Hairy and enhancer of split 1 is a primary effector of NOTCH2 signaling and induces osteoclast differentiation and function

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\textsuperscript{1,2}Hairy and enhancer of split 1 is a primary effector of NOTCH2 (HES1) mice, which harbor a mutation replicating that found in Hajdu–Cheney syndrome, exhibit marked osteopenia because of increased osteoclast number and bone resorption. Hairy and enhancer of split 1 (HES1) is a Notch target gene and a transcriptional modulator that determines osteoclast cell fate decisions. Transcript levels of \textit{Hes1} increase in \textit{Notch2}tm1.1Ecan bone marrow–derived macrophages (BMMs) as they mature into osteoclasts, suggesting a role in osteoclastogenesis. To determine whether HES1 is responsible for the phenotype of \textit{Notch2}tm1.1Ecan mice and the skeletal manifestations of Hajdu–Cheney syndrome, \textit{Hes1} was inactivated in \textit{Ctsk}-expressing cells from \textit{Notch2}tm1.1Ecan mice. \textit{Ctsk} encodes the protease cathepsin K, which is expressed preferentially by osteoclasts. We found that the osteopenia of \textit{Notch2}tm1.1Ecan mice was ameliorated, and the enhanced osteoclastogenesis was reversed in the context of the \textit{Hes1} inactivation. Microcomputed tomography revealed that the downregulation of \textit{Hes1} in \textit{Ctsk}-expressing cells led to increased bone volume/total volume in female mice. In addition, cultures of BMMs from Csk\textsuperscript{-/-}\textsuperscript{CreWT};Hes1\textsuperscript{-/-} mice displayed a decrease in osteoclast number and size and decreased bone-resorbing capacity. Moreover, activation of HES1 in Ctsk-expressing cells led to osteopenia and enhanced osteoclast number, size, and bone resorptive capacity in BMM cultures. Osteoclast phenotypes and RNA-Seq of cells in which HES1 was activated revealed that HES1 modulates cell–cell fusion and bone-resorbing capacity by supporting sealing zone formation. In conclusion, we demonstrate that HES1 is mechanistically relevant to the skeletal manifestation of \textit{Notch2}tm1.1Ecan mice and is a novel determinant of osteoclast differentiation and function.

Osteoclasts are multinucleated giant cells that are responsible for bone resorption and essential to maintain bone homeostasis. Osteoclasts are derived from the differentiation and fusion of mononuclear cells of the myeloid lineage by the actions of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-\kappa B ligand (RANKL) (1, 2). RANKL triggers downstream signaling to induce the expression of transcription factors required for osteoclastogenesis, such as nuclear factor of activated T cells, cytoplasmic 1 (NFATc1) (3–6). An imbalance of physiological or pathological conditions causing dysregulation of osteoclast differentiation and function leads to diseases associated with alterations in bone mass (7, 8).

Hajdu–Cheney syndrome (HCS) (Online Mendelian Inheritance in Man: 102500) is a rare and devastating disorder characterized by numerous skeletal manifestations, including craniofacial developmental defects, short stature, bone loss with fractures, and acroosteolysis associated with inflammation of the distal phalanges (9–12). HCS is associated with mutations or short deletions in exon 34 of NOTCH2 upstream of the PEST domain, which is required for the ubiquitination and degradation of NOTCH2 (12–16). The HCS pathogenic variants lead to the premature termination of a protein product lacking sequences necessary for the proteasomal degradation of the NOTCH2 intracellular domain so that the protein is stable and a gain-of-NOTCH2 function ensues. Autosomal dominant inheritance as well as de novo heterozygous mutations have been reported (12–16).

Our laboratory created a knock-in mouse model harboring a Notch2\textsuperscript{G935C>T} mutation reproducing HCS and termed \textit{Notch2}tm1.1Ecan (also known as Notch2\textsuperscript{G223195}) (17, 18). The homozygous mutation is associated with craniofacial developmental abnormalities and is lethal, and heterozygous \textit{Notch2}tm1.1Ecan mutant mice exhibit profound osteopenia and short limbs, reproducing functional outcomes of the human disease and establishing the first model for the study of HCS (12, 17). \textit{Notch2}tm1.1Ecan mice have increased bone resorption secondary to a direct effect of the gain-of-NOTCH2 function on osteoclastogenesis as well as the increased expression of RANKL by cells of the osteoblast lineage (17). These are unique functional properties of NOTCH2, which are distinct from those reported for other Notch receptors (19, 20). Indeed, NOTCH1 inhibits osteoclastogenesis directly, and NOTCH3 is not expressed in the myeloid lineage; although, by inducing RANKL in cells of the osteoblast lineage, it enhances osteoclastogenesis indirectly (21–23). Low levels of NOTCH4 are expressed in the myeloid lineage, and it is not known to play a role in osteoblastogenesis (24).

