Hajdu Cheney Mouse Mutants Exhibit Osteopenia, Increased Osteoclastogenesis, and Bone Resorption**

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Notch receptors are determinants of cell fate and function and play a central role in skeletal development and bone remodeling. Hajdu Cheney syndrome, a disease characterized by osteoporosis and fractures, is associated with NOTCH2 mutations resulting in a truncated stable protein and gain-of-function. We created a mouse model reproducing the Hajdu Cheney syndrome by introducing a 6955C→T mutation in the Notch2 locus leading to a Q2319X change at the amino acid level. Notch2Q2319X heterozygous mutants were smaller and had shorter femurs than controls; and at 1 month of age they exhibited cancellous and cortical bone osteopenia. As the mice matured, cancellous bone volume was restored partially in male but not female mice, whereas cortical osteopenia persisted in both sexes. Cancellous bone histomorphometry revealed an increased number of osteoclasts and bone resorption, without a decrease in osteoblast number or bone formation. Osteoblast differentiation and function were not affected in Notch2Q2319X cells. The pre-osteoclast cell pool, osteoclast differentiation, and bone resorption in response to receptor activator of nuclear factor κB ligand in vitro were increased in Notch2Q2319X mutants. These effects were suppressed by the γ-secretase inhibitor LY450139. In conclusion, Notch2Q2319X mice exhibit cancellous and cortical bone osteopenia, enhanced osteoclastogenesis, and increased bone resorption.

Notch proteins are four single-pass transmembrane receptors that play a critical role in cell fate decisions (Fig. 1) (1–4). Notch regulates cell renewal and plays a role in skeletal development and homeostasis and in osteoblast and osteoclast differentiation (4–8). Jagged1 and -2 and DeltaLike1, -3, and -4 are the five classic Notch ligands (4). Notch-ligand interactions result in the proteolytic cleavage and release of the Notch intracellular domain (NICD), which translocates to the nucleus to form a complex with recombination signal-binding protein for immunoglobulin κJ region (Rbpjk) and Mastermind-like to regulate transcription (9–12). This canonical signaling pathway leads to the transcription of Hairy Enhancer of Split (Hes)1, -5, and -7 and Hes related with YRPW motif (Hey)1, -2, and -L.

Skeletal cells express Notch1, Notch2, and low levels of Notch3 transcripts (13–15). Activation of Notch in undifferentiated and differentiated osteoblasts inhibits cell differentiation and function and causes osteopenia (16, 17). In contrast, activation of Notch1 in osteocytes causes a pronounced increase in bone mass due to a suppression of bone resorption (18). Results from the conditional inactivation of Notch1 and Notch2 in the developing skeleton confirmed the inhibitory role of Notch in osteoblastogenesis (6, 19). Whereas substantial work has characterized the consequences of Notch1 gain-of-function in the skeleton, there is limited knowledge on the function of Notch2 in the postnatal skeleton. This knowledge is particularly important because Notch1 and Notch2 do not have redundant functions, and Notch1 inhibits, whereas Notch 2 enhances, osteoclastogenesis (13, 20–24).

Hajdu Cheney syndrome is a devastating disease characterized by focal bone lysis of distal phalanges and by generalized osteoporosis (25, 26). Hajdu Cheney syndrome is transmitted as an autosomal dominant disease, although sporadic cases occur. Whole exome sequencing in families affected by Hajdu Cheney syndrome revealed the presence of point mutations or short deletions in exon 34 of NOTCH2 leading to the creation of a stop codon and the premature termination of the protein product upstream from the PEST (proline (P), glutamic acid (E), serine (S), and (T) threonine) domain (27–31). Because the PEST domain is necessary for the ubiquitinylation and degradation of Notch, the mutations lead to a stable NICD protein and persistence of NOTCH2 signaling because the sequences required for the formation of the Notch transcriptional complex are preserved (Fig. 1). Despite the pronounced skeletal abnormalities reported in Hajdu Cheney syndrome, little is known regarding the mechanisms underlying the bone loss or the effects of Notch2 gain-of-function in the skeletal. Information obtained from iliac crest bone biopsies has been inconclusive and reported in a small number of subjects with Hajdu Cheney syndrome (32–35).

The purpose of this work was to gain understanding on the Hajdu Cheney syndrome skeletal phenotype and the mechanisms involved. To this end, we created a mouse model reproducing the NOTCH2 mutation (6949C→T) found in a subject affected by the disease and presenting with pronounced osteoporosis and fractures (28, 30). To create a Hajdu Cheney syn-
Hajdu Cheney Mutants

