C., Blacklow, S. C., Kung, A. L., Gilliland, G. D., Verdine, G. L., and Bradner, J. E. (2009) Direct inhibition of the NOTCH transcription factor complex. *Nature* 462, 182–188
29. Wu, Y., Caim-Hom, C., Choy, L., Hagenbeek, T. J., de Leon, G. P., Chen, Y., Finkle, D., Venook, R., Wu, X., Ridgway, J., Schahin-Reed, D., Dow, G. J., Shelton, A., Stavicki, S., Watts, R. J., et al. (2010) Therapeutic antibody targeting of individual Notch receptors. *Nature* 464, 1052–1057
30. Canalis, E., Sanjay, A., Yu, J., and Zanotti, S. (2017) An antibody to Notch2 reverses the osteopenic phenotype of Hajdu-Cheney mutant male mice. *Endocrinology* 158, 730–742
31. Yu, J., Siebel, C. W., Schilling, L., and Canalis, E. (2020) An antibody to Notch3 reverses the skeletal phenotype of lateral meningocele syndrome in male mice. *J. Cell. Physiol.* 235, 210–220
32. Bennett, C. F., Baker, B. F., Pham, N., Swayne, E., and Geary, R. S. (2017) Pharmacology of α-secretase drugs. *Annu. Rev. Pharmacol. Toxicol.* 57, 81–105
33. Cerritelli, S., and Mouch, C. J. (2009) α-secretase: H2 enzymes in eukaryotes. *FEBS J.* 276, 1494–1505
34. Murray, S. F., Jazayeri, A., Mattes, M. T., Yasumura, D., Yang, H., Peralta, R., Watt, A., Freier, S., Hung, G., Adamson, P. S., Guo, S., Monia, B. P., LaVail, M. M., and McCaleb, M. L. (2015) Allele-specific inhibition of rhodopsin with an antisense oligonucleotide slows photoreceptor cell degeneration. *Invest. Ophthalmol. Vis. Sci.* 56, 6362–6375
35. Shy, M. E. (2018) Antisense oligonucleotides offer hope to patients with Charcot-Marie-Tooth disease type 1A. *J. Clin. Invest.* 128, 110–112
36. Carroll, J. B., Warby, S. C., Southwell, A. L., Doty, C. N., Greenlee, S., Sotko, N., Hung, G., Bennett, C. F., Freier, S. M., and Hayden, M. R. (2011) Potent and selective antisense oligonucleotides targeting single-nucleotide polymorphisms in the Huntington disease gene/allele-specific silencing of mutant huntingtin. *Mol. Ther.* 19, 2178–2185
37. Limbroth, V., Barkhof, F., Desem, N., Diamond, M. P., Tachas, G., and ATL1102 Study Group. (2014) CD49d antisense drug ATL1102 reduces
57. Zhang, G., Guo, B., Wu, H., Tang, T., Zhang, B. T., Zheng, L., He, Y., Yang, Z., Pan, X., Chow, H., To, K. Li, Y., Li, D., Wang, X., Wang, Y., et al. (2012) A delivery system targeting bone formation surfaces to facilitate RNAi-based anabolic therapy. *Nat. Med.* 18, 307–314 CrossRef Medline

58. Bouxsein, M. L., Boyd, S. K., Christiansen, B. A., Goldberg, R. E., Jepsen, K. J., and Müller, R. (2010) Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J. Bone Miner. Res.* 25, 1468–1486 CrossRef Medline

59. Glatt, V., Canalis, E., Stadmeyer, L., and Bouxsein, M. L. (2007) Age-related changes in trabecular architecture differ in female and male C57BL/6j mice. *J. Bone Miner. Res.* 22, 1197–1207 CrossRef Medline

60. Dempster, D. W., Compston, J. E., Dzernzer, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., Ott, S. M., Recker, R. R., and Parfitt, A. M. (2013) Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* 28, 2–17 CrossRef Medline

61. Parfitt, A. M., Dzernzer, M. K., Glorieux, F. H., Kanis, J. A., Malluche, H., Meunier, P. J., Ott, S. M., and Recker, R. R. (1987) Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* 2, 595–610 CrossRef Medline

62. Yesil, P., Michel, M., Chwalek, K., Pedack, S., Jany, C., Ludwig, B., Bornstein, S. R., and Lammert, E. (2009) A new collagenase blend increases the number of islets isolated from mouse pancreas. *Islets* 1, 185–190 CrossRef Medline