Cultures of bone marrow macrophages (BMMs) revealed that the expression of \textit{Hes1}, a Notch target gene, is enhanced as cells mature as osteoclasts, and the increased expression is of greater magnitude in cultures from \textit{Notch2}tm1.1Ecan mice
Hes1 and osteoclast

Importantly, other Notch target genes, such as Hes3, Hes5, Hey1, Hey2, and HeyL, are either expressed at very low levels or not detected in BMMs from control or mutant mice. This observation suggests that hairless and enhancer of split 1 (HES1) may be an important regulator of osteoclastogenesis and is in part responsible for the HCS phenotype. HES1 is a transcriptional modulator that plays a role in the differentiation of embryonic stem and mesenchymal cells. Although HES1 is considered a transcriptional repressor, transcription factors can function as either positive or negative regulators of transcription in a cell-context-dependent manner. In addition, calcium/calmodulin-dependent protein kinase 2 can convert HES1 from an inactivated to de-repressed state. HES1 was mechanistically relevant to the HCS phenotype and is in part responsible for the HCS phenotype.

Skeletal phenotypes were determined by microcomputed tomography (μCT) and histomorphometry and cellular effects by study of osteoclast differentiation and resorption activity in vitro.

Results

**Inactivation of Hes1 reverses the effect of the Hajdu–Cheney mutation on osteoclastogenesis**

To determine whether HES1 plays a role in the osteopenia of the Hajdu–Cheney syndrome, Hes1 mutant mice were isolated. BMMs were cultured in the presence of M-CSF and RANKL for 2 days and transduced with Ad-Cre to delete loxP flanked sequences or Ad-GFP as control. Excision of the RosaHes1 sequences resulted in a 50% decrease in osteoclast number compared with control cultures transduced with Ad-GFP (Fig. 3). Conversely, deletion of Hes1 resulted in a 50% reduction in Hes1 mRNA levels and a 50% decrease in osteoclast number compared with control cultures transduced with Ad-GFP (Fig. 3). The results demonstrate that HES1 is a determinant of osteoclast differentiation in vitro.

**HES1 is a determinant of osteoclastogenesis in vitro**

To ascertain the function of HES1 in cells of the osteoclast lineage, BMMs from either Hes1loxP/loxP or RosaSTOP/Hes1 mice were isolated. BMMs were cultured in the presence of M-CSF and RANKL for 2 days and transduced with Ad-Cre to delete loxP flanked sequences or Ad-GFP as control. Excision of the STOP cassette in RosaHes1 cells resulted in a 20-fold induction of Hes1 mRNA and a 1.7-fold increase in osteoclastogenesis compared with RosaSTOP/Hes1 cultures transduced with Ad-GFP (Fig. 3). The results demonstrate that HES1 is a determinant of osteoclast differentiation in vitro.

**Inactivation of Hes1 in osteoclasts of female mice increases BV in vivo**

To confirm a role of HES1 in osteoclastogenesis and bone homeostasis, Hes1 was inactivated in vivo in Ctsk-expressing cells. For this purpose, CtskCre/Wt;Hes1loxP/loxP mice were crossed with Hes1loxP/loxP mice to generate CtskCre/Wt;Hes1loxP/loxP controls. CtskCre/Wt;Hes1loxP/loxP mice appeared healthy, and their weight and femoral length were
CtskCre-mediated recombination was documented in genomic DNA from tibiae of CtskCre/WT;Hes1Δ/Δ mice with a consequent decrease in Hes1 mRNA. Confirming the results observed in the context of the Notch2tm1.1Ecan mutant mice, inactivation of Hes1 in 2- or 4-month-old male mice did not result in an obvious skeletal phenotype, although trabecular number and connectivity were modestly increased (Table 2). In contrast, 2-month and particularly 4-month-old female mice harboring the inactivation of Hes1 exhibited a significant increase in femoral BV/TV (Table 2 and Fig. 4). Femoral μCT of 4-month-old female CtskCre/WT;Hes1Δ/Δ mice revealed an 85% increase in BV/TV associated with an increase in trabecular number and connectivity density and a decrease in structure model index (SMI) compared with controls. Bone histomorphometry of 4-month-old CtskCre/WT;Hes1Δ/Δ female mice demonstrated an ~50% decrease in osteoclast number and ~35% decrease in eroded surface, compared with littermate controls, confirming that HES1 is required for osteoclast differentiation and function in vivo (Table 3 and Fig. 5). Osteoblast number and bone formation were not affected by the Hes1 deletion.

**Inactivation of Hes1 decreases osteoclast differentiation in vitro**

To confirm that the phenotype of CtskCre/WT;Hes1Δ/Δ mice was due to a decrease in osteoclast differentiation, BMMs derived from CtskCre/WT;Hes1Δ/Δ and control littermates were cultured in the presence of M-CSF and RANKL. CtskCre/WT;Hes1Δ/Δ cultures revealed a 42% decrease in osteoclast number when compared with cells from littermate controls (Fig. 6). The number of osteoclasts with high number of nuclei was decreased in CtskCre/WT;Hes1Δ/Δ cultures compared with controls, indicating that the size of osteoclasts was reduced because of a decrease in the fusion capacity of CtskCre/WT;Hes1Δ/Δ cells. Mature osteoclasts have a distinct cytoskeletal structure, namely the sealing zone, a circular actin-rich

