Sustained Notch2 signaling in osteoblasts, but not in osteoclasts, is linked to osteopenia in a mouse model of Hajdu-Cheney syndrome

Received for publication, March 15, 2017, and in revised form, June 5, 2017 Published, Papers in Press, June 7, 2017, DOI 10.1074/jbc.M117.786129

Stefano Zanotti,‡†‡‡ Jungeun Yu,‡ Archana Sanjay,‡ Lauren Schilling,‡ Chris Schoenherr,‡ Aris N. Economides,‡ and Ernesto Canalis‡†‡

From the Departments of Orthopaedic Surgery and Medicine, and the UConn Musculoskeletal Institute, UConn Health, Farmington, Connecticut 06030 and Regeneron Pharmaceuticals, Tarrytown, New York 10591

Individuals with Hajdu-Cheney syndrome (HCS) present with osteoporosis, and HCS is associated with NOTCH2 mutations causing deletions of the proline-, glutamic acid-, serine-, and threonine-rich (PEST) domain that are predicted to enhance NOTCH2 stability and cause gain-of-function. Previously, we demonstrated that mice harboring Notch2 mutations analogous to those in HCS (Notch2HCS) are severely osteopenic because of enhanced bone resorption. We attributed this phenotype to osteoclast sensitivity to the receptor activator of nuclear factor-κB ligand and increased osteoclastic tumor necrosis factor superfamily member 11 (TNFsf11) expression. Here, to determine the individual contributions of osteoclasts and osteoblasts to HCS osteopenia, we created a conditional-by-inversion (Notch2COIN) model in which Cre recombination generates a Notch2ΔPEST allele expressing a Notch2 mutant lacking the PEST domain. Germ line Notch2COIN inversion phenocopied the Notch2HCS mutant, validating the model. To activate Notch2 in osteoclasts or osteoblasts, Notch2COIN mice were bred with mice expressing Cre from the Lyz2 or the BGLAP promoter, respectively. These crosses created experimental mice harboring a Notch2ΔPEST allele in Cre-expressing cells and control littermates expressing a wild-type Notch2 transcript. Notch2COIN inversion in Lyz2-expressing cells had no skeletal consequences and did not affect the capacity of bone marrow macrophages to form osteoclasts in vitro. In contrast, Notch2COIN inversion in osteoblasts led to generalized osteopenia associated with enhanced bone resorption in the cancellous bone compartment and with suppressed endocortical mineral apposition rate. Accordingly, Notch2 activation in osteoblast-enriched cultures from Notch2COIN mice induced TNFsf11 expression. In conclusion, introduction of the HCS mutation in osteoblasts, but not in osteoclasts, causes osteopenia.

Notch signaling plays a fundamental role in cell fate determination (1). Interactions of the four Notch receptors with cognate ligands of the Jagged and Delta-like families lead to the proteolytic cleavage of the receptor and the release of the Notch intracellular domain (NICD)2 from the cellular membrane (2). Subsequently, the NICD translocates to the nucleus and forms a complex with recombination signal-binding protein for the immunoglobulin κ (Rbpjk), mastermind-like (Maml), and additional DNA-associated proteins to elicit a transcriptional response (3). These events result in the induction of Notch target genes, such as Hes1, Hey1, Hey2, and HeyL (4). Although this signaling mechanism is shared by the Notch paralogs, each receptor has distinct functions (5). The reason appears to be related to the differential cellular pattern of expression of the receptors, structural differences between the paralogs, and interactions of the individual NICDs with Rbpjk (6–8).

Bone remodeling is the process whereby the coordinated activities of osteoclasts and osteoblasts preserve skeletal integrity (9). Osteoclasts are multinucleated bone-resorbing cells formed by the fusion of mononuclear myeloid precursors in the presence of receptor activator of nuclear factor κB ligand (Rankl), a protein encoded by TNFsf11, and macrophage colony-stimulating factor (M-CSF) (10). Osteoblasts are bone-forming cells of mesenchymal origin that regulate bone resorption by secreting Rankl and its decoy receptor, osteoprotegerin (9, 11). Notch1 and Notch2 exhibit distinct functions in skeletal cells, and tight regulation of their activity is essential to maintain bone remodeling (12). Notch1 inhibits osteoclastogenesis and osteoblastogenesis, whereas Notch2 inhibits osteoblast differentiation/function but stimulates osteoclastogenesis (13–19).

Hajdu-Cheney syndrome (HCS) is a rare and devastating disease with multiple systemic manifestations, including osteopo-

The abbreviations used are: NICD, Notch intracellular domain; Ad, adenovirus; α-MEM, α-minimum essential medium; ATCC, American Type Culture Collection; BMM, bone marrow-derived macrophage; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; FLP, flippase; FRT, FLP recombination target; HCS, Hajdu-Cheney syndrome; kb, kilobase; L66, lux66; L71, lux71; L72, lux72; IDT, Integrated DNA Technologies; M-CSF, macrophage colony-stimulating factor; μC, microcomputed tomography; PEST, proline- (P), glutamic acid- (E), serine- (S), and (T) threonine-rich; qRT-PCR, quantitative reverse transcription-PCR; β-glA, β-glutelin polyadenylation signal; Rankl, receptor activator of nuclear factor κB ligand; Rbpjk, recombination signal binding protein for immunoglobulin κ J region; Trap, tartrate-resistant acid phosphatase; SM, structure model index.

This work was supported by National Institutes of Health Grant DK045227 from the NIDDK, C. S. and A. E. receive stock options from Regeneron Pharmaceuticals. 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: Dept. of Orthopaedic Surgery, UConn Health, Farmington, CT 06030-5456. Tel.: 860-679-7978; Fax: 860-679-1474; E-mail: canalis@uchc.edu.

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Edited by Jeffrey E. Pessin

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Figure 1. Engineering of the Notch2\(^{\text{COIN}}\) allele. A. A genomic structure and size of the Notch2 locus with the position of the 34 exons indicated by vertical black bars for coding sequences or white boxes for untranslated regions (UTR). B. Position of the AAG codon (underlined) for lysine 2384 in exon 34. The sequence of the insertion site of the COIN module is in lowercase, and gray and white boxes indicate the coding sequence and the 3'-UTR (r[glpA]), respectively. C. Structure of exon 34 and of the targeting construct correctly integrated. From 5' to 3': lox71 (L71), rabbit \(\beta\)-globin polyadenylation signal, eGFP-coding sequence, internal ribosome entry site (Gtx ires), human influenza hemagglutinin (HA) tag coding sequence, 3'-splice region from the second intron of the rabbit \(\beta\)-globin gene (white curved arrow), and lox66 (L66) that constitute the COIN module and a flippase (FLP) recognition site (FRT)-flanked neo cassette downstream of the human UbP promoter (FRT-hUbP-neo-FRT). Removal of the neo cassette by FLP recombination is indicated (gray dotted lines). D. Representation of the silent COIN module in the antisense orientation and of the splicing event (black dotted lines) that excises the COIN module from the nascent transcript, allowing expression of a wild-type Notch2 mRNA and protein. E. Generation of the Notch2\(^{\text{PEST}}\) allele by Cre recombinase-mediated permanent inversion of the COIN module, and illustration of the splicing event (black dotted lines) that occurs during the maturation of the Notch2\(^{\text{PEST}}\) transcript. The latter is translated into a Notch2 mutant lacking the PEST domain. The position of the silent lox72 (L72) sequence and of the wild-type lox\(P\) site created by Cre recombination of L71 and L66 is indicated. Images are scaled either in kilobase (kb) or bp.

To study the consequences of the Notch2 truncation in specific skeletal cell lineages, Notch2 conditional mice were crossed with appropriate Cre drivers to introduce the mutation in cells of the osteoclast (Lyz2\(^{\text{Cre}}\)) or osteoblast (BGLAP-Cre) lineages. Mutant and control mice were examined for skeletal phenotypic changes by microcomputed tomography (\(\mu\)CT) and bone histomorphometry, and the potential mechanisms of Notch2 action were explored.

Results

Generation of a conditional HCS mouse model

To induce the HCS mutation in selected cell populations, Notch2\(^{\text{COIN}}\) mice were created by inserting an artificial COIN intron into exon 34 of the murine Notch2 locus (Fig. 1A). As a result, exon 34 was split into two exons at a position corresponding to lysine 2384, which is upstream of the PEST domain and downstream of the domains required for the transcriptional activation of Notch2 (NCBI protein database NP035058; Fig. 1B). The COIN module is composed of a gene trap-like lox66 HA-e GFP-polyA_lox71 cassette encoding for a hemagglutinin (HA)-internal ribosome entry site and enhanced green fluorescent protein (eGFP) and placed in the antisense strand. The cassette is preceded by a 3’-splice region derived from the second intron of rabbit HBB2 and followed by the polyadenyl-
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The COIN element contains a neo selection cassette downstream of the Ubp promoter and the EM7 prokaryotic promoter and upstream of the polyadenylation region of Pgk1 flanked by flippase (FLP) recognition target (FRT) sequences (Frt-neo-Pgk1polyA-Frt) (Fig. 1C) (30–32). Prior to Cre recombinase, the COIN module is removed by splicing of the precursor mRNA to generate a Notch2COIN transcript that is indistinguishable from the Notch2WT mRNA (Fig. 1D). In the presence of Cre recombinase, which recognizes the lox71 and lox66 mutant sites in a mirror image configuration, the lox66_HA-egfp-polyA_lox71 cassette is brought into the sense strand, causing the irreversible conversion of the COIN allele. The resulting allele encodes for a bicistronic message that is translated into an HA-tagged Notch2 mutant truncated at lysine 2384 and thereby lacking the PEST domain and eGFP (Fig. 1E). This allele was termed Notch2APEST.