Experimental Procedures

Hajdu Cheney Mutant Mice—To create a mouse model of Hajdu Cheney syndrome, we reproduced the mutation reported in a subject with the disease (28, 30). In the individual, a C at nucleotide 6949 from the translational start of NOTCH2 mutated into a T (6949C→T) leading to the creation of a premature stop codon in exon 34. The mutation corresponding to the 6949C→T substitution (6955C→T) was introduced into the mouse Notch2 locus by homologous recombination. A targeting vector containing 4.6 kb of 5′-homology arm from exon 30 to exon 33 of Notch2, a phosphoglycerate kinase promoter-driven neomycin (neo) selection cassette flanked by loxP sites ∼400 nucleotides upstream of exon 34, a 6955C→T mutation, and a 3′-homology arm of 3.0 kb, was used (Fig. 1). Embryonic (ES) cells from 129Sv/C57BL/6j embryos were electroporated, and G418-resistant colonies were selected. Targeted clones were verified by long range polymerase chain reaction (PCR) of genomic DNA. To ensure proper integration of the 5′-homology arm, we used forward F1 5′-GGTTGACAGGTATGCA-GTGCAGCG-3′ and reverse R1 5′-GGCTTCTGAGGCGGAAAGAACCAG-3′ primers followed by nested forward F2 5′-GCACATAACACAGGTACGCTGAG-3′ and reverse R2 5′-GATCGGAAATTGGGCTCGAGATTT-3′ primers. To ensure proper integration of the 3′-homology arm, forward F3 5′-GGCTCTGAGGCGGAAAACCCAG-3′ and reverse R3 5′-CAATGGGGAGCCGTCATCATCGG-3′ primers were used (Fig. 1B). The presence of the 6955C→T mutation in the selected clone was confirmed by DNA sequencing (GENEWIZ, South Plainfield, NJ) (Fig. 1). Targeted ES clones were used for aggregations to generate chimeric mice at the Gene Targeting and Transgenic Facility of University of Connecticut Health Center. Chimeric male mice were bred with female mice expressing Cre under the control of the hypoxanthine-guanine phosphoribosyltransferase (Hprt) promoter to remove the PKGneo cassette (36). The removal of the cassette was verified by PCR, and the Hprt-Cre transgene was segregated by crossing with C57BL/6j wild type mice. Genomic DNA was obtained from ear punches of F1 pups, and the Notch2 mutation was confirmed by DNA sequencing (GENEWIZ) as shown in Fig. 1C. Genotyping of Notch2Q2319X mice was conducted in tail DNA extracts by PCR using forward primer Nch2Lox gtF 5′-CCCTTCTCTCTGTTGCCTAG-3′ and reverse primer Nch2Lox gtr 5′-CTCAGAGGCAAGGCTTACTG-3′. In this study, we characterized 129Sv/C57BL/6j mutant mice and sex-matched littermate controls obtained by crossing heterozygous Notch2Q2319X Hajdu Cheney mutants with wild type mice. Studies were approved by the Institutional Animal and Care Use Committees of Saint Francis Hospital and Medical Center and University of Connecticut Health Center.

Microcomputed Tomography (μCT)—Bone microarchitecture of femurs from experimental and control mice was determined using a microcomputed tomography instrument (μCT 40; Scanco Medical AG, Bassersdorf, Switzerland), which was calibrated periodically using a phantom provided by the manufacturer (37, 38). Femurs were scanned in 70% ethanol at high resolution, energy level of 55 peak kV, 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 and a slice thickness of 6 μm and were chosen for analysis. Trabecular bone volume fraction 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. 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 structure model index, using a Gaussian filter (σ = 0.8) and user-defined thresholds (37, 38). 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, endosteal perimeter, and material density was performed using a Gaussian filter (σ = 0.8, support = 1), and user-defined thresholds.

Bone Histomorphometric Analysis—Static and dynamic cancellous bone histomorphometry was carried out on experimental and control mice after they were injected with calcein, 20 mg/kg, and demeclocycline, 50 mg/kg, at an interval of 2 days in 1-month-old or of 5 days in 3-month-old animals. Five micron longitudinal sections of undecalcified femurs embedded in methyl methacrylate were cut on a microtome (Microm, Richmond, WI) (Fig. 1). Slides were left unstained for fluorescence microscopy or stained with hematoxylin/eosin to establish cellular parameters and analyzed at a magnification of ×400 using OsteoMeasureXP software. Stained sections were used to draw the cortical bone, marrow space, osteoid, and cell surfaces as well as to count osteocytes within the cortex and osteoblasts and osteoclasts along the endocortical surface. Data from
1-month-old mice were generated from an all-inclusive cortical section, whereas data from 3-month-old mice were obtained from approximately half of a cortical section. Osteocyte number was expressed as cells/bone area measured. The terminology and units used for cancellous and cortical bone are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (39, 40).

**Biochemical Parameters of Bone Turnover**—Serum levels of C-terminal collagen cross-links, procollagene type I N-terminal propeptide, and tartrate-resistant acid phosphatase form 5b (TRACP5b) were measured by enzyme-linked immunosorbent assay (ELISA) (Immunodiagnostic Systems, Bolton, England). Serum from Notch2<sup>Q2319X</sup> and control littermate mice was obtained following a modification of a previously described assay (ELISA) (Immunodiagnostic Systems, Bolton, England). Serum from Notch2<sup>Q2319X</sup> and control littermate mice was obtained following an overnight fast, and assays were conducted according to the manufacturer’s instructions.