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**Figure 1. Hes1 inactivation reverses the effect of the Hajdu-Cheney mutation on osteoclastogenesis.** BMMs derived from 2-month-old Notch2tm1.1Ecan;Hes1loxP/loxP and Hes1loxP/loxP littermate controls were cultured for 2 days with M-CSF at 30 ng/ml and RANKL at 10 ng/ml and transduced with adenoviruses carrying CMV-Cre (Ad-Cre) or adenoviruses carrying GFP (Ad-GFP) as control at MOI 100 and cultured for two additional days in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml until the formation of multinucleated TRAP-positive cells. A, total RNA was extracted, and gene expression was determined by quantitative RT–PCR. Data are expressed as Notch26955C, Notch2, and Hes1, corrected for Rpl38 copy number. B, representative images of TRAP-stained multinucleated cells are shown. The scale bars in the right corner represent 500 μm. C, TRAP-positive cells with more than three nuclei were considered osteoclasts and counted. Values are means ± SD; n = 4 technical replicates for WT (open circles) and Notch2tm1.1Ecan (closed circles) cells in the context of Hes1loxP/loxP (white bar) or Hes1Δ/Δ (gray bar) deleted alleles. Representative data are shown from two independent experiments. *Significantly different between Notch2tm1.1Ecan and control, p < 0.05. #Significantly different between Hes1Δ/Δ and Hes1loxP/loxP, p < 0.05. BMM, bone marrow–derived macrophage; M-CSF, macrophage colony-stimulating factor; MOI, multiplicity of infection; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate resistant acid phosphatase.
**Table 1**

Cancellous bone histomorphometry of 2-month-old Hes1loxP/loxP, Notch2tm1.1Ecan;Hes1loxP/loxP, CtskCre/WT;Hes1Δ/Δ and CtskCre/WT;Notch2tm1.1Ecan;Hes1Δ/Δ male mice

| Distal femur trabecular bone | WT | Notch2tm1.1Ecan;Hes1loxP/loxP | CtskCre/WT;Hes1Δ/Δ | CtskCre/WT;Notch2tm1.1Ecan;Hes1loxP/loxP | n = 4 |
|-----------------------------|----|-----------------------------|---------------------|------------------------------------------|------|
| BV/TV (%)                   | 32.1 ± 2.8 | 14.2 ± 3.6                  | 37.4 ± 1.9          | 24.9 ± 7.4                               |      |
| Trabecular separation (μm)  | 117 ± 5    | 236 ± 53                   | 94 ± 7              | 130 ± 40                                 |      |
| Trabecular number (1/mm)    | 5.8 ± 0.1  | 3.9 ± 1.0                   | 6.7 ± 0.5           | 6.0 ± 1.2                                 |      |
| Trabecular thickness (μm)   | 55 ± 5     | 39 ± 4                      | 56 ± 5              | 41 ± 6                                   |      |
| Osteoblast surface/bone surface (%) | 14.7 ± 0.9 | 19 ± 1.3                     | 13 ± 1.9           | 18 ± 4.3                                 |      |
| Osteoblasts/bone perimeter (1/mm) | 9.1 ± 0.9 | 11.3 ± 0.6                     | 8.5 ± 1.0         | 10.1 ± 1.6                                |      |
| Osteoclast surface/bone surface (%) | 10.4 ± 3.7 | 20.1 ± 3.4                      | 12.1 ± 2.2       | 9.1 ± 4.1                                 |      |
| Osteoclasts/bone perimeter (1/mm) | 3.2 ± 1.3 | 6.1 ± 1.2                      | 3.6 ± 0.8         | 2.8 ± 1.7                                 |      |
| Eroded surface/bone surface (%) | 10.3 ± 1.3 | 13.4 ± 2.2                      | 11.9 ± 0.8       | 7.1 ± 1.1                                 |      |
| Mineral apposition rate (μm/day) | 3.3 ± 1.0 | 2.8 ± 0.2                      | 2.6 ± 0.1         | 2.4 ± 0.2                                 |      |
| Mineralizing surface/bone surface (%) | 31.2 ± 2.8 | 27.7 ± 3.5                      | 35.1 ± 4.4       | 32.3 ± 2.3                                |      |
| Bone formation rate (μm²/μm³/day) | 1.0 ± 0.3 | 0.8 ± 0.1                      | 0.9 ± 0.1         | 0.8 ± 0.1                                 |      |

Bone histomorphometry was performed on distal femurs from 2-month-old Hes1loxP/loxP, Notch2tm1.1Ecan;Hes1loxP/loxP, CtskCre/WT;Hes1Δ/Δ and CtskCre/WT;Notch2tm1.1Ecan;Hes1loxP/loxP male mice. Values are means ± SD.

*Significantly different between Notch2tm1.1Ecan and control, p < 0.05.