To ensure equivalent expression of the Notch2COIN and Notch2WT alleles, the microarchitecture of the distal femur in 1-month-old Notch2COIN/COIN male and female mice and wild-type C57BL/6J controls of the same sex and age was analyzed. Cancellous bone volume and cortical thickness were not different between Notch2COIN/COIN mice and controls, demonstrating that homozygosity for the Notch2COIN allele has no appreciable effect on femoral microarchitecture (data not shown).

Inversion of the Notch2COIN allele in the germ line causes osteopenia

To validate the Notch2COIN mouse as a model of HCS, the skeletal phenotype of Notch2APEST/WT mice created by inversion of the COIN module in the germ line was determined. To this end, Notch2COIN/WT male mice were crossed with Hprt-Cre female mice to create Notch2APEST/WT mice; these were crossed with wild-type mice to create Notch2APEST/WT heterozygous and control wild-type littermates. COIN inversion was documented by the presence of the Notch2APEST allele in DNA from tails of Notch2APEST/WT mice, and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis of total RNA from tibiae confirmed the expression of the Notch2APEST transcript in mutant mice but not in control littermates (Fig. 2, A and B). Notch2WT transcript levels were ~50% lower in Notch2APEST/WT mice than in wild-type littermates, and this is consistent with a systemic heterozygous Notch2APEST inversion and comparable expression levels of the Notch2APEST and Notch2WT alleles (Fig. 2B).

One-month-old germ line Notch2APEST/WT male mice appeared normal, albeit a small reduction (~5%: p < 0.05) in femoral length was noted. Analysis of the distal femur by μCT revealed that, compared with sex-matched littermate controls, Notch2APEST/WT male mice had a 50% decrease in trabecular bone volume secondary to a reduced number and thickness of trabeculae. Connectivity density was lower, and structure model index (SMI) was higher in Notch2APEST/WT mice than in controls, indicating a prevalence of rod-like trabeculae (Table 1 and Fig. 2C).

Femoral microarchitecture assessed by μCT of 1-month-old Notch2APEST/WT (Notch2APEST) and sex-matched wild-type littersmates (control)

μCT was performed at the femoral distal end for trabecular or midshaft for cortical bone. Values are means ± S.D.

| Control | Notch2APEST |
|---------|-------------|
| Bone volume/total volume (%) | 87.6 ± 0.5 | 85.6 ± 1.6* |
| Trabecular separation (µm) | 134 ± 19 | 129 ± 18 |
| Trabecular no. (1/mm³) | 7.7 ± 1.0 | 5.9 ± 0.4* |
| Trabecular thickness (µm) | 31 ± 1 | 25 ± 2 |
| Connectivity density (1/mm³) | 1300 ± 152 | 720 ± 94* |
| Structure model index | 1.6 ± 0.2 | 2.5 ± 0.1* |
| Density of material (mg HA/cm³) | 923 ± 12 | 930 ± 27 |
| Femoral midshaft cortical bone | n = 4 | n = 5 |
| Porosity (%) | 12.4 ± 0.5 | 14.4 ± 1.6* |
| Cortical thickness (µm) | 110 ± 5 | 97 ± 6* |
| Total area (mm²) | 1.76 ± 0.10 | 1.50 ± 0.09* |
| Bone area (mm²) | 0.59 ± 0.03 | 0.51 ± 0.04* |
| Periosteal perimeter (µm) | 4.7 ± 0.1 | 4.3 ± 0.1* |
| Endocortical perimeter (µm) | 3.8 ± 0.1 | 3.5 ± 0.1* |
| Density of material (mg HA/cm³) | 1001 ± 11 | 999 ± 8 |

* Data are significantly different between control and Notch2APEST, p < 0.05 by t test.

Figure 2. Inversion of the Notch2COIN allele in the germ line causes osteopenia. One-month-old male Notch2APEST/WT mutants (black bars; Notch2APEST) were compared with wild-type littermate controls (white bars) of the same sex. A, DNA was extracted from tail, and Notch2COIN inversion was documented by gel electrophoresis of PCR products obtained with primers specific for the Notch2APEST allele. Arrows indicate the position of the 250-bp amplicon. B, total RNA was extracted from tibiae, and expression of the Notch2WT and Notch2WT mRNA was measured by qRT-PCR. Transcript levels are reported as copy number corrected for Rpl38 mRNA levels; data for Notch2WT were normalized to corrected expression in control. Values are means ± S.D.; n = 4 for control, n = 5 for Notch2APEST, all biological replicates. Two technical replicates were used for each qPCR.

Table 1

Table 1: Femoral microarchitecture assessed by μCT of 1-month-old Notch2APEST/WT (Notch2APEST) and sex-matched wild-type littersmates (control)
to the phenotypic manifestations of Notch2HCS mutant mice (27).

**Inversion of the Notch2COIN allele in the osteoclast lineage does not cause a skeletal phenotype**

To establish whether the osteosare phenotype of the Notch2HCS mutants is secondary to direct effects in cells of the osteoclast lineage, the Notch2COIN allele was introduced into Lyz2Cre/WT heterozygous mice. Subsequently, Lyz2Cre/WT, Notch2COIN/COIN mice were crossed with Notch2COIN/COIN mice for the creation of Lyz2Cre/WT;Notch2APEST/PEST experimental mice and Notch2COIN/COIN littermate controls. In an alternative mating scheme, the Notch2APEST inversion was carried out in the context of Lyz2Cre homozygosity. To this end, Lyz2Cre/Cre;Notch2COIN/COIN mice were crossed with Lyz2Cre/Cre, Notch2COIN/WT mice for the creation of Lyz2Cre/Cre;Notch2APEST/PEST experimental and Lyz2Cre/Cre;Notch2WT/WT control mice. In preliminary studies, we documented that 1- and 4-month-old Lyz2Cre and 1-month-old Lyz2Cre/Cre mice did not have a skeletal phenotype as determined by μCT of distal femurs, when compared with wild-type controls (data not shown). COIN inversion was demonstrated in cultures of bone marrow-derived macrophages (BMMs) from 1-month-old Lyz2Cre/WT,Notch2APEST/PEST and Lyz2Cre/Cre;Notch2APEST/PEST mice, and expression of the Notch2APEST mRNA was detected in total RNA from their parietal bones (Fig. 3, A, B, D, and E). These results demonstrate that the Haja-Cheney mutation was introduced and transcribed in Lyz2-expressing cells. Femoral microradiography of male and female at 1- or 4-month-old Lyz2Cre/WT;Notch2APEST/PEST mice or 1-month-old Lyz2Cre/Cre;Notch2APEST/PEST mice was different from that of wild-type sex-matched littermate controls (Tables 2 and 3). In addition, BMM cultures from either Lyz2Cre/WT, Notch2APEST/PEST or Lyz2Cre/Cre;Notch2APEST/PEST mice and control littermates formed a similar number of osteoclasts in vitro (Fig. 3, C and F).

These results demonstrate that the induction of a dual Notch2 mutant allele in cells of the osteoclastic lineage has no skeletal consequences and that the osteosarcoma phenotype of the global Notch2HCS mutant mice should be attributed to an effect in alternate cells (27).