**Osteoblast-enriched Cell Cultures**—The parietal bones of 3- to 5-day-old control and Notch2<sup>Q2319X</sup> mutant mice were exposed to type II collagenase from Clostridium histolyticum (Worthington) pretreated with N-α-tosyl-L-lysyl-chloromethyl ketone hydrochloride at 17 μg/ml (Calbiochem) (41). Bones were digested for 20 min at 37 °C; cells were extracted in five consecutive reactions, and cells from the last three digestions were pooled and seeded at a density of 10,000 cells/cm<sup>2</sup>, as described (42). Osteoblast-enriched cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with non-essential amino acids (both from Gibco and Life Technologies, Inc.), 20 mM HEPES, 100 μg/ml ascorbic acid (both from Sigma), and 10% heat-inactivated fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA) in a humidified 5% CO<sub>2</sub> incubator at 37 °C. To promote maturation, confluent osteoblasts were exposed to DEMEM supplemented with 10% heat-inactivated FBS, 100 μg/ml ascorbic acid, and 5 mM β-glycerophosphate (Sigma).

**Transient Transfections**—Osteoblast-enriched cells were transfected with a plasmid containing six dimeric CSL or Rbpj<sub>α</sub> promoter directs the expression of β-galactosidase (CMV/β-galactosidase; Clontech) was used to correct for transfection efficiency. Cells were exposed to X-tremeGENE 9/DNA mix for 16 h, and the medium was replaced after 24 h. Subsequently, cells were harvested in reporter lysis buffer (Promega, Madison, WI) and lysed by freezing at −80 °C and thawing at 37 °C. Luciferase and β-galactosidase activities were determined, respectively, with the luciferase assay system kit (Promega) and gallocyanin blue (Life Technologies, Inc.) in accordance with manufacturer’s instructions on an Opticomp luminometer (MGM Instruments, Hamden, CT).

**Osteocyte-enriched Cultures**—Osteocyte-enriched cells were obtained following a modification of a previously described method (46). Femurs were removed aseptically from 1-month-old experimental and control mice; the surrounding tissues were dissected; the proximal epiphysal end was excised, and the bone marrow was removed by centrifugation. The distal epiphysis was excised, and femurs were digested for 20 min at 37 °C with type II bacterial collagenase pretreated with N-α-tosyl-β-lysyl-chloromethyl ketone hydrochloride and subsequently exposed to 5 mM EDTA for 20 min at 37 °C. The resulting osteocyte-enriched cortical femurs were cultured individually in DEMEM supplemented with nonessential amino acids (both from Life Technologies, Inc.), 100 μg/ml ascorbic acid, and 10% FBS for 3 days at 37 °C in a humidified 5% CO<sub>2</sub> incubator. Cells were cultured in the absence or presence of the γ-secretase inhibitor LY450139 (Selleck Chemicals, Houston, TX), dissolved in dimethyl sulfoxide (DMSO), and tested at 1 μM (47). An equal amount of DMSO was added to control cultures.

**Bone Marrow Cell Cultures and Osteoclast Formation**—Bone marrow cells were isolated from the femurs by a modification of previously published methods (48, 49). Briefly, bone marrow cells were collected by centrifugation and washed twice with α-minimum essential medium (MEM) (Life Technologies, Inc.) and cultured overnight in α-MEM containing 10% FBS. Nonadherent cells were collected, and bone marrow mononuclear cells were isolated using Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. The interface between Ficoll-Hypaque and medium was collected, and cells were seeded at a density of 1 × 10<sup>6</sup> cells/cm<sup>2</sup> and cultured in α-MEM with 10% heat-inactivated FBS (GE Healthcare) in the presence of macrophage colony-stimulating factor (M-CSf) at 30 ng/ml and receptor activator of nuclear factor κB ligand (Rankl) at 1–30 ng/ml (both from R&D Systems, Minneapolis, MN) for 6 days. In a subsequent experiment, total bone marrow cells were isolated from femurs by centrifugation, cultured in α-MEM in the presence of M-CSf at 100 ng/ml for 6 days, and switched to α-MEM containing M-CSf at 30 ng/ml and Rankl at 10–30 ng/ml for 8 days. Cells were cultured in the absence or presence of the γ-secretase inhibitor LY450139 at 1 μM or DMSO (47). In both experiments, cultured medium was changed every 3 days and cells were fixed for 30 s at room temperature prior to tartrate-resistant acid phosphatase (TRAP) enzyme histochemistry using a commercial kit (Sigma), in accordance with the manufacturer’s instructions. TRAP-positive cells that contained more than three nuclei were considered osteoclast-like cells.