#Significantly different between Hes1Δ/Δ and Hes1loxP/loxP, p < 0.05.
Hes1 and osteoclast

Figure 3. HES1 is required for osteoclastogenesis in vitro. BMMs derived from 2-month-old Rosa\textsuperscript{Hes1} (A and B) or Rosa\textsuperscript{Hes1}ΔΔ mice (C and D) were cultured in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml for 2 days. Cells were transduced with Ad-Cre (open circles), to recombineloxP flanked sequences, or Ad-GFP (open circles) as a control and then cultured for two additional days. A and C, representative images of TRAP-stained multinucleated cells. The scale bar in the right corner represents 500 μm. B and D, Hes1 transcript levels were measured by quantitative RT–PCR in total RNA from osteoclasts. Transcript levels are reported as copy number corrected for Rpl38 (left). TRAP-positive cells with more than three nuclei were considered osteoclasts (right). Values are means ± SD; n = 3 or 4 technical replicates for control (open circles) and either Hes1\textsuperscript{ΔΔ} or recombined Rosa\textsuperscript{Hes1} (closed circles) cells. Representative data are shown from two independent experiments. *Significantly different between Hes1\textsuperscript{ΔΔ} and control, p < 0.05; or recombined Rosa\textsuperscript{Hes1} and control, p < 0.05. BMM, bone marrow–derived macrophage; HES1, hairy and enhancer of split 1; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate resistant acid phosphatase.

structure formed by podosomes in a cluster to create a ring that is tightly adherent to the bone matrix for efficient bone resorption (33). Phalloidin staining of osteoclasts from Ctsk\textsuperscript{Cre/WT};Hes1\textsuperscript{ΔΔ} mice cultured on bone slices revealed smaller sealing zones than controls and a ~30% decrease in the perimeter of the sealing zone (Fig. 6). Ctsk\textsuperscript{Cre/WT};Hes1\textsuperscript{ΔΔ} osteoclasts also exhibited a ~60% decrease in total bone resorption area, indicating a decrease in osteoclast resorptive activity (Fig. 6).

Induction of HES1 in osteoclasts causes osteopenia

To determine the effect of the HES1 induction on osteoclastogenesis in vivo, homozygous Rosa\textsuperscript{STOP}\textsuperscript{Hes1} mice were crossed with Ctsk\textsuperscript{Cre/WT} mice for the creation of Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} experimental mice and Rosa\textsuperscript{STOP}\textsuperscript{Hes1} littermate controls. Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice appeared healthy, and their weight was not different from that of littermate controls (Fig. S3). Ctsk\textsuperscript{Cre/WT}–mediated recombination was demonstrated in genomic DNA from tibiae of Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice, and Hes1 mRNA levels were increased in bone extracts from Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice.

Femoral architecture of 10-week-old male and female Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice revealed a 30% decrease in BV/TV associated with a decrease in connectivity and an increase in SMI in Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice that reached statistical significance in female but not in male mice (Table 4). Bone histomorphometry of 10-week-old female Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice demonstrated a 1.7-fold increase in osteoclast surface and number, and approximately twofold increase in eroded surface, when compared with littermate controls, confirming that HES1 increases osteoclast differentiation and function in vivo (Table 5 and Fig. 7).

Induction of HES1 enhances osteoclast differentiation in vitro

To verify that the phenotype of Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice was due to a direct effect in cells of the osteoclast lineage, BMMs from Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} and control littermates were cultured in the presence of M-CSF and RANKL. BMMs from Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice exhibited a 4.5-fold increase in osteoclast number in comparison to cells from littermate controls (Fig. 8). In addition, osteoclasts with a high number of nuclei were significantly increased in Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} cultures compared with controls, indicating that the size of osteoclasts was larger because of highly activated fusion in Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} cells. Phalloidin staining of osteoclasts from Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} mice cultured on bone slices confirmed larger cells with sealing zones that were 25% larger than in cells from control littermates (Fig. 8). Accordingly, Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} osteoclasts exhibited a sixfold increase in total resorption pit area (Fig. 8), indicating enhanced bone resorptive capacity in Ctsk\textsuperscript{Cre/WT};Rosa\textsuperscript{Hes1} osteoclasts.