**Inversion of the Notch2COIN allele in osteoblasts causes osteopenia**

To determine whether the osteopenia observed in mice carrying the HCS mutation is driven by an effect in cells of the osteoblastic lineage, the Notch2APEST mutation was created in Bglap-expressing cells. For this purpose, Bglap-Cre+/−; Notch2COIN/COIN and Notch2COIN/COIN mice were crossed to create Bglap-Cre;Notch2APEST/PEST mice and littermate Notch2COIN/COIN controls. As reported previously, Bglap-Cre transgenics do not have a skeletal phenotype when compared with wild-type mice (15). Inversion of the COIN allele was detected in DNA from parietal bones of Bglap-Cre; Notch2APEST/PEST mice at 1 and 4 months of age but not in littermate controls (Fig. 4A). Accordingly, the Notch2APEST transcript was detected only in bones from Bglap-Cre; Notch2APEST/PEST mice, documenting the induction of the Notch2 activation in osteoblasts causes osteopenia

**Figure 3. Inversion of the Notch2COIN allele in Lyz2-expressing cells has no skeletal consequences.** Documentation of Notch2COIN inversion, analysis of gene expression, and osteostogenesis in 1-month-old Lyz2Cre/WT; Notch2APEST/PEST or Lyz2Cre/Cre; Notch2APEST/PEST (black bars; Notch2APEST/PEST), and sex-matched Notch2COIN/COIN or Lyz2Cre/Cre;Notch2WT/WT (white bars) controls, respectively. A and D, BMMs from 1-month-old Lyz2Cre/WT;Notch2APEST/PEST (A) or Lyz2Cre/Cre;Notch2APEST/PEST (D) mice and respective controls were cultured for 72 h in the presence of M-CSF at 30 ng/ml. DNA was extracted, and Notch2COIN inversion was demonstrated by gel electrophoresis of PCR products obtained with primers specific for the Notch2APEST allele. The arrows indicate the position of the 250-bp amplon. B and E, Notch2APEST transcript levels were measured by qRT-PCR in total RNA from the parietal bones of Lyz2Cre/WT;Notch2APEST/PEST (B) or Lyz2Cre/Cre;Notch2APEST/PEST (E) mice and respective controls. Transcript levels are reported as copy number corrected for Rpl38 RNA levels. Values are means ± S.D.; n = 4–6 biological replicates. Values are means ± S.D.; n = 4 for both controls, n = 4 for Lyz2Cre/WT; Notch2APEST/PEST, n = 6 for Lyz2Cre/Cre; Notch2APEST/PEST, all biological replicates. Two technical replicates were used for each qPCR. C and F, BMMs from 1-month-old Lyz2Cre/WT;Notch2APEST/PEST (C) or Lyz2Cre/Cre;Notch2APEST/PEST (F) mice and respective controls were cultured for 72 h in the presence of M-CSF at 30 ng/ml and then with the addition of Rankl at 10 ng/ml until the formation of osteoclasts. Trap activity was assessed by enzyme histochemistry, and data are expressed as number of osteoclasts per well. Values are means ± S.D.; n = 4 for Notch2COIN/COIN, n = 3 for Lyz2Cre/WT;Notch2APEST/PEST, n = 5 for Lyz2Cre/WT;Notch2APEST/PEST, and n = 4 for Lyz2Cre/Cre; Notch2APEST/PEST mice, all biological replicates.
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Table 2
Femoral microarchitecture assessed by μCT of 1- and 4-month-old Lyz2Cre/WT,Notch2ΔPEST/ΔPEST (Notch2 ΔPEST) mice and sex-matched Notch2COIN/COIN littersmates (control)

μCT was performed at the femoral distal end for trabecular or midshaft for cortical bone. Values are means ± S.D.

|            | Control | 1 Month | Notch2ΔPEST | 4 Months | Notch2ΔPEST |
|------------|---------|---------|-------------|----------|-------------|
|            | Males   |         | Females     |          |             |
| Distal femur trabecular bone |         |         |             |          |             |
| Bone volume/total volume (%)  | 5.7 ± 1.8 | 5.7 ± 1.5 | 6.8 ± 1.6  | 6.7 ± 1.4 | 5.4 ± 1.8  |
| Trabecular separation (μm)    | 224 ± 36  | 220 ± 21 | 226 ± 27   | 290 ± 16  | 298 ± 23   |
| Trabecular no. (1/mm)         | 4.6 ± 0.7 | 4.6 ± 0.5 | 4.5 ± 0.5  | 3.5 ± 0.2 | 3.4 ± 0.2  |
| Trabecular thickness (μm)     | 24 ± 2    | 25 ± 1   | 25 ± 1     | 42 ± 3    | 38 ± 3     |
| Connectivity density (1/mm³)  | 306 ± 130 | 263 ± 101 | 257 ± 97   | 117 ± 27  | 96 ± 55    |
| Structure model index         | 2.8 ± 0.2 | 2.7 ± 0.2 | 2.8 ± 0.2  | 2.6 ± 0.3 | 2.7 ± 0.4  |
| Density of material (mg HA/cm³) | 787 ± 13 | 783 ± 15 | 781 ± 15   | 973 ± 15  | 971 ± 22   |
| Femoral midshaft cortical bone |         |         |             |          |             |
| Bone volume/total volume (%)  | 940 ± 2.5 | 827 ± 3.3 | 833 ± 3.0  | 99.5 ± 0.2 | 99.4 ± 0.2 |
| Porosity (%)                   | 16.0 ± 2.5 | 17.3 ± 3.3 | 16.7 ± 3.0 | 0.5 ± 0.2 | 0.6 ± 0.2 |
| Cortical thickness (μm)        | 84 ± 12   | 83 ± 15  | 87 ± 10    | 170 ± 7   | 170 ± 4    |
| Total area (mm²)               | 1.4 ± 0.17| 1.45 ± 0.10| 1.56 ± 0.09| 1.97 ± 0.13| 2.10 ± 0.10|
| Bone area (mm²)                | 0.4 ± 0.07| 0.45 ± 0.05| 0.45 ± 0.05| 1.05 ± 0.07| 1.17 ± 0.09|
| Periosteal perimeter (mm)      | 4.2 ± 0.3 | 4.3 ± 0.1 | 4.4 ± 0.1  | 5.0 ± 0.2 | 5.1 ± 0.1  |
| Endocortical perimeter (mm)    | 3.6 ± 0.2 | 3.6 ± 0.3 | 3.6 ± 0.3  | 4.0 ± 0.5 | 4.0 ± 0.4  |
| Density of material (mg HA/cm³) | 952 ± 25 | 960 ± 27 | 958 ± 29   | 1217 ± 15 | 1226 ± 20  |

Table 3
Femoral microarchitecture assessed by μCT of 1-month-old Lyz2Cre/WT,Notch2ΔPEST/ΔPEST (Notch2 ΔPEST) and Notch2COIN/COIN mice (control) of the same sex and age

μCT was performed at the femoral distal end for trabecular or midshaft for cortical bone. Values are means ± S.D.

|            | Control | 1 Month | Notch2ΔPEST | 4 Months | Notch2ΔPEST |
|------------|---------|---------|-------------|----------|-------------|
|            | Males   |         | Females     |          |             |
| Distal femur trabecular bone |         |         |             |          |             |
| Bone volume/total Volume (%)  | 8.6 ± 3.2 | 8.16 ± 3.9 | 7.6 ± 4.3  | 5.7 ± 1.3 | 9.6 ± 4.4  |
| Trabecular separation (μm)    | 172 ± 31 | 181 ± 39 | 194 ± 39   | 212 ± 18  | 175 ± 30   |
| Trabecular no./mm (1)         | 6.0 ± 1.1 | 5.3 ± 1.2 | 5.3 ± 1.2  | 4.8 ± 0.4 | 5.9 ± 1.1  |
| Trabecular thickness (μm)     | 27 ± 1   | 27 ± 1   | 26 ± 3     | 26 ± 1    | 27 ± 4     |
| Connectivity density (1/mm³)  | 376 ± 290 | 304 ± 328 | 304 ± 328  | 176 ± 101 | 404 ± 337  |
| Structure model index         | 2.8 ± 0.2 | 2.8 ± 0.1 | 2.8 ± 0.1  | 2.7 ± 0.3 | 2.5 ± 0.1  |
| Density of material (mg HA/cm³) | 1011 ± 6 | 987 ± 15 | 987 ± 15   | 979 ± 34  | 992 ± 16   |
| Femoral midshaft cortical bone |         |         |             |          |             |
| Bone volume/total volume (%)  | 81.6 ± 3.9 | 84.1 ± 2.2 | 84.1 ± 2.2 | 84.1 ± 0.6 | 85.6 ± 2.5 |
| Porosity (%)                   | 18.5 ± 3.9 | 15.9 ± 2.2 | 15.9 ± 2.2 | 15.9 ± 0.6 | 14.4 ± 2.5 |
| Cortical thickness (μm)        | 97.1 ± 13 | 105.6 ± 10| 105.6 ± 10| 97.6 ± 6.6 | 111 ± 16   |
| Total area (mm²)               | 1.55 ± 0.07| 1.51 ± 0.13| 1.51 ± 0.13| 1.50 ± 0.17| 1.55 ± 0.15|
| Bone area (mm²)                | 0.54 ± 0.03| 0.53 ± 0.08| 0.53 ± 0.08| 0.49 ± 0.01| 0.57 ± 0.06|
| Periosteal perimeter (mm)      | 4.4 ± 0.1 | 4.3 ± 0.2 | 4.3 ± 0.2  | 4.3 ± 0.2 | 4.4 ± 0.2  |
| Endocortical perimeter (mm)    | 3.6 ± 0.1 | 3.5 ± 0.1 | 3.5 ± 0.1  | 3.5 ± 0.3 | 3.5 ± 0.3  |
| Density of material (mg HA/cm³) | 1047 ± 3 | 1066 ± 7 | 1067 ± 7   | 1061 ± 14 | 1077 ± 49  |

decreased in female mutant mice, and cortical bone was porous (Fig. 5B and Table 4). At 4 months of age, the skeletal phenotype of BGLAP-Cre;Notch2ΔPEST/ΔPEST female mice was less pronounced, and cancellous bone volume/total volume was 30% lower than in control littersmates (p < 0.071). A modest cortical osteopenia with cortical thinning and increased porosity was observed in BGLAP-Cre;Notch2ΔPEST/ΔPEST 4-month-old mice of both sexes (Fig. 6B and Table 4).