**In Vitro Bone Resorption Assay**—Bone marrow cells were isolated from femurs by centrifugation, expanded in the presence of M-CSf at 100 ng/ml for 5 days, and seeded at a density of 30,000 cells/cm<sup>2</sup> on bovine cortical bone slices in α-MEM with 10% FBS and M-CSf at 30 ng/ml and Rankl at 30 ng/ml each for 16 days. Culture medium was replaced every 4 days with fresh medium containing M-CSf and Rankl. To visualize resorption pits, bone slices were sonicated to remove osteoclasts and stained with 1% toluidine blue in 1% sodium borate. To evaluate the ability of osteoclasts to resorb bone, the number of individual pits and resorption area were measured on images acquired with an Olympus DP72 camera using CellSens Dimension software version 1.6 (Olympus Corp., Center Valley, PA) (50).
Flow Cytometry—Bone marrow cells were obtained by flushing tibiae and collecting cells in α-MEM. After washing in α-MEM, the red blood cells were lysed with ammonium chloride/potassium lysis buffer (Life Technologies, Inc.), and the cell preparation was filtered through a nylon mesh and counted. Dead cells were excluded by their inability to incorporate propidium iodide. Anti-mouse antibodies used for flow cytometric analysis were anti-CD45R (B220) for B-cell lineage cells, anti-CD3 for T-cell lineage cells, anti-CD11b (Mac-1) for macrophage lineage cells, anti-CD117 (c-Kit), a hematopoietic stem cell marker, and anti-CD115 (the M-CSF receptor) (all from BD Biosciences). Antibodies conjugated to fluorochromes, or biotinylated, and secondary step reagents were obtained commercially (eBioscience, San Diego, CA). Labeling of bone marrow cells for flow cytometric analysis was performed by standard staining procedures in Hanks’ balanced salt solution (Life Technologies, Inc.) containing 0.01% gelatin, 5% heat-inactivated fetal bovine serum (FBS), and 5% fetal calf serum (Care, Inc.), member 11 (Rpl38) or glyceraldehyde-3-phosphate dehydrogenase (Gapdh) (Table 1). Notch2 cDNA was amplified by qPCR in the presence of specific primers (Table 1), a TET-labeled DNA probe of sequence 5’-CATTGCCTAGGGCAGC-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 (59). RpL38 or Gapdh cDNA was amplified as described above. Notch2*6955C→T 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 cloned into pcDNA3.1(+) (Life Technologies, Inc.) by isothermal single reaction assembly using commercially available reagents (New England Biolabs, Ipswich, MA) (60).

Amplification reactions were conducted in a CFX96 qRT-PCR detection system (Bio-Rad), and fluorescence was moni-
PCR cycle at the annealing step. Data are expressed as copy number corrected for *Rpl38* or *Gapdh* copy number, estimated by comparison with a serial dilution of *Rpl38* (from ATCC) or *Gapdh* (from R. Wu, Ithaca, NY), respectively (61, 62). To establish changes in gene expression in osteoblast-enriched cultures, data were obtained from three experiments and controls normalized to 1.

**Statistics**—Data are expressed as means ± S.D. Statistical differences were determined by unpaired Student’s *t* test or analysis of variance with Holm-Sidak post hoc analysis for pairwise or multiple comparisons.

**Results**

**Generation and General Appearance of Hajdu Cheney Notch2<sup>Q2319X</sup> Mutant Mice**—To introduce a Hajdu Cheney syndrome mutation into the Notch2 locus, a targeting vector containing a 6955C→T substitution in exon 34 (Fig. 1) was introduced into ES cells derived from an F1 129Sv/C57BL/6j.
embryo by electroporation. A targeted clone was used to generate mutant mice, which were studied following the removal of the selection cassette. Breeding between heterozygous Hajdu Cheney Notch2Q2319X mutant mice resulted in perinatal lethality, whereas matings of heterozygous mutants with wild type mice resulted in no apparent lethality and a similar distribution of 56% wild type and 44% mutant mice in the offspring. Therefore, heterozygous Hajdu Cheney mutant mice were compared with wild type littermates following heterozygous crossings with wild type mice, all in a 129Sv/C57BL/6j genetic background. At 1 month of age, Notch2Q2319X heterozygous mice weighed ~20% less than littermate controls, but their general appearance was not substantially different from controls. As the mice matured, there was less difference in the size of mutant and wild type mice, and mutant mice weighed ~10% less than wild types at 3 months of age. Femoral length was 12% shorter in Notch2Q2319X heterozygous mice of both sexes than controls at 1 month of age, but at 3 months of age, femoral length in Notch2Q2319X mice was not different from controls in male mice and was only 5% shorter in female mice (Fig. 2).

Femoral microarchitecture of Hajdu Cheney Mutant Mice—μCT of the distal femur revealed that male Notch2Q2319X mutant mice had a 50% decrease in trabecular bone volume at 1 month of age, whereas female mice had a 20% decrease in trabecular bone volume (Table 2 and Fig. 3). The osteopenia was due to a reduction in the number of trabeculae and to a lesser extent to a decrease in trabecular thickness. The decreased cancellous bone volume in male mice was associated with decreased connectivity. The decrease in trabecular number was sustained and observed in 3-month-old Notch2Q2319X mutant mice resulting in a non-significant decrease in cancellous bone volume of 30% in male mice and a significant decrease of 50% in female mice (Table 2). There were pronounced changes in the cortical bone structure of Notch2Q2319X mutants of both sexes; cortical bone was thin and porous, and total area as well as bone area were reduced (Table 2 and Fig. 3). The decrease in cortical bone and overall bone size was more pronounced in Notch2Q2319X at 1 month of age, although cortical bone architecture remained affected at 3 months of age in Notch2Q2319X mutants of both sexes.