63. McCarthy, T. L., Centrella, M., and Canalis, E. (1988) Further biochemical and molecular characterization of primary rat parietal bone cell cultures. *J. Bone Miner. Res.* 3, 401–408 CrossRef Medline

64. Halleux, C., Kramer, I., Allard, C., and Kneissel, M. (2012) Isolation of mouse osteocytes using cell fractionation for gene expression analysis. *Methods Mol. Biol.* 816, 55–66 CrossRef Medline

65. Canalis, E., Schilling, L., and Zanotti, S. (2017) Effects of sex and Notch signaling on the osteocyte cell pool. *J. Cell. Physiol.* 232, 363–370 CrossRef Medline

66. Lee, S. H., Rho, J. Y., Jeong, D., Sul, J. Y., Kim, T., Kim, N., Kang, J. S., Miyamoto, T., Suda, T., Lee, S. K., Pignolo, R. I., Koczon-Jaremko, B., Lorenzo, J., and Choi, Y. (2006) v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoblast fusion and increased bone formation. *Nat. Med.* 12, 1403–1409 CrossRef Medline

67. Wang, Y., Lebowitz, D., Sun, C., Tang, H., Grynpas, M. D., and Glogauer, M. (2008) Identifying the relative contributions of Rac1 and Rac2 to osteoclastogenesis. *J. Bone Miner. Res.* 23, 260–270 CrossRef Medline

68. Nazarenko, I., Pires, R., Lowe, B., Obaidy, M., and Rashtchian, A. (2002) Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Res.* 30, 2089–2195 CrossRef Medline

69. Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., and Rashtchian, A. (2002) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res.* 30, e37 CrossRef Medline

70. Iso, T., Sartorelli, V., Chung, G., Shichinohe, T., Kedes, L., and Hamamori, Y. (1997) Proto-oncogene of int-3, a mouse Notch homologue. *J. Biol. Chem.* 272, 213–224 CrossRef Medline

71. Nakagawa, O., Nakagawa, M., Richardson, J. A., Olson, E. N., and Srivastava, D. (1999) HRT1, HRT2 and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev. Biol.* 216, 72–84 CrossRef Medline

72. Wong, B. R., Rho, J., Arron, J., Robinson, E., Orlindick, J., Chao, M., Kala-chikov, S., Cayani, E., Bartlett F. S 3rd, Frankel, W. N., Lee, S. Y., and Choi, Y. (1997) TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. *J. Biol. Chem.* 272, 25190–25194 CrossRef Medline

73. Nye, J. S., Kopan, R., and Axel, R. (1994) An activated Notch suppresses anaplastic lymphoma kinase activity in T cells. *Cell* 25, 386–395 CrossRef Medline

74. Zambetti, G., and Stein, G. (1989) Structure of the rat osteocalcin gene and promoter. *J. Biol. Chem.* 264, 7327–7335 CrossRef Medline

75. McCarty, C. M., Shen, L., and Zambetti, G. P. (1999) A conserved sequence element in the 5′ flanking region of the rat osteocalcin gene is required for tissue-specific expression of the osteocalcin gene in vivo. *J. Biol. Chem.* 274, 18586–18594 CrossRef Medline

76. Tabas, I., Valenti, L., Lavine, J. E., and Pajvani, U. B. (2018) Hepatocyte-specific Notch2 inactivation causes increased trabecular bone mass at specific sites of the appendicular skeleton. *Proc. Natl. Acad. Sci. U. S. A.* 115, 7844–7849 CrossRef Medline

77. Zhang, M., Comfort, N., Wang, B., Amacker, J., and海报, H. (2020) Novel ASOs and Hajdu-Cheney disease. *J. Biol. Chem.* 295, 3952–3964 CrossRef Medline
regulation of vitamin D-dependent expression. Proc. Natl. Acad. Sci. U.S.A. 86, 1143–1147 CrossRef Medline

76. Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., 3rd., and Smith, H. O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345 CrossRef Medline

77. Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., and Hedgpeth, J. (2000) 3’-Minor groove binder–DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28, 655–661 CrossRef Medline

78. Kouadjo, K. E., Nishida, Y., Cadrin-Girard, J. F., Yoshioka, M., and St-Amand, J. (2007) Housekeeping and tissue-specific genes in mouse tissues. BMC Genomics 8, 127 CrossRef Medline