Cancellous bone histomorphometry of the distal femur of 1-month-old female BGLAP-Cre;Notch2ΔPEST/ΔPEST mice confirmed the decreased bone volume/tissue volume secondary to a reduced number of trabeculae. Eroded surface and osteoclast numbers were increased, whereas the numbers of osteoblasts and bone formation rates were not different from control littersmates (Table 5). Cortical bone histomorphometry revealed a suppressed endocortical mineral deposition.
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Figure 4. Inversion of the Notch2COIN allele in osteoblasts leads to Notch2 activation in vivo. Documentation of Notch2COIN inversion and analysis of gene expression in BGLAP-Cre;Notch2ΔPEST/ΔPEST (black bars; Notch2ΔPEST) and Notch2COIN/COIN littermate controls (white bars). A, DNA was extracted from the parietal bones of 1- and 4-month-old male mice, and Notch2COIN inversion was demonstrated by gel electrophoresis of PCR products obtained with primers specific for the Notch2ΔPEST allele. The arrows indicate the position of the 250-bp amplicon. B, gene expression was measured by qRT-PCR in total RNA from tibiae of Notch2COIN/COIN (black bars; Notch2ΔPEST) and Notch2COIN/COIN (white bars; Notch2COIN) newborns. Cultures were infected with an adenoviral vector expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter, and parallel cultures infected with an adenoviral vector where the CMV promoter governs GFP expression (Ad-CMV-GFP) served as controls. Ad-CMV-Cre, but not Ad-CMV-GFP, infection led to the inversion of the COIN module and expression of the Notch2ΔPEST mRNA associated with induction of Hey1 and HeyL, demonstrating activation of Notch signaling (Fig. 6, A and B). In accordance with the enhanced bone resorption

apposition rate in BGLAP-Cre;Notch2ΔPEST/ΔPEST mice (Table 6).

Inversion of the Notch2COIN allele in osteoblasts induces Tnfsf11

To determine the mechanisms responsible for the skeletal phenotype of the BGLAP-Cre;Notch2ΔPEST/ΔPEST mice, osteoblast-enriched cells were obtained from the parietal bones of Notch2COIN/COIN newborns. Cultures were infected with an adenoviral vector expressing Cre recombinase under the control of the cytomegalovirus (CMV) promoter, and parallel cultures infected with an adenoviral vector where the CMV promoter governs GFP expression (Ad-CMV-GFP) served as controls. Ad-CMV-Cre, but not Ad-CMV-GFP, infection led to the inversion of the COIN module and expression of the Notch2ΔPEST mRNA associated with induction of Hey1 and HeyL, demonstrating activation of Notch signaling (Fig. 6, A and B). In accordance with the enhanced bone resorption
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Table 4

| Femoral microarchitecture assessed by μCT of 1- and 4-month-old BGLAP-Cre;Notch2ΔPEST/ΔPEST (Notch2ΔPEST) mice and sex-matched Notch2COIN/COIN littermates (control) |
|-------------------------------------------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                                                                                   | 1 Month          | 4 Months        | 1 Month          | 4 Months        |
|                                                                                                   | Control          | Notch2ΔPEST     | Control          | Notch2ΔPEST     |
| **Males**                                                                                         |                  |                 |                  |                 |
| Distal femur trabecular bone                                                                      |                  |                 |                  |                 |
| Bone volume/total volume (%)                                                                      | 10.0 ± 4.4       | 10.4 ± 5.5      | 13.3 ± 2.5       | 11.7 ± 2.8      |
| Trabecular separation (μm)                                                                        | 176 ± 32         | 186 ± 29        | 207 ± 30         | 226 ± 48        |
| Trabecular no. (1/mm)                                                                             | 5.9 ± 1.0        | 5.6 ± 0.9       | 4.9 ± 0.7        | 4.6 ± 0.9       |
| Trabecular thickness (μm)                                                                          | 28 ± 4           | 29 ± 6          | 44 ± 5           | 40 ± 3          |
| Connectivity density (1/mm³)                                                                       | 500 ± 262        | 532 ± 224       | 341 ± 124        | 361 ± 159       |
| Structure model index                                                                             | 2.6 ± 0.5        | 2.4 ± 0.5       | 2.0 ± 0.2        | 2.1 ± 0.3       |
| Density of material (mg HA/cm³)                                                                    | 799 ± 15         | 791 ± 11        | 941 ± 10         | 927 ± 11*       |
| Femoral midshaft cortical bone                                                                    |                 |                 |                  |                 |
| Bone volume/total volume (%)                                                                      | 83.7 ± 2.9       | 83.6 ± 1.6      | 88.5 ± 1.0       | 87.0 ± 0.9*     |
| Porosity (%)                                                                                      | 16.3 ± 2.9       | 16.5 ± 1.5      | 11.5 ± 1.0       | 13.0 ± 0.9*     |
| Cortical thickness (μm)                                                                            | 95 ± 13          | 93 ± 14         | 179 ± 7          | 158 ± 9*        |
| Total area (mm²)                                                                                   | 1.52 ± 0.14      | 1.65 ± 0.16     | 2.2 ± 0.2        | 2.2 ± 0.3       |
| Bone area (mm²)                                                                                   | 0.48 ± 0.07      | 0.51 ± 0.10     | 1.00 ± 0.08      | 1.03 ± 0.33     |
| Periosteal perimeter (mm)                                                                          | 4.4 ± 0.2        | 4.5 ± 0.2       | 5.2 ± 0.2        | 5.3 ± 0.4       |
| Endocortical perimeter (mm)                                                                        | 3.6 ± 0.1        | 3.8 ± 0.1*      | 3.8 ± 0.2        | 3.9 ± 0.4       |
| Density of material (mg HA/cm³)                                                                    | 967 ± 27         | 961 ± 29        | 1198 ± 12        | 1182 ± 11*      |
| **Females**                                                                                       |                  |                 |                  |                 |
| Distal femur trabecular bone                                                                      |                  |                 |                  |                 |
| Bone volume/total volume (%)                                                                      | 9.3 ± 3.0        | 4.3 ± 2.3*      | 5.7 ± 1.0        | 4.0 ± 1.7*      |
| Trabecular separation (μm)                                                                          | 175 ± 32         | 293 ± 56*       | 309 ± 37         | 377 ± 64*       |
| Trabecular no. (1/mm)                                                                             | 5.9 ± 1.0        | 3.6 ± 0.8*      | 3.3 ± 0.4        | 2.8 ± 0.5*      |
| Trabecular thickness (μm)                                                                          | 27 ± 1           | 25 ± 4          | 43 ± 3          | 39 ± 4*         |
| Connectivity density (1/mm³)                                                                       | 517 ± 244        | 187 ± 178*      | 103 ± 32         | 79 ± 45         |
| Structure model index                                                                             | 2.6 ± 0.2        | 3.0 ± 0.3*      | 2.7 ± 0.1        | 2.7 ± 0.3       |
| Density of material (mg HA/cm³)                                                                    | 791 ± 11         | 782 ± 11*       | 947 ± 16         | 921 ± 13*       |
| Femoral midshaft cortical bone                                                                    |                 |                 |                  |                 |
| Bone volume/total volume (%)                                                                      | 83.3 ± 1.3       | 79.6 ± 3.2*     | 87.6 ± 1.1       | 86.3 ± 0.7*     |
| Porosity (%)                                                                                      | 16.7 ± 1.3       | 20.4 ± 3.2*     | 12.4 ± 1.1       | 13.7 ± 0.7*     |
| Cortical thickness (μm)                                                                            | 93 ± 7           | 78 ± 8*         | 171 ± 16         | 152 ± 7*        |
| Total area (mm²)                                                                                   | 1.54 ± 0.15      | 1.44 ± 0.18     | 1.73 ± 0.14      | 1.60 ± 0.07     |
| Bone area (mm²)                                                                                   | 0.48 ± 0.06      | 0.40 ± 0.05*    | 0.85 ± 0.12      | 0.74 ± 0.05*    |
| Periosteal perimeter (mm)                                                                          | 4.4 ± 0.2        | 4.2 ± 0.3       | 4.7 ± 0.2        | 4.5 ± 0.1       |
| Endocortical perimeter (mm)                                                                         | 3.7 ± 0.2        | 3.6 ± 0.2       | 3.3 ± 0.1        | 3.3 ± 0.1       |
| Density of material (mg HA/cm³)                                                                    | 965 ± 29         | 941 ± 34        | 1235 ± 7         | 1211 ± 15*      |

* Data are significantly different between control and Notch2ΔPEST, p < 0.05 by unpaired t test.