Mechanisms of HES1 action on osteoclastogenesis

To understand the molecular mechanisms associated with the effect of HES1 on osteoclast differentiation, total RNA...
from Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> mice and control osteoclasts was examined by RNA-Seq analysis. Ingenuity pathway analysis (IPA) revealed that ~200 genes associated with cellular functions, including movement, spreading, cell–cell contact, and organization of the cytoskeleton, were upregulated in cells from Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> mice (Fig. S4). Known fusion markers of osteoclastogenesis, such as Ocstamp, Destamp, and Atp6v0d2, were significantly increased in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts compared with controls (Fig. 9) (34–39). Tetraspanins, a family of 32 distinct members, are known to affect cell–cell fusion, motility, and sealing zone formation (40–42). Among the tetraspanins, the transcripts of Cd9, Cd63, Cd82, Tspan5, Tspan7, and Tspan10 were expressed in osteoclasts and significantly increased in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts. Analysis of altered canonical pathway in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts by IPA revealed upregulation of integrin signaling in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts (Fig. S5). Osteoclasts express αvβ3 integrins, and they play a role in the adhesion of osteoclasts to bone matrix, cytoskeletal organization, and sealing zone formation (33, 43, 44). Expression of genes associated with integrin signaling, including itgb3 (integrin β3), Src, Syk, Rac2, Vav3, Vcl and Dock5, was upregulated in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts (Fig. 9). The transcriptional repressors of Nfatc1, including Bcl6, Mafb, Id11, and Irf8, were decreased, and Nfatc1 was increased in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts; B lymphocyte–induced maturation protein 1 (Blimp1) was not affected (45–48). Interleukin (IL) 1β, known to induce osteoclast differentiation in physiological conditions and following inflammation, and its receptor Il1r1, were markedly upregulated in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts (Fig. 9) (49–51). The levels of other osteoclastogenic markers, such as Oscar, Calcr, Car2, and Acp5, also were increased in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts. The mRNA expression of Bcl6, Mafb, Nfatc1, Atp6v0d2, Ocstamp, and Acp5 was demonstrated by quantitative RT–PCR (qRT–PCR) (Fig. 9). In accordance with these results, Nfatc1 protein levels were increased in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> osteoclasts (Fig. 9). HES1 protein levels were increased in differentiated osteoclasts, and the increase was greater in Ctsk<sup>Cre</sup>/WT;Rosa<sup>Hes1</sup> cells.

**Discussion**

The present work uncovers a new function of HES1 on osteoclast differentiation and bone remodeling. The deletion
of Hes1 in Ctsk-expressing cells decreased the osteoclastogenic potential of preosteoclasts, whereas its induction enhanced osteoclastogenesis. Osteoclast phenotypes and RNA-Seq analysis revealed that HES1 regulates cell–cell fusion and the formation of the sealing zone. The gene subsets of fusion markers, integrin signaling, and structural proteins for sealing zone formation were significantly upregulated in osteoclasts overexpressing HES1. These results indicate that HES1 has a direct role in osteoclast differentiation and function. Our study also reveals that the expression of Nfatc1 and that of inhibitors of osteoclastogenesis acting as transcriptional brakes of Nfatc1, such as Irf8, Bcl6, Mafb, and Id1, were regulated by HES1. It is possible that HES1 interacts with transcriptional repressors of osteoclastogenesis in a manner analogous to BLIMP1, although the expression of Blimp1 was not affected by HES1 (47, 52). It is probable that HES1 acts as a transcriptional repressor of inhibitors of osteoclastogenesis and as a consequence causes enhanced Nfatc1 expression. Under selected

![Figure 4. Inactivation of Hes1 in Ctsk-expressing cells increases bone volume in female mice.](image)

Representative microcomputed tomography image of femurs from 2- (A) and 4-month-old (B) male and female CtskCreWT;Hes1Δ/Δ mice and Hes1loxP/loxP sex-matched control littersmates. The scale bar in the right corner represents 1 mm.

![Figure 5. Inactivation of Hes1 in Ctsk-expressing cells decreases osteoclast number and bone resorption in vivo.](image)

Representative static (upper panels) and dynamic (lower panels) cancellous bone histomorphometry of the distal femur from 4-month-old CtskCreWT;Hes1Δ/Δ female mice and Hes1loxP/loxP sex-matched control littersmates. The scale bar in the right corner represents 50 μm.

**Table 3**

Cancellous bone histomorphometry of 4-month-old CtskCreWT;Hes1Δ/Δ female mice and sex-matched littermate controls

|                          | Control            | Hes1Δ/Δ          |
|--------------------------|--------------------|------------------|
|                          | n = 4–5            | n = 6–8          |
| BV/TV (%)                | 9.3 ± 2.3          | 13.6 ± 2.2*      |
| Trabecular separation (μm)| 317 ± 69           | 229 ± 38*        |
| Trabecular number (1/mm) | 3.0 ± 0.7          | 3.9 ± 0.5*       |
| Trabecular thickness (μm)| 31 ± 3.2           | 34 ± 4.0         |
| Osteoblast surface/bone surface (%) | 15.9 ± 2.2 | 15.3 ± 2.0        |
| Osteoblasts/bone perimeter (1/mm) | 12.4 ± 2.6  | 12.6 ± 1.6        |
| Osteoclast surface/bone surface (%) | 24.4 ± 8.1 | 12.6 ± 3.5*       |
| Osteoclasts/bone perimeter (1/mm) | 8.3 ± 2.1  | 4.7 ± 1.1*        |
| Eroded surface/bone surface (%) | 16.5 ± 6.5 | 10.6 ± 2.4*       |
| Mineral apposition rate (μm/day) | 1.7 ± 0.1 | 1.8 ± 0.4         |
| Mineralizing surface/bone surface (%) | 31.1 ± 5.1  | 32.8 ± 2.5        |
| Bone formation rate (μm²/μm²/day) | 0.5 ± 0.1   | 0.6 ± 0.1         |

Bone histomorphometry was performed on distal femurs from 4-month-old CtskCreWT;Hes1Δ/Δ female mice and sex-matched littermate controls. Values are means ± SD.