Cancellous bone histomorphometric analysis of femurs from Notch2Q2319X mutant mice at 1 month of age confirmed the microarchitectural findings and demonstrated decreased bone volume/tissue volume secondary to a decrease in trabecular number in male and female mutant mice (Table 3 and Fig. 3). At 1 month of age, there was an increase in osteoblast surface/bone surface and eroded surface in Notch2Q2319X mutant mice, and there was no change in osteoblast number/perimeter, osteoblast surface, bone formation rate, or osteocyte number/bone area. This indicates that enhanced bone resorption without a coupled bone-forming response was responsible for the skeletal phenotype. At 3 months of age, the cellular phenotype evolved. The numbers of osteoclasts and eroded surface were no longer significantly increased, and osteoblast surface and mineral apposition rates were increased in male mice, a possible reflection of increased bone remodeling or a compensatory bone-forming response to the enhanced bone resorption noted at 1 month of age. An increase in osteoblast number and mineralizing surface was not observed in female mice. Although trabecular number was decreased by ~30% in 3-month-old male mice, bone volume/total volume was only modestly affected.
possibly because, over time, bone formation compensated for the initial increase in bone resorption.

Cortical bone histomorphometry confirmed a decrease in cortical thickness and revealed no difference in osteocyte number (Table 4). There was an increase in endocortical osteoclast number and eroded surface in 1-month-old Notch2Q2319X mutants, but the osteoblast number was not different from controls. At 3 months of age, osteoblast, osteoclast, and osteocyte number were not different between mutant and control mice. The identity of cells in the periosteal surface could not be determined with confidence. Mineral apposition rate was not determined in Notch2Q2319X mutants because limited areas contained well defined double labels. This may suggest that bone formation was impaired in cortical bone.

Although cancellous and cortical bone histomorphometry revealed increased bone resorption in Notch2Q2319X mutants, serum levels of the biochemical markers of C-terminal collagen cross-links and procollagen type 1 N-terminal propeptide were not different between Notch2Q2319X mice and control littermates of both sexes at 1 and 3 months of age, and TRACP5b values were variable (data not shown). This may indicate that bone remodeling was not increased because a bone-forming response was delayed and observed by bone histomorphometry only in 3-month-old male mutant mice or may indicate limited sensitivity and variability of serum assays used to determine bone remodeling (63, 64).

Mechanisms Operational in Notch2Q2319X Mutant Mice—To explore mechanisms that may explain the phenotype of Notch2Q2319X mutant mice, RNA was extracted from femurs from mutant and control mice. qRT-PCR revealed expression of Notch2Q2319X (Notch2Q2319X) and moderately increased mRNA levels of Notch target genes Hey1, Hey2, and HeyL, but not Hes1, in femurs from mutant mice confirming activation of Notch signaling in skeletal tissue (Fig. 4). In accordance with
Cortical bone histomorphometry was performed at mid-diaphysis in femurs from 1- and 3-month-old mutant mice and control littermates (control). Data from 1-month-old mice were generated from analysis of an all-inclusive cortical section, and data from 3-month-old mice were obtained from analysis of an approximate half-cortical section so that bone volume/total volume and total bone area were not calculated but are provided by μCT in Table 2. Osteocyte number was counted in either full or semi-sections and expressed as cells/bone area measured. Values are means ± S.D.

| Table 4: Cortical histomorphometry of 1- and 3-month-old Notch2Q2319X mutant male mice and littermate controls |
|---------------------------------------------------------------|
| Control n = 6 | Notch2Q2319X n = 4 | Control n = 4 | Notch2Q2319X n = 4 |
|----------------|---------------------|----------------|---------------------|
| Bone volume/tissue volume (%) | 38.1 ± 7.5 | 29.7 ± 4.8 | 219 ± 70 | 157 ± 54 |
| Cortical thickness (μm) | 206 ± 30 | 168 ± 11* | 219 ± 70 | 157 ± 54 |
| Bone area (mm²) | 0.54 ± 0.11 | 0.42 ± 0.06 | 219 ± 70 | 157 ± 54 |
| Osteocyte no./bone area (mm²) | 1531 ± 385 | 1405 ± 263 | 219 ± 70 | 157 ± 54 |

* Significantly different from controls, p < 0.05 by unpaired t test.

FIGURE 4. NotchQ2319X, Hey1, Hey2 and HeyL, Hes1, Tnfsf11 (Rankl), and Tnfsf11b (osteoprotegerin) mRNA levels in femoral bones from 1-month-old Hajdu Cheney Notch2Q2319X mutant mice (black bars) and control littermate mice (white bars). Transcript levels are expressed as copy number corrected for Gapdh. Values are means ± S.D.; n = 8 for control; n = 9 for Notch2Q2319X for all transcripts. * significantly different between Notch2Q2319X mutants and control, p < 0.05 by unpaired t test.

The resorptive phenotype observed in 1-month-old mice, there was an increased expression of Tnfsf11, encoding for Rankl, in femurs from Notch2Q2319X mutant mice, and no changes in Tnfsf11b, encoding for osteoprotegerin.