Discussion

In this study, the individual contributions of the osteoclast and osteoblast lineages to the bone loss observed in Notch2HCS mutant mice were explored by the conditional introduction of the HCS genetic defect in selected cell lineages. The mutations associated with the disease occur within exon 34 of NOTCH2, and conditional insertion of a premature STOP codon in the homologous region of the murine Notch2 locus was achieved by the creation of a COIN allele. The COIN module can be introduced directly into coding exons without disrupting the expression or function of the targeted allele, a goal that cannot be accomplished with traditional Cre-loxP approaches (28). Absence of an appreciable phenotype in Notch2ΔPEST/ΔPEST mice documented the skeletal equivalency of the wild-type and engineered Notch2 alleles prior to Cre-mediated inversion. The Notch2ΔPEST mutants generated by germ line inversion of the COIN module expressed the Notch2ΔPEST transcript and exhibited a 50% reduction in wild-type Notch2 mRNA, indicating comparable expression levels of maternal and paternal Notch2. Notch2ΔPEST germ line mice exhibited generalized osteopenia and reduced bone size and length, phenocopying global Notch2HCS mutants. These results validated the COIN strategy and confirmed that generalized expression of a Notch2 mutant lacking the PEST domain causes bone loss (27). Although these findings should be extrapolated with caution to the human condition, they support the concept that de novo or inherited dominant NOTCH2 gain-of-function mutations are responsible for the bone loss in subjects with HCS (33).

Selective introduction of the HCS mutation in osteoblasts, but not in cells of the myeloid lineage, led to generalized bone loss. The reduction in cancellous bone volume was observed only in female mice and was more pronounced in younger BGLAP-Cre;Notch2ΔPEST/ΔPEST mice. The bone loss was attributed to enhanced bone resorption uncoupled from a bone-forming response and suppressed endocortical bone formation. These features are consistent with the skeletal phenotype of global Notch2HCS mutants and demonstrate that a direct effect in osteoblasts is largely responsible for the osteopenia associated with the HCS mutation in mice (27). Absence of a phenotype in Lyz2Cre/WT;Notch2ΔPEST/ΔPEST and Lyz2Cre/Cre;Notch2ΔPEST/ΔPEST mice is congruent with the observation that the Notch2 deletion in Lyz2-expressing cells has no consequences on skeletal homeostasis (18). These results indicate that either the activation or inactivation of Notch2 in myeloid cells in vivo has no skeletal consequences and that the effect of Notch2 on bone resorption is secondary to its actions on alter-
mice, Notch2\textsuperscript{APEST/APEST} osteoblasts expressed increased levels of Tnfs11 mRNA suggesting that osteoblast-derived Rankl is responsible for the enhanced bone resorption in vivo in HCS mutant mice. These findings are in agreement with those in a subject with HCS and severe osteoporosis who was reported to present with elevated levels of circulating RANKL (27, 35). However, a limitation of this work was the inability to detect Rankl protein by Western blot analysis in either control or Notch2\textsuperscript{APEST/APEST} osteoblasts. This is possibly related to low levels of Rankl expression and the lack of available antibodies with sufficient sensitivity to detect murine Rankl in osteoblasts. There was an absence of a bone-forming response to the increased bone resorption implying that Notch2 inhibits bone formation.

Moreover, Notch2 gain-of-function suppresses endocortical mineral apposition rate, an effect that possibly contributes to the cortical osteopenic phenotype. The role of Notch2 as an inhibitor of bone formation is supported by previous studies demonstrating that deletion of Notch2 in Runx2-expressing cells increases trabecular bone volume due to enhanced osteoblast differentiation and activity (18). Further support for an inhibitory role of Notch2 on bone formation is derived from studies showing that the dual inactivation of Notch1 and Notch2 in cells of the osteoblastic lineage increases bone mass (36, 37).

It is important to mention that some discrepancies exist between the phenotypes of the BGLAP-Cre;Notch2\textsuperscript{APEST/APEST} mice and of the global Notch2HCS mutants (27). The osteoblast-selective mutation did not affect femoral length, and this was expected because the BGLAP-Cre transgene is not expressed in chondrocytes, cells that govern longitudinal bone growth. Direct inhibitory effects of Notch2 on endochondral bone formation are accountable for the reduced femoral length of the Notch2HCS mutants (38, 39). Cancellous bone osteopenia was detected only in female BGLAP-Cre;Notch2\textsuperscript{APEST/APEST} mice, although both sexes were affected by the global Notch2HCS mutation (27). These sex-related differences may be secondary to the more pronounced expression of the BGLAP-Cre transgene in female than in male mice. Alternatively, a higher rate of bone remodeling in young female than in male mice, a known attribute of the C57BL/6 genetic background, might have sensitized female mice to a greater activation of Notch2 in osteoblasts (40, 41).
**Notch2 activation in osteoblasts causes osteopenia**

osteopenia was milder in *BGLAP-Cre;Notch2^ΔPEST/ΔPEST* than in the *Notch2HCS* mice, and low expression of the *BGLAP-Cre* transgene during embryonic skeletal development might account for the less pronounced phenotype of the conditional mice (42). It is of interest that the *BGLAP-Cre; Notch2^ΔPEST/ΔPEST* mice did not display the increase in endocortical bone resorption observed in the global *Notch2HCS* mutants. This difference may also account for the modest cortical bone phenotype of the conditional mice and suggests that the presence of the HCS mutation in both osteoclasts and osteoblasts might be necessary to recapitulate the cortical bone-resorptive phenotype and osteopenia of the *Notch2HCS* mouse (27).

The conditional HCS model described in this study reaffirmed that Notch2, like Notch1, increases the transcript levels of *Hey1*, *Hey2*, and *HeyL*, thereby confirming that both paralogs are able to activate Rbpjk-mediated Notch signaling in skeletal cells. The increase in mRNA levels for the Notch target genes reflects activation of the Notch canonical pathway but does not imply that Hey proteins mediate the effects of Notch2 in bone. In fact, either generalized or skeletal misexpression of *Heys* has a small impact on skeletal microarchitecture (43–46). The current observations also indicate that Notch1 and Notch2 have distinct skeletal functions because Notch1 induces osteoprotegerin and inhibits bone resorption, whereas Notch2 induces Rankl and stimulates the resorptive event.

In conclusion, osteoblast expression of a *Notch2* mutant lacking the PEST domain causes osteopenia in mice.

**Experimental procedures**

**Creation of the Notch2^COIN* mouse**

The targeting vector containing the COIN element was electroporated into embryonic stem (ES) cells, and the cassette was used for the selection of G418-resistant cells from 129sv/J/C57BL/6j embryos at the Gene Targeting and Transgenic Facility of UConn Health. Targeted clones were verified by long-range PCR of genomic DNA. Correct integration of the 5′-homology arm was verified with forward F1 5′-GGAGGTT-GCTTACCACTCC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTTGAAAACCTTGCTCCCTC-3′ and reverse R1 5′-CACCTG...-TGGTCAAGGGCAGGAGATCAACAG-3′ primers followed by nested forward F3 5′-CACTTGAGAGCAAGGCTG-3′ and reverse R2 5′-CCAAAACCCGGCGCGGAGGC-3′. Proper integration of the 3′-homology arm was ensured with forward F3 5′-CCAAAACCCGGCGCGGAGGC-3′ and reverse R3 5′-CACTTGAGAGCAAGGCTG-3′ primers followed by nested forward F4 5′-CCTTCTCTTTTCTTACTAGTACCC-3′ and reverse R4 5′-GGTGCAAGGGCAGGAGATCAACAG-3′ primers (all primers from Integrated DNA Technologies, IDT, Coralville, IA). Positive ES clones were used for morula aggregations and the creation of chimeras, and the Frt-neo-PgkIpolyA-Frt cassette was removed by FLP recombination following crosses of male chimeras with mice expressing FLP under the control of the Rosa26 promoter (*Rosa26^FLP*; The Jackson Laboratory, Bar Harbor, ME) (47, 48). Excision of the cassette was verified by PCR in ear punches of F1 pups, and the *Rosa26^FLP* allele segregated by breeding with C57BL/6j wild-type mice. Correct integration of the COIN module into the Notch2 locus was confirmed in the progeny by loss of wild-type allele assay.

**Induction of the HCS mutation in the germ line, osteoclasts, or osteoblasts**

To test whether the *Notch2^COIN* and *Notch2^WT* alleles are functionally equivalent, the skeletal phenotype of *Notch2^COIN/COIN* mice was compared with the phenotype of wild-type C57BL/6j controls of the same age and sex. To achieve systemic inversion of the *Notch2^COIN* allele, F1 heterozygous *Notch2^COIN/WT* male mice were bred with female mice expressing Cre under the control of the Hprt promoter (Hprt^CRE) (49). This resulted in the germ line inversion of the COIN module and consequent creation of mice heterozygous for the *Notch2^ΔPEST* allele (*Notch2^ΔPEST/WT*). The latter were crossed with wild-type C57BL/6j mice to generate *Notch2^ΔPEST/WT* experimental and wild-type control cohorts.