*Significantly different from control, p < 0.05.
cellular conditions, HES1 can act as a transcriptional activator so that one cannot exclude a direct effect of HES1 on the transcriptional activation of Nfatc1 (30). In accordance with our observations, γ-secretase inhibitors, known to prevent Notch activation, were found to inhibit osteoclast cell fusion and the formation of the podosomal actin belt structure by suppressing HES1/mitogen-activated protein kinase/AKT-mediated induction of NFATc1 in vitro (53). However, it is

Figure 6. Number, size, and resorptive capacity are decreased in CtskCreWT, Hes1Δ/Δ osteoclasts. BMMs derived from 2-month-old CtskCreWT, Hes1Δ/Δ (closed circles) mice and control littermates (open circles) were cultured for 4 days in the presence of M-CSF at 30 ng/ml and of RANKL at 10 ng/ml in cell culture–coated plates (A and B) or bone discs (C–F). A representative image of TRAP-stained multinucleated cells in cell culture–coated plates is shown. The scale bar in the right corner represents 500 μm. B, Hes1 transcript levels were measured by quantitative RT–PCR in total RNA from osteoclasts. Transcript levels are reported as copy number corrected for Rpl38 (left). TRAP-positive cells with more than three nuclei were considered osteoclasts and counted (middle). TRAP-positive cells with differential counting of nuclei/osteoclast are shown (right). C, representative images of Alexa Fluor 594 phalloidin-stained multinucleated cells on bone discs are shown. The scale bar in the right corner represents 100 μm. D, the perimeter of sealing zones was measured in n = 145 osteoclasts from control and n = 139 osteoclasts from CtskCreWT, Hes1Δ/Δ cultures. E, representative images of toluidine blue–stained resorption pits. The scale bar in the right corner represents 200 μm. F, the total resorption pit area was measured (%). Values are means ± SD; n = 3 or 4 biological replicates for control and CtskCreWT, Hes1Δ/Δ. *Significantly different between CtskCreWT, Hes1Δ/Δ and control, p < 0.05. BMM, bone marrow–derived macrophage; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate resistant acid phosphatase.

Table 4
Femoral microarchitecture assessed by μCT of 10-week-old CtskCreWT, RosaHes1 mice and sex-matched littermate controls

| μCT parameters                  | Control        | RosαHes1 | Control | RosαHes1 |
|---------------------------------|----------------|----------|---------|----------|
| **Males**                       | n = 7          | n = 9    | n = 6   | n = 6    |
| Distal femur trabecular bone    |                |          |         |          |
| BV/TV (%)                       | 12.5 ± 2.6     | 10.5 ± 4.1 | 4.7 ± 0.7 | 3.3 ± 0.6 |
| Trabecular separation (μm)      | 206 ± 26       | 225 ± 25  | 313 ± 33 | 346 ± 54 |
| Trabecular number (1/mm)        | 4.9 ± 0.6      | 4.5 ± 0.5  | 3.3 ± 0.3 | 3.0 ± 0.4 |
| Trabecular thickness (μm)       | 44 ± 5         | 42 ± 6    | 39 ± 3   | 39 ± 4   |
| Connectivty density (1/mm³)     | 280 ± 82       | 214 ± 83  | 92 ± 19  | 46 ± 18  |
| Structure model index           | 2.0 ± 0.3      | 2.1 ± 0.5  | 2.7 ± 0.2 | 3.0 ± 0.3 |
| Density of material (mg HA/cm³) | 980 ± 10       | 973 ± 9.7  | 995 ± 9  | 993 ± 16 |
| Femoral midshaft cortical bone  |                |          |         |          |
| BV/TV (%)                       | 89.4 ± 0.1     | 88.8 ± 0.8 | 87.3 ± 0.8 | 87.8 ± 1.1 |
| Porosity (%)                    | 10.6 ± 0.1     | 11.2 ± 0.8 | 12.7 ± 0.8 | 12.2 ± 1.1 |
| Cortical thickness (μm)         | 191 ± 14       | 186 ± 17  | 152 ± 10 | 159 ± 17 |
| Total area (mm²)                | 2.3 ± 0.2      | 2.2 ± 0.3  | 1.8 ± 0.1 | 1.7 ± 0.1 |
| Bone area (mm²)                 | 1.1 ± 0.1      | 1.1 ± 0.2  | 0.8 ± 0.1 | 0.8 ± 0.1 |
| Periosteal perimeter (mm)       | 5.4 ± 0.3      | 5.2 ± 0.4  | 4.7 ± 0.2 | 4.6 ± 0.2 |
| Endocortical perimeter (mm)     | 3.9 ± 0.2      | 3.7 ± 0.2  | 3.5 ± 0.2 | 3.4 ± 0.1 |
| Density of material (mg HA/cm³) | 1181 ± 12      | 1187 ± 18 | 1195 ± 14 | 1195 ± 16 |

μCT was performed on distal femurs for trabecular bone and midshaft for cortical bone. Values are means ± SD.

a Significantly different from control, p < 0.05.
mice had an increase in osteoclast number and bone resorption in vivo. Representative static (upper panels) and dynamic (lower panels) cancellous bone histomorphometry of the distal femur from 10-week-old Ctsk\textsuperscript{Cre}\textsuperscript{WT};RosaHes1\textsuperscript{tm1.1Ecan} female mice and sex-matched littermate controls. The scale bar in the right corner represents 50 μm. HES1, hairy and enhancer of split 1.