Osteoblast-enriched and Osteocyte-enriched Cell Cultures—To understand the consequences of the Hajdu Cheney Notch2Q2319X mutation in skeletal cells, osteoblast-enriched calvarial cells from mutant mice and littermate controls were cultured. Osteoblasts from Notch2Q2319X mice expressed Notch2Q2319X transscripts, which were not detected in wild type littermate controls. The activity of the target genes Hey1, Hey2, and HeyL and the activity of the transiently transfected 12×CSL-Luc and Hey2-Luc (both p < 0.05) and Hey1-Luc (p > 0.05) reporter constructs were increased in Notch2Q2319X osteoblasts confirming activation of Notch signaling (Fig. 5). In accordance with the histomorphometric findings revealing no changes in osteoblast number or function in 1-month-old Notch2Q2319X mice, Notch2Q2319X mutant cells expressed no significant changes in Bglap and Alpl mRNA levels (7, 17). In accordance with the increase in bone resorption observed in Notch2Q2319X mice, expression of Tnfsf11 was increased in osteoblast- (Fig. 5) and osteocyte-enriched preparations. This increase in osteocyte Tnfsf11 was dependent on activation of Notch signaling because it was prevented by the addition of the γ-secretase inhibitor LY450139. Tnfsf11/Rpl38 copy number was (means ± S.D.; n = 4 for all cultures) 1.2 ± 0.8 in control and 2.8 ± 0.7 (p < 0.05) in Notch2Q2319X osteocyte-enriched cultures. In the presence of the LY450139, the values were 1.3 ± 0.6 in control and 1.1 ± 0.6 in Notch2Q2319X cultures (not significant). In agreement with the lack of an effect of Notch2Q2319X mutant on osteocyte number in cancellous and cortical bone, Casp3 mRNA expression was not affected in osteocyte-rich cultures suggesting that osteocyte apoptosis was not enhanced in Notch2Q2319X mutants. Casp3/Rpl38 copy number was (means ± S.D.) 2.4 ± 1.6 (n = 7) in control and 2.4 ± 2.5 (n = 9) in Notch2Q2319X cultures.

In Vitro Osteoclast Formation and Flow Cytometry—To investigate the cause of the increase in osteoclast number and bone resorption, the number of osteoclast precursors was determined in bone marrow cells from Notch2Q2319X heterozygous and control littermates by flow cytometric analysis. There was a 25% increase in the fraction of B220+ CD3− CD11b−/−/CD115 (c-Fms)high CD117 (c-Kit)high cells in Notch2Q2319X mice compared with controls, and most of the early osteoclastogenic activity resides in these cells (50, 51). Further analysis of the monocyte/macrophage population present in the bone marrow B220+ CD3− CD11b+ CD115+ fraction revealed no difference in additional osteoclast precursor cells between Notch2Q2319X and control mice. Somatic NOTCH2 gain-of-function mutations in exon 34 upstream from the PEST domain have been reported in B-cell lymphomas, and the B-cell fraction was analyzed by flow cytometry (65–67). There was no difference in the population of B-cells (B220+ fraction) in the bone marrow from Notch2Q2319X mice (means ± S.D.; n = 4), 34.8% ± 2.4, when compared with controls 31.9% ± 2.4.

To determine whether the Notch2Q2319X mutation resulted in enhanced osteoclastogenesis, non-adherent bone marrow mononuclear cells isolated by Ficoll-Hypaque gradient centri-
FIGURE 5. Calvarial osteoblast-enriched cells from Notch2Q2319X mutant (black bars) and wild type (white bars) littermate controls were isolated and cultured. A, 12× CSL-Luc, Hey1-Luc, and Hey2-Luc reporter constructs were transiently co-transfected with a CMV β-galactosidase construct; data are expressed as luciferase/β-galactosidase activity. Values are means ± S.D.; n = 6 for all data sets. B, total RNA was extracted, and gene expression was measured by qRT-PCR in the presence of specific primers and probes. Data are expressed as Notch2<sup>Q2319X</sup>-Hey1, Hey2, HeyL, Alpl, Bglap, Tnfsf11 (Rank1), and Tnfsf11b (osteoprotegerin) copy numbers and corrected for Rpl38. Values are means ± S.D.; number of observations for control, n = 12 at 0 days, n = 10 at 3 days, and n = 8 at 7 days; number of observations for Notch2<sup>Q2319X</sup>, n = 12 at 0 and at 3 days and n = 8 at 7 days for all transcripts. For mRNA expression, data were obtained from three experiments and controls normalized to 1. *, significantly different between Notch2<sup>Q2319X</sup> mutant and wild type control cells, p < 0.05 by unpaired t test.

Osteoclasts formed 5 days following cellular exposure to Rankl, and the number of osteoclasts was greater in Notch2<sup>Q2319X</sup> cultures than in controls (Fig. 7). The γ-secretase inhibitor LY450139 decreased the formation of osteoclasts in the initial phases of osteoclast differentiation in control cultures, although not after 8 days of Rankl exposure. These findings confirm previous work suggesting that Notch activation is required for basal osteoclastogenesis (20). LY450139 precluded the effect of the Notch2<sup>Q2319X</sup> mutation on osteoclastogenesis demonstrating that Notch activation is required for this effect of Notch2. Notch2<sup>Q2319X</sup> osteoclasts expressed Notch2 mutant transcripts, whereas control cells did not. There was an increase in the levels of Hes1 mRNA, confirming activation of Notch signaling, and of Acp5 (encoding for TRAP) mRNA in Notch2<sup>Q2319X</sup> cells; LY450139 suppressed the expression of both genes in control and mutant cells (Fig. 7). Neither control nor Notch2<sup>Q2319X</sup> mutant cells expressed detectable levels of Hey1, -2, or -L transcripts (data not shown). These results demonstrate that, in the context of the Notch2<sup>Q2319X</sup> mutation, there is an increase in the pre-osteoclast cell pool as well as in its ability to differentiate into mature osteoclasts, and Notch activation is required for these effects.