C57BL/6j mice where the Cre coding sequence was inserted into the endogenous *Lyz2* locus (*Lyz2^CRE*; The Jackson Laboratory) were used to express Cre recombinase in cells of the myeloid lineage (50, 51). To induce inversion of the COIN module in osteoclast precursor, homozygous *Notch2^COIN* mice heterozygous for the *Lyz2^CRE* allele (*Lyz2^CRE/WT*; *Notch2^COIN/COIN*) were bred with *Notch2^COIN/COIN* mice to create *Lyz2^CRE/WT*; *Notch2^ΔPEST/WT* mice. In an alternate mating scheme, heterozygous *Notch2^COIN* mice homozygous for the *Lyz2^CRE* allele (*Lyz2^CRE/C^ E; *Notch2^COIN/WT*) were inter-mated to create *Lyz2^CRE/WT*; *Notch2^ΔPEST/WT* experimental and *Lyz2^CRE/WT*; *Notch2^WT/WT* control mice.

C57BL/6j mice harboring a transgene where the Cre recombinase coding sequence was cloned downstream a 3.9-kb human *BGLAP* promoter fragment (*BGLAP-Cre*; The Jackson Laboratory) were used to induce inversion of the COIN module in osteoblasts (42). Hemizygous *BGLAP-Cre* transgenics homozygous for the *Notch2^COIN* allele (*BGLAP-Cre; *Notch2^COIN/COIN*) were bred with *Notch2^COIN/COIN* mice to generate *BGLAP-Cre;Notch2^ΔPEST/ΔPEST* experimental and *BGLAP-Cre;Notch2^WT/WT* littermate control cohorts.

Allelic composition was determined by PCR analysis in tail DNA with primers specific for the Hprt^WT, Hprt^CRE, Notch2^WT, Notch2^COIN, Notch2^ΔPEST, Ly2^CRE*, and Ly2^WT* alleles and for the *BGLAP-Cre* transgene. Inversion of the COIN module was documented by PCR analysis in DNA from BMMs or parietal bones (all primers were from IDT; Table 7). The generation and establishment of the *Notch2^COIN* mouse line were approved by the Institutional Animal Care and Use Committee of UConn Health and of Saint Francis Hospital Medical Center. All other studies were approved by the Institutional Animal Care and Use Committee of UConn Health.

**Microcomputed tomography**

Femoral microarchitecture was determined using a microcomputed tomography instrument (Scanco µCT 40; Scanco Medical AG, Bussersdorf, Switzerland), which was calibrated periodically using a phantom provided by the manufacturer (41, 52). Femurs were scanned in 70% ethanol at high resolution, energy level of 55 peak kV, intensity of 145 µA, and inte-
mation rate of 200 ms. A total of 100 slices at midshaft and 160 slices at the distal metaphysis was acquired at an isotropic voxel size of 216 μm$^3$ and a slice thickness of 6 μm and chosen for analysis. Trabecular bone volume fraction (bone volume/total volume) and microarchitecture were evaluated starting ~1.0-mm proximal from the femoral condyles. Contours were manually drawn every 10 slices, a few voxels away from the endocortical boundary, to define the region of interest for analysis. Whereas the remaining slice contours were iterated automatically. Total volume, bone volume, bone volume fraction, trabecular thickness, trabecular number, connectivity density, SMI, and material density were measured in trabecular regions using a Gaussian filter (σ = 0.8) and user-defined thresholds (41, 52). For analysis of cortical bone, contours were iterated across 100 slices along the cortical shell of the femoral midshaft, excluding the marrow cavity. Analysis of bone volume/total volume, porosity, cortical thickness, total cross-sectional and cortical bone area, periosteal and endosteal perimeter, and material density were conducted using a Gaussian filter (σ = 0.8, support = 1) with operator-defined thresholds.

**Bone histomorphometry analysis**

Bone histomorphometry was carried out in 1-month-old mice injected with 20 mg/kg calcein and 50 mg/kg demeclocycline at a 2-day interval and sacrificed 2 days after demeclocycline administration. Femurs were dissected, fixed in 70% ethanol, and embedded in methyl methacrylate. For cancellous bone analysis, bones were sectioned at a thickness of 5 μm along the sagittal plane on a Microm microtome (Richards-Allan Scientific, Kalamazoo, MI) and ground methyl methacrylate and cut through the mid-diaphysis along the transverse plane with an EXAKT Precision Saw, ground using an EXAKT 400 CS Micro Grinding System (Exakt Technologies, Oklahoma City, OK), and surface-polished to a thickness of ~15 μm (Alizee Pathology, Baltimore, MD). Parameters of cortical bone morphometry were measured at a magnification of ×400 using OsteoMeasureXP software (Osteometrix). Stained sections were used to draw the cortical bone, marrow space, and cell surfaces, as well as to measure osteoblasts and osteoclasts along the endocortical surface. Mineral apposition rate was measured in unstained sections under UV light, using a triple diamidino-2-phenylindole/fluorescein/Texas Red set long pass filter, and bone formation rate was calculated.

For cortical histomorphometry, femurs were embedded in methyl methacrylate and cut through the mid-diaphysis along the transverse plane with an EXAKT Precision Saw, ground using an EXAKT 400 CS Micro Grinding System (Exakt Technologies, Oklahoma City, OK), and surface-polished to a thickness of ~15 μm (Alizee Pathology, Baltimore, MD). Parameters of cortical bone morphometry were measured at a magnification of ×400 using OsteoMeasureXP software (Osteometrix). Stained sections were used to draw the cortical bone, marrow space, and cell surfaces, as well as to measure osteoblasts and osteoclasts along the endocortical surface. Mineral apposition rate was measured in unstained sections under UV light, using a triple diamidino-2-phenylindole/fluorescein/Texas Red set long pass filter. Terminology and units used for cancellous and cortical bone histomorphometry are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (53, 54).

**Culture of BMMs and osteoclast formation**

To obtain BMMs, the marrow was removed by flushing with a 26-gauge needle, and erythrocytes were lysed in 150 mM NH$_4$Cl, 10 mM KHCO$_3$, and 0.1 mM EDTA (pH 7.4). Cells were centrifuged, and the sediment was suspended in α-minimum essential medium (α-MEM) in the presence of 10% fetal bovine serum (FBS; both from Thermo Fisher Scientific, Waltham, MA) and recombinant human M-CSF at 30 ng/ml. M-CSF cDNA and expression vector were obtained from D. Fremont (St. Louis, MO), and M-CSF was purified as reported previously (55). Cells were seeded at a density of 300,000 cells/cm$^2$ and cultured for 3–4 days. Inversion of the COIN module was documented by PCR of genomic DNA using primers specific for the Notch2$^{COIN}$ allele (Table 7). For osteoclast formation, cells were collected following treatment with 0.05% trypsin/EDTA for 5 min and seeded at a density of 47,000 cells/cm$^2$ in α-MEM

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**Table 7**

| Allele | Strand | Sequence 5’–3’ | Amplicon size (bp) |
|--------|--------|----------------|-------------------|
| Notch2$^{COIN}$ | Forward | CCAGGCCCGGACTGAAAGCATGCAG | 330 |
| Notch2$^{WT}$ | Reverse | CACCCCGCTTGGTGGAGGGACGAG | 100 |
| Hprt$^{WT}$ | Forward | GCTGACATGGTCGGAGGAAGAGA | 200 |
| Hprt$^{PEST}$ | Reverse | CAGCTGCTGCGAGATGAATTA | 100 |
| Lyz2$^{WT}$ | Forward1 | TTACAGTGGGCGAGCTGAC | Lyz2$^{WT}$ = 350 |
| Lyz2$^{C25}$ | Forward2 | CCCAGGAAATGGCCAGATACG | Lyz2$^{C25}$ = 700 |
| BGLAP-Cre | Reverse | GCTGCTGCGAGATGAATTA | 200 |
| Fabp1 | Forward | CAGCAGCATTTGAGGAGGCGTAA | 700 |
| Notch2$^{APEST}$ | Reverse | GTGACGCCGCGAACATGCTTC | 350 |

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Notch2 activation in osteoblasts causes osteopenia
**Notch2 activation in osteoblasts causes osteopenia**

**Table 8**
Primes used for qRT-PCR determinations

| Gene       | Strand | Sequence 5′–3′* | GenBank™ accession no. |
|------------|--------|----------------|-----------------------|
| Hes1       | Forward| ACCAAAGCAGCGCTCTGGAACGAAAGT | NM_008235            |
|            | Reverse| ATTTTGCCCTCTCTTCTT   |                       |
| Hey1       | Forward| ATCTCAACATCATCCACCTCCAAGC | NM_010423            |
|            | Reverse| GGGCGGCTTGGTGGAGGG   |                       |
| Hey2       | Forward| AGGAGAAAGATTTAAGGCGAC | NM_013904            |
|            | Reverse| GGTAGTCGCCAGATTTAAGCTT |                       |
| HeyL       | Forward| CAGTAAAGCTTCTGAGATTCGAC | NM_013905            |
|            | Reverse| AGCCTGAGAAGCCCTTCTTCTC |                       |
| Notch2WT   | Forward| CCTATGTGACACTACTATCAT | NM_010928*           |
|            | Reverse| TTAGAGAGGCGTCTACTGT    |                       |
| Notch2PEST | Forward| GCTTTCCCACCTTACAT    | Not applicable        |
|            | Reverse| TATTCGCGGACACTGTAAG |                       |
| Rpl38      | Forward| AGAACAGAGGGATAGTGTAAGGTTTCTC | NM_001048057; NM_001048058; NM_023372 |
|            | Reverse| CTGCTTGACCTCCTCTCTCTCTT |                       |
| Tnfsf11    | Forward| TATAGAATCCTGAGACTTCCTAGAAAC  | NM_011613            |
|            | Reverse| CCCCTGAAGGCTCTGTTTCTTACC |                       |

* This recognizes a fragment coding for the PEST domain of Notch2.

with 10% FBS, M-CSF at 30 ng/ml, and recombinant murine Rankl at 10 ng/ml. Rankl cDNA and expression vector were obtained from M. Glogauer (Toronto, Canada), and GST-tagged Rankl was expressed and purified as described (56). Cultures were carried out until formation of multinucleated tartrate-resistant acid phosphatase (Trp)-positive cells. 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.