Table 5

| Distal femur trabecular bone                        | Control (n = 3–4) | Rosa\textsuperscript{Hes1} Hes1\textsuperscript{tm1.1Ecan} (n = 3–5) |
|----------------------------------------------------|-------------------|---------------------------------------------------------------|
| BV/TV (%)                                            | 11.2 ± 1.4        | 8.2 ± 1.6          |
| Trabecular separation (μm)                          | 286 ± 42          | 383 ± 60           |
| Trabecular number (1/mm)                            | 3.1 ± 0.4         | 2.4 ± 0.4          |
| Trabecular thickness (μm)                           | 35 ± 3.0          | 34 ± 2.9           |
| Osteoblast surface/bone percentage (%)              | 17.2 ± 5.6        | 16.9 ± 3.8         |
| Osteoblasts/bone perimeter (1/mm)                   | 11.3 ± 3.1        | 12.4 ± 1.5         |
| Osteoclast surface/bone percentage (%)              | 10.8 ± 1.7        | 18.7 ± 1.4         |
| Osteoclasts/bone perimeter (1/mm)                   | 4.1 ± 0.6         | 6.8 ± 0.8          |
| Eroded surface/bone surface (%)                     | 12.3 ± 2.8        | 23.1 ± 4.3         |
| Mineral apposition rate (μm/day)                    | 1.7 ± 0.3         | 1.4 ± 0.7          |
| Mineralizing surface/bone percentage (%)            | 17.7 ± 7.7        | 18.7 ± 2.8         |
| Bone formation rate (μm\textsuperscript{3}/μm\textsuperscript{2}/day) | 0.3 ± 0.2 | 0.3 ± 0.1 |

Bone histomorphometry was performed on distal femurs from 10-week-old Ctsk\textsuperscript{Cre}\textsuperscript{WT};RosaHes1\textsuperscript{tm1.1Ecan} female mice and sex-matched littermate controls. Values are means ± SD.

\*Significantly different from control, p < 0.05.

important to note that \textgamma-secretase inhibitors can target many substrates, and their effect is not specific to Notch signaling (54, 55).

Although HES1 had a pronounced effect on osteoclast differentiation and function \textit{in vitro}, this effect was restricted to female mice \textit{in vivo}. The Hes1 inactivation caused an 85% increase in BV in mature female mice, and the induction of HES1 in Ctsk-expressing cells caused an osteopenic phenotype. The inactivation of Hes1 in male mice did not result in a prominent skeletal phenotype; however, it opposed the osteopenic and resorptive phenotype of Notch2\textsuperscript{tm1.1Ecan} mice harboring an HCS mutation causing a gain-of-NOTCH2 function. The absence of a phenotype in male mice facilitated the interpretation of the rescue of the Notch2\textsuperscript{tm1.1Ecan} phenotype by the Hes1 deletion. Since female Hes1-inactivated mice had an increase and Notch2\textsuperscript{tm1.1Ecan} a decrease in BV, one would expect Notch2\textsuperscript{tm1.1Ecan}Hes1\textsuperscript{tm1.1Ecan} female mice to have an intermediate BV. So that an increase in the BV of Notch2\textsuperscript{tm1.1Ecan} would not necessarily represent a rescue of the osteopenic phenotype and that HES1 was a mediator of NOTCH2. It is not readily apparent why the Hes1 inactivation caused a phenotype in female but not in male mice, and the observation stresses the importance of examining phenotypes in mice of different sexes independently (56, 57). It is not unusual to observe sex-specific phenotypes in genetically engineered mice (58–60). Possible explanations for the prevalence of a phenotype in female mice include genetic influences, a loss of the inhibitory actions of estrogens on osteoclastogenesis in the context of the Hes1 inactivation as well as the earlier NF-κB–NFATc1 activation and osteoclastogenesis that occurs in female mice (57, 61, 62).

In previous work, we demonstrated that HES1 is induced as osteoclasts mature, particularly in the context of the Notch2\textsuperscript{tm1.1Ecan} mutation (17). A plausible explanation for the modest skeletal phenotype of the Hes1 inactivation in male mice is that under basal conditions HES1 levels are low and play a modest role in skeletal physiology, and only following Notch activation, HES1 plays a significant role in bone homeostasis. This explanation is substantiated by the amelioration of the Notch2\textsuperscript{tm1.1Ecan} osteopenic phenotype following the Hes1 inactivation. The Notch2\textsuperscript{tm1.1Ecan} osteopenic phenotype was not fully reversed, and this is explained by the effects of NOTCH2 enhancing RANKL expression by cells of the osteoblast lineage since these are independent of the induction of HES1 in the myeloid lineage (17, 24). Other Notch target genes, such as Hey1, Hey2, and HeyL, are not expressed in cells of the myeloid lineage and as a consequence could not be responsible for the stimulatory effects of NOTCH2 on osteoclastogenesis (19). HES3 and HES5 could compensate for the effects of HES1, but their expression in osteoclasts is low and their role in osteoclastogenesis is unknown (63). Whereas, HES1 mediates direct effects of NOTCH2 on osteoclastogenesis, it is not likely to mediate the effects of NOTCH1, known to inhibit and not enhance osteoclast maturation, or NOTCH3, since this Notch receptor is not expressed in the myeloid lineage and its effects on osteoclastogenesis are indirect (21, 23). NOTCH4 is expressed at low levels in the myeloid lineage and not known to play a role in osteoclast differentiation (19, 24).