To determine the effect of the Notch2<sup>Q2319X</sup> mutation on bone resorption, bone marrow mononuclear cells from control and Notch2<sup>Q2319X</sup> mutants were expanded in the presence of
of age and was accompanied by a shortening of the femoral length suggesting a possible effect on endochondral bone formation and an influence by the Notch2<sup>Q2319X</sup> mutation on skeletal development. The phenotype of 1-month-old mice harboring the Notch2<sup>Q2319X</sup> mutation could be attributed to an increase in osteoclast number and bone resorption. It is of interest that there was a subsequent increase in osteoblast number and mineral apposition rate observed in 3-month-old male, but not female, mutant mice possibly representing a delayed compensatory response or increased bone remodeling. This response may explain a partial recovery in cancellous and cortical bone architecture. Although 1-month-old female mice also exhibited a cancellous bone osteopenic phenotype, it was less pronounced than in male mice, but as female mice matured the osteopenia became more evident. Biochemical markers of bone turnover were not affected in Notch2<sup>Q2319X</sup> mutant mice, but this can be explained by limited sensitivity of the assays and variability of results (63, 64).

The phenotype of the Notch2<sup>Q2319X</sup> mutant mouse recapitulates aspects of Hajdu Cheney syndrome, a progressive disorder characterized by a high degree of phenotypical pleiotropy. Some of the facial features of the syndrome appear in the first few months of life, but the clinical manifestations of the syndrome, including osteoporosis and acral osteolysis, are progressive and more evident during adolescence and adulthood (68, 69). It is of interest that Notch2<sup>Q2319X</sup> mutant Hajdu Cheney mice did not exhibit detectable acral osteolysis or obvious neurological manifestations reported in humans affected by the disease. However, our work is limited to the study of young mice and additional phenotypic manifestations may appear in aging mice.

The phenotype observed in Hajdu Cheney Notch2<sup>Q2319X</sup> mutant mice is distinct from the one reported following the activation of Notch1 in osteoblasts (7, 16). Notch1 activation in immature and mature osteoblasts leads to pronounced osteopenia due to an arrest of osteoblast maturation and function. Because the Notch2<sup>Q2319X</sup> global mutation affects all cell lineages, an osteopenic phenotype secondary to a decrease in osteoblast number or function was conceivable. However, there was no evidence that Hajdu Cheney mutants had decreased osteoblast number or function in cancellous bone, and in vitro experiments revealed no changes in the expression of osteoblast gene markers in cultures of calvarial osteoblasts from Notch2<sup>Q2319X</sup> mutants. Importantly, the phenotype of 1-month-old mice revealed a lack of a bone-forming response to enhanced bone resorption, possibly invoking a direct or indirect inhibition of this process under conditions of Notch2 activation. In this context, we have found that Notch2 induces Nfatc2 in skeletal cells, and Nfatc2 suppresses osteoblast differentiation and function and may contribute to the uncoupling of a bone-forming response to the increase in bone resorption (70, 71). Histomorphometric analysis of cortical bone confirmed an increase in osteoclast number and bone resorption in the endocortical surface of 1-month-old Notch2<sup>Q2319X</sup> mutants.

The osteopenic phenotype of the Hajdu Cheney Notch2<sup>Q2319X</sup> mutant mouse can be explained by a increase in

**Discussion**

Our findings indicate that a global Notch2<sup>Q2319X</sup> gain-of-function mutation causes osteopenia affecting both cancellous and cortical bone. The phenotype appeared as early as 1 month of age and was accompanied by a shortening of the femoral length suggesting a possible effect on endochondral bone formation and an influence by the Notch2<sup>Q2319X</sup> mutation on skeletal development. The phenotype of 1-month-old mice harboring the Notch2<sup>Q2319X</sup> mutation could be attributed to an increase in osteoclast number and bone resorption. It is of interest that there was a subsequent increase in osteoblast number and mineral apposition rate observed in 3-month-old male, but not female, mutant mice possibly representing a delayed compensatory response or increased bone remodeling. This response may explain a partial recovery in cancellous and cortical bone architecture. Although 1-month-old female mice also exhibited a cancellous bone osteopenic phenotype, it was less pronounced than in male mice, but as female mice matured the osteopenia became more evident. Biochemical markers of bone turnover were not affected in Notch2<sup>Q2319X</sup> mutant mice, but this can be explained by limited sensitivity of the assays and variability of results (63, 64).