**Osteoblast-enriched cell cultures**

The parietal bones of 3–5-day-old Notch2COIN/COIN mice were exposed to 1.2 units/ml Liberase™ TL (Sigma) for 20 min at 37 °C, and cells were extracted in five consecutive reactions (57). Cells from the last three digestions were pooled and seeded at a density of 10,000 cells/cm², as described (40). Osteoblast-enriched cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with non-essential amino acids (both from Sigma), in accordance with manufacturer’s instructions. The integrity of the RNA was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad), and only RNA with a quality indicator number equal to or higher than 7.0 was used for subsequent analysis (59, 60). Equal amounts of RNA were reverse-transcribed using the iScript RT-PCR kit (Bio-Rad) and amplified in the presence of specific primers (all primers from IDT; Table 8) with the 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 Hes1 (from American Type Culture Collection, ATCC; Manassas, VA), Hey1 or Hey2 (T. Iso, Los Angeles, CA), HeyL (D. Srivastava, Dallas, TX), or Tnfsf11 (Source BioScience, Nottingham, UK) (61–64).

To monitor for the efficiency of the COIN inversion, primers designed to amplify a sequence of the Notch2 transcript coding for the PEST domain of Notch2 were used (Table 8). These primers allow detection by qRT-PCR of the transcripts for Notch2WT and Notch2PEST but not for Notch2PEST, because the latter lacks the sequences coding for the PEST domain. Notch2WT and Notch2PEST copy numbers were measured by comparing with a serial dilution of Notch2 cDNA (Thermo Fisher Scientific). Notch2PEST transcripts were detected with primers that generate an amplicon straddling the artificial splice junction generated within exon 34 of the targeted Notch2 locus upon inversion of the COIN module (Table 8). Primers are specific for the Notch2PEST mRNA and do not recognize the wild-type Notch2 transcript or the Notch2COIN mRNA prior to the COIN inversion. Notch2PEST copy number was estimated by comparison with a serial dilution of an ~200 bp 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) (65).

**RNA integrity and qRT-PCR**

Total RNA was extracted from osteoblast-enriched cells with the RNeasy kit (Qiagen, Valencia, CA) and from homogenized bones with the micro RNeasy kit (Qiagen), in accordance with manufacturer’s instructions. The integrity of the RNA was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad), and only RNA with a quality indicator number equal to or higher than 7.0 was used for subsequent analysis (59, 60). Equal amounts of RNA were reverse-transcribed using the iScript RT-PCR kit (Bio-Rad) and amplified in the presence of specific primers (all primers from IDT; Table 8) with the 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 Hes1 (from American Type Culture Collection, ATCC; Manassas, VA), Hey1 or Hey2 (T. Iso, Los Angeles, CA), HeyL (D. Srivastava, Dallas, TX), or Tnfsf11 (Source BioScience, Nottingham, UK) (61–64).

To monitor for the efficiency of the COIN inversion, primers designed to amplify a sequence of the Notch2 transcript coding for the PEST domain of Notch2 were used (Table 8). These primers allow detection by qRT-PCR of the transcripts for Notch2WT and Notch2PEST but not for Notch2PEST, because the latter lacks the sequences coding for the PEST domain. Notch2WT and Notch2PEST copy numbers were measured by comparing with a serial dilution of Notch2 cDNA (Thermo Fisher Scientific). Notch2PEST transcripts were detected with primers that generate an amplicon straddling the artificial splice junction generated within exon 34 of the targeted Notch2 locus upon inversion of the COIN module (Table 8). Primers are specific for the Notch2PEST mRNA and do not recognize the wild-type Notch2 transcript or the Notch2COIN mRNA prior to the COIN inversion. Notch2PEST copy number was estimated by comparison with a serial dilution of an ~200 bp 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) (65).
Amplification reactions were conducted in CFX96 qRT-PCR detection systems (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step. Data are expressed as copy number corrected for Rpl38 expression estimated by comparison with a serial dilution of Rpl38 (ATCC) (66).

Statistics

Data are expressed as means ± S.D. Statistical differences were determined by Student’s t test or two-way analysis of variance with Holm–Šidak post hoc analysis for pairwise or multiple comparisons, respectively.

Author contributions—S. Z. designed research studies, conducted experiments, analyzed data, and wrote the manuscript. J. Y. conducted experiments and analyzed data. A. S. conducted experiments and analyzed data. L. S. conducted the analysis of skeletal phenotypes. C. S. and A. N. E. designed and created the Notch2COIN targeting construct. E. C. designed research studies, analyzed data, and wrote the manuscript.

Acknowledgments—We thank D. Fremont for M-CSF cDNA, M. Glogauer for Rank1 cDNA, T. Iso for Hey1 and Hey2 cDNAs, D. Srivastava for Hey1 cDNA, David Bridgewater and Tabitha Eller for technical assistance, and Mary Yurczak for secretarial support.