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3 S. Zanotti and E. Canalis, unpublished observations.
bone resorption and osteoclastogenesis, an effect that has been reported selectively for Notch2 but not for Notch1 (13, 20). Notch2 enhances osteoclastogenesis by interacting with NF-κB on Nfatc1 regulatory elements in cells of the osteoclast lineage (20). A recent study conducted in Rbpj−/− null mice demonstrated that Rbpj−/− inhibits osteoclastogenesis, an effect similar to that reported for Notch1 (72). This would suggest that canonical Notch signaling is responsible for the inhibitory effect of Notch1 on osteoclastogenesis because Rbpj−/− is required for the activation of this signaling pathway. It may also suggest that the induction of osteoclastogenesis by Notch2 operates by non-canonical signaling pathways and that the direct interactions between the Notch2 intracellular domain and NF-κB on the Nfatc1 promoter do not require activation of Notch canonical signaling. It is also possible that interactions between the Notch2 intracellular domain and Rbpj are distinct from those of Notch1, and they may result in different downstream events than those reported for Notch1 (73). It is of interest that Notch2, like Notch1, induced the expression of the canonical target genes Hes1, Hey1, Hey2, and HeyL in skeletal cells indicating a capacity to activate Notch canonical signaling. However, induction of Notch target genes may simply represent activation of Notch signaling and not necessarily imply that the canonical target genes are responsible for the effects observed. Although there was a non-preferential increase in canonical Notch target genes, Hey1, Hey2, and HeyL in Notch2Q2319X mutant osteoblasts, this was not the case in cells of the osteoclast lineage that expressed Hes1 but not Hey1,
Hey2, and HeyL. Confirming previous work, the induction of Hes1 in osteoclasts was dependent not only on Notch2 activation but also on the degree of osteoclast maturation (20). Moreover, the phenotype observed in Notch2Q2319X male mice is in accordance with the known effects of Hes1 on osteoclastogenesis and bone resorption (74). This may suggest that Hes1 is responsible for selected actions of Notch2 or of the Notch2Q2319X mutants in the skeleton.

Although these studies demonstrate induction of Notch target gene mRNA and transactivation, both representing enhanced Notch activation by the Notch2Q2319X mutation, we were not able to determine the mechanism responsible for the gain-of-function. Technical difficulties prevented us from determining Notch2 protein levels and demonstrating a more stable Notch2 protein product in Notch2Q2319X mutant cells. This is because antibodies to detect cleaved Notch2 are not available (75). We suggest that stabilization of the truncated Notch2 protein is the cause of enhanced Notch2 signaling in mice. The mutation in the mouse, like in humans, was created upstream from the PEST domain, which is required for protein degradation (76). Moreover, mutations in the same region of exon 34 of either Notch1 or Notch2 are associated with Notch gain-of-function and signal activation (66, 78, 79). Another limitation of the studies presented is the use of a global knock-in Notch2Q2319X mutation. Although the intent was to reproduce the human syndrome, we cannot exclude systemic effects of the mutant Notch2Q2319X on the skeleton.

It is important to note that Notch operates by distinct mechanisms in different cellular compartments. In bone Notch2Q2319X mutants expressed increased levels of Rankl without changes in osteoprotegerin expression so that the Rankl/osteoprotegerin ratio was increased at the mRNA level, possibly contributing to the resorptive phenotype observed. It is of interest that Rankl expression was increased in both osteoblasts and osteocytes of mutant mice, and this increase was dependent on Notch activation because it was not observed in osteocyte-rich cultures treated with a γ-secretase inhibitor. The increased number of osteoclasts and bone resorption may be secondary to a diversity of mechanisms in addition to the enhanced Rankl expression. The osteoclast cell precursor pool was increased in Notch2Q2319X mutants by ~25%. Moreover, their capacity to differentiate into mature osteoclasts, capable of resorbing bone, in response to Rankl was enhanced, and these mechanisms serve to explain the increased osteoclast number and bone resorption in Notch2Q2319X mutants. These results are consistent with the reported stimulatory effects of Notch2 on osteoclastogenesis and are congruent with a mutation causing a Notch2 gain-of-function.

There was no change in the number of osteocytes in either cancellous or cortical bone of Notch2Q2319X mutants, and caspase 3 expression in osteocytes was not increased, suggesting that osteocyte apoptosis was not affected and probably not responsible for the increased cancellous and cortical bone resorption. Although osteocytes are a rich source of Rankl and osteocyte apoptosis precedes osteoclast recruitment during unloading, inhibition of osteocyte apoptosis prevents the increase in Rankl but does not stop bone resorption suggesting that additional mechanisms play a role in the regulation of bone resorption by these cells (77, 80–82). The cortical porosity observed is likely secondary to the increased osteoclastogenesis and bone resorption in Notch2Q2319X mutants.

Although one needs to be cautious with the extrapolation of these results to human disease, an increase in bone resorption could explain the pronounced osteoporosis suffered by subjects with Hajdu Cheney syndrome. The osteolytic lesions observed in these patients reflect a localized resorptive event as well as an inflammatory process (68). If enhanced bone resorption is responsible for the disease, anti-resorptive therapy could prove beneficial to patients with Hajdu Cheney syndrome; however, clinical data on its effectiveness are sparse. Although enhanced bone resorption was observed, additional undiscovered mechanisms may contribute to the bone loss.

In conclusion, Notch2Q2319X mutant mice replicating the mutation found in subjects with Hajdu Cheney syndrome exhibit marked osteopenia; enhanced osteoclastogenesis and bone resorption are in part responsible for the phenotype observed.

Author Contributions—E. C. designed the research studies, analyzed the data, and wrote the manuscript. L. S. conducted the experiments. S. P. Y, designed and created the mouse model. K. L. conducted the experiments. S. Z. acquired and analyzed the data, conducted the experiments, and edited the manuscript.

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