References

1. Fortini, M. E. (2009) Notch signaling: the core pathway and its posttranslational regulation. Dev. Cell 16, 633–647
2. Kopan, R., and Ilagan, M. X. (2009) The canonical Notch signaling pathway: unfolding the activation mechanism. Cell 137, 216–233
3. Kovall, R. A. (2007) Structures of CSL, notch and mastermind proteins: piecing together an active transcription complex. Curr. Opin. Struct. Biol. 17, 117–127
4. Iso, T., Kedes, L., and Hamamori, Y. (2003) HES and HERP families: multiple effectors of the Notch signaling pathway. J. Cell. Physiol. 194, 237–255
5. Groot, A. J., Habets, R., Yahyanejad, S., Hodin, C. M., Reiss, K., Saftig, P., et al. (2016) Osteoblast-specific Notch2 inactivation causes increased trabecular bone mass at specific sites of the appendicular skeleton. Bone 87, 136–146
6. Van Esch, H., FORGE Canada Consortium, Michaud, J. L., and Samuels, E. M. (2011) Mutations in NOTCH2 in families with Hajdu-Cheney syndrome. Hum. Mutat. 32, 1114–1117
7. Simpson, M. A., Irving, M. D., and Robertson, S. P. (2012) Serpentine fibula polycystic kidney syndrome is part of the phenotypic spectrum of Hajdu-Cheney syndrome. Eur. J. Hum. Genet. 20, 122–124
8. Yuan, Z., Friedmann, D. R., VanderWielen, B. D., Collins, K. J., and Kovall, R. A. (2007) Structures of CSL, notch and mastermind proteins: piecing together an active transcription complex. Curr. Opin. Struct. Biol. 17, 117–127
9. Liu, Z., Brunskill, E., Varnum-Finney, B., Zhang, C., Zhang, A., Jay, P. Y., et al. (2013) Mechanisms of ana- bolic therapies for osteoporosis. N. Engl. J. Med. 375, 905–916
10. Teitelbaum, S. L. (2007) Osteoclasts: what do they do and how do they do it? Am. J. Pathol. 170, 427–435
11. Bianco, P., and Gehron Robey, P. (2000) Marrow stromal stem cells. J. Clin. Invest. 105, 1663–1668
12. Zanotti, S., and Canalis, E. (2016) Notch signaling and the skeleton. Endocr. Rev. 37, 223–253
13. 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
14. Canalis, E., Adams, D. J., Boskey, A., Parker, K., Zanotti, S., and Teitelbaum, S. L. (2008) NOTCH1 regulates osteoclastogenesis differentially regulates cancellous and cortical bone remodeling. J. Biol. Chem. 283, 25614–25625
15. Canalis, E., Parker, K., Feng, J. Q., and Zanotti, S. (2013) Osteoclast lineage-specific effects of Notch activation in the skeleton. Endocrinology 154, 632–634
16. Engin, F., Yao, Z., Yang, T., Zhou, G., Bertin, T., Jiang, M. M., Chen, Y., Wang, L., Zheng, H., Sutton, R. E., Boyce, B. F., and Lee, B. (2008) Dimorphic effects of Notch signaling in bone homeostasis. Nat. Med. 14, 299–305
17. Fukushima, H., Nakao, A., Okamoto, F., Shin, M., Kajiy, H., Sakano, S., Bigas, A., Jimi, E., and Okabe, K. (2008) The association of Notch2 and NF-κB accelerates RANKL-induced osteoclastogenesis. Mol. Cell. Biol. 28, 6402–6412
18. Yorgun, T., Vollersten, N., Riedel, C., Jesche, A., Peters, S., Busse, B., Ameling, M., and Schinke, T. (2016) Osteoblast-specific Notch2 inactivation causes increased trabecular bone mass at specific sites of the appendicular skeleton. Bone 87, 136–146
19. Zanotti, S., Smerdel-Ramoya, A., Stadmeyer, L., Durant, D., Radtke, F., and Canalis, E. (2008) Notch inhibits osteoblast differentiation and causes osteo- porosis. Endocrinology 149, 3890–3899
20. Cheney, W. D. (1965) Acto-osteolysis. Am. J. Roentgenol. Radium. Ther. Nucl. Med. 94, 595–607
21. Hajdu, N., and Kauntze, R. (1948) Cranio-skeletal dysplasia. Br. J. Radiol. 21, 42–48
22. 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) Serpentine fibula polycystic kidney syndrome is part of the phenotypic spectrum of Hajdu-Cheney syndrome. Eur. J. Hum. Genet. 20, 122–124
23. Isidor, B., Lindenbaum, P., Pichon, O., Bézieau, S., Dina, C., Jacquemont, S., Martin-Coignard, D., Thauvin-Robinet, C., Le Meerer, M., Mandel, J. L., David, A., Faire, L., Cormier-Daire, V., Redon, R., and Le Caigence, C. (2011) Truncating mutations in the last exon of NOTCH2 cause a rare skeletal disorder with osteoporosis. Nat. Genet. 43, 306–308
24. Majewski, J., Schwartztentruber, J. A., Caqueret, A., Patry, L., Marcadier, J., Frys, J., Boycott, K. M., De-Marie, L. G., McKiernan, F. E., Marik, L., Van Esch, H., FORGE Canada Consortium, Michaud, J. L., and Samuelu, M. E. (2011) Mutations in NOTCH2 in families with Hajdu-Cheney syndrome. Hum. Mutat. 32, 1114–1117
25. Simpson, M. A., Irving, M. D., Asilmaz, E., Gray, M. I., 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
26. 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
27. 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
28. Economides, A. N., Frendewey, D., Yang, P., Dominguez, M. G., Dore, A. T., Loboz, I. B., Persaud, T., Rohas, J., McClain, J., Lengyl, P., Droogert, G., Chernomorsky, R., Stevens, S., Auerbach, W., Dechiara, T. M., et al. (2013) Conditional loss by inversion provide a universal method for the generation of conditional alleles. Proc. Natl. Acad. Sci. U.S.A. 110, 63179–63188
29. Yang, M., Trettel, L. B., Adams, D. J., Harrison, J. R., Canalis, E., and Kream, B. E. (2010) Col3.6-HSD2 transgenic mice: A glucocorticoid loss-of-function mouse model spanning early and late osteoblast differentiation. Bone 47, 573–582
30. Adra, C. N., Boer, P. H., and McBurney, M. W. (1987) Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. Gene 60, 65–74
31. Beck, E., Ludwig, G., Auerwald, E. A., Reuss, B., and Schaller, H. (1982) Nucleotide sequence and exact localization of the neomycin phospho- transferase gene from transposon Tn5. Gene 19, 327–336
32. Yoshikawa, Y., Kode, A., Xu, L., Mosialou, I., Silva, B. C., Ferron, M., Clemens, T. L., Economides, A. N., and Kousteni, S. (2011) Genetic evi-
Notch2 activation in osteoblasts causes osteopenia
dence points to an osteocalcin-independent influence of osteoblasts on energy metabolism. J. Bone Miner. Res. 26, 2012–2025
33. Canalis, E., and Zanotti, S. (2016) Hajdu-Cheney syndrome, a disease associated with NOTCH2 mutations. Curr. Osteoporos. Rep. 14, 126–131
34. Canalis, E., Sanjay, A., Yu, J., and Zanotti, S. (2017) An antibody of Notch2 reverses the osteoporotic phenotype of Hajdu Cheney mutant male mice. Endocrinology 158, 730–742
35. Adami, G., Rossi, 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
36. Hilton, M. J., Tu, X., Wu, X., Bai, S., Zhao, H., Kobayashi, T., Kronenberg, H. M., Teitelbaum, S. L., Ross, F. P., Kopan, R., and Long, F. (2008) Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. Nat. Med. 14, 306–314
37. Zanotti, S., and Canalis, E. (2014) Notch1 and Notch2 expression in osteoblast precursors regulates femoral microarchitecture. Bone 62, 22–28
38. Dong, Y., Jesse, A. M., Kohn, A., Gunnell, L. M., Hu, G., Zuscik, M. J., O’Keefe, R. J., and Hilton, M. J. (2010) RBPj-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. Development 137, 1461–1471
39. Mead, T. J., and Yutzey, K. E. (2009) Notch pathway regulation of chondrocyte differentiation and proliferation during appendicular and axial skeleton development. Proc. Natl. Acad. Sci. U.S.A. 106, 14420–14425
40. Canalis, E., Zanotti, S., and Smerdel-Ramoya, A. (2014) Connective tissue growth factor is a target of Notch signaling in cells of the osteoblastic lineage. Bone 64, 273–280
41. Canalis, E., Zanotti, S., and Smerdel-Ramoya, A. (2013) Hairy and enhancer of split-related LysMcre mice. J. Bone Miner. Res. 28, 624–632
42. Tu, X., Chen, J., Lim, J., Karner, C. M., Lee, S. Y., He, C., Wiese, C., Surendran, K., Kopan, R., Gessler, M., and Long, F. (2012) Physiological notch signaling maintains bone homeostasis via RBPjk and Hey upstream of NFATc1. PLoS Genet. 8, e1002577
43. Canalis, E., and Zanotti, S. (2013) Hairy and enhancer of split-related with YRPW Motif (HEY)2 regulates bone remodeling in mice. J. Biol. Chem. 288, 21547–21557
44. Canalis, E., and Zanotti, S. (2017) Hairy and enhancer of split-related with YRPW Motif-like (HeyL) is dispensable for bone remodeling in mice. J. Cell. Biochem. 118, 1819–1826
45. Buchholz, F., Angrand, P. O., and Stewart, A. F. (1996) A simple assay to determine the functionality of Cre or FLP recombination targets in genomic manipulation constructs. Nucleic Acids Res. 24, 3118–3119
46. Buchholz, F., Angrand, P. O., and Stewart, A. F. (1998) Improved properties of FLP recombinase evolved by cycling mutagenesis. Nat. Biotechnol. 16, 657–662.
47. Tang, S. H., Silva, F. J., Tsark, W. M., and Mann, J. R. (2002) A Cre/loxP-deleter transgenic line in mouse strain 129S1/SvImJ. Genesis 32, 199–202
48. Clausen, B. E., Burkhartt, C., Reith, W., Renkawitz, R., and Förster, I. (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res. 8, 265–277
49. Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Förster, I., and Akira, S. (1999) Enhanced TH1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity 10, 39–49
50. Bouxsein, M. L., Boyd, S. K., Christiansen, B. A., Guldberg, 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
51. Dempster, D. W., Compston, J. E., Drezner, 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
52. Parfitt, A. M., Drezner, 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
53. Lee, S. H., Rho, J., Jeong, D., Sul, J. Y., Kim, T., Kim, N., Kang, J. S., Miyamoto, T., Suda, T., Lee, S. K., Pignolo, R. J., Koczon-Jaremko, B., Lorenzo, J., and Choi, Y. (2006) v-ATPase V0 subunit d2-deficient mice exhibit impaired osteoclast fusion and increased bone formation. Nat. Med. 12, 1403–1409
54. Wang, Y., Lebowitz, D., Sun, C., Thang, 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
55. Yasul, 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
56. Zanotti, S., Smerdel-Ramoya, A., and Canalis, E. (2013) Nuclear factor of activated T-cells (Nfatc2) inhibits Notch signaling in osteoblasts. J. Biol. Chem. 288, 624–632
57. Nazareno, 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
58. Nazareno, 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
59. Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C., and Niehrs, C. (1998) Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 357–362
60. Iso, T., Sartoelli, A., Chung, G., Shichinohe, T., Kedes, L., and Hamamoto, Y. (2001) HERP, a new primary target of Notch regulated by ligand binding. Mol. Cell. Biol. 21, 6071–6079
61. Liu, J., Stewart, C., Puchacz, E., Mackowiak, S., Shalhoub, V., Collart, D., Zambetti, G., and Stein, G. (1989) Structure of the rat osteocalcin gene and regulation of vitamin D-dependent expression. Proc. Natl. Acad. Sci. U.S.A. 86, 1143–1147
62. 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 cardiomyocytes, somitic, and pharyngeal arch segments. Dev. Biol. 216, 72–84
63. Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., Smith, M., and Stein, G. (2000) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods 6, 343–345
64. Koudajo, 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