In the present work, we confirm that Notch2\textsuperscript{tm1.1Ecan} mice are osteopenic because of direct effects of NOTCH2 in cells of the myeloid lineage. The stimulatory effect of NOTCH2 on osteoclastogenesis has been attributed to direct interactions of the NOTCH2 intracellular domain with NF-κB in the context of Nfatc1 regulatory regions and increased Nfatc1 transcription (64). However, recent work from our laboratory has demonstrated that NOTCH2 has NF-κB–independent effects on tumor necrosis factor α (TNFα)–induced osteoclastogenesis, and some of these effects are secondary to the activation of AKT and Il1b expression (25, 65). The present work demonstrates that the direct effects of NOTCH2 on osteoclastogenesis are HES1 dependent confirming previous work from this laboratory revealing that the enhancement of the osteolytic actions of TNFα by the Notch2\textsuperscript{tm1.1Ecan} mutation depend on the induction of HES1 (25).

HES1 is known to inhibit phosphatase and tensin homolog and as a consequence enhance phosphoinositide 3-kinase–
AKT signaling (66). AKT signaling is required for cell–cell fusion during osteoclast differentiation, and inhibitors of AKT lead to a decrease in Destamp transcripts and osteoclast size (67). However, the levels of phosphatase and tensin homolog transcripts and the phosphorylation levels of AKT were not different between CtskCreWT;RosaHes1 osteoclasts and controls (data not shown). Although RANKL and TNFα share and activate similar downstream molecules, mechanisms triggering osteoclastogenesis are different in part because Nfatc1 expression of Notch2tm1.1Ecan mice and control littermates (open circles) were cultured for 4 days in the presence of M-CSF at 30 ng/ml and of RANKL at 10 ng/ml in cell culture–coated plates (A and B) or bone discs (C–F). A, representative images of TRAP-stained multinucleated cells in cell culture–coated plates are shown. The scale bar in the right corner represents 500 μm. B, Hes1 transcript levels were measured by quantitative RT–PCR in total RNA from osteoclasts. Transcript levels are reported as copy number corrected for Rpl38. C, TRAP-positive cells with more than three nuclei were considered osteoclasts and counted (middle). TRAP-positive cells with differential counting of nuclei/osteoclast are shown (right). C, representative images of Alexa Fluor 594 phalloidin-stained multinucleated cells on bone discs are shown. The scale bar in the right corner represents 100 μm. D, the perimeter of sealing zones was measured in n = 68 osteoclasts from control and in n = 131 osteoclasts from CtskCreWT;RosaHes1 cultures. E, representative images of toluidine blue–stained resorption pits. The scale bar in the right corner represents 200 μm. F, the total resorption pit area was measured (%). Values are means ± SD; n = 3 biological replicates for control and CtskCreWT;RosaHes1, *Significantly different between CtskCreWT;RosaHes1 and control, p < 0.05. BMM, bone marrow–derived macrophage; M-CSF, macrophage colony-stimulating factor; RANKL, receptor activator of NF-κB ligand; TRAP, tartrate resistant acid phosphatase.

In conclusion, HES1 plays a critical role in osteoclastogenesis and bone resorption and is mechanistically relevant to the skeletal phenotype of an experimental model of HCS.

**Experimental procedures**

**Genetically modified mice**

Notch2ΔΔ mice harboring a 6955C>T substitution in the Notch2 locus have been characterized in previous studies and were backcrossed into a C57BL/6 background for eight
and more generations (17, 18, 73). Hes1loxP/loxP (Hes1<tm1Iimayo) mice, where loxP sequences are knocked into the first intron and downstream of the 3’ UTR of Hes1 alleles, were obtained from RIKEN (RBRC06047; Wako Saitama) in a 26Sor background (26). In Rosa<sup>STOP</sup>/Hes1 mice, Hes1 coding sequences are cloned into the Rosa-26 locus downstream of a Neo-STOP cassette flanked by loxP sequences, so that HES1-RES-GFP is expressed following the excision of the cassette by Cre recombination. To induce or delete Hes1 in differentiated cells of the osteoclast lineage, mice harboring sequences coding for the Cre recombinase knocked-in into the Ctsk locus (Ctsk<sup>Cre</sup>) were used in a C57BL/6 background (74). Rosa<sup>STOP</sup>/Hes1 (Gt(Rosa)26So<r1>Hes1.1EFP)Imayo>) were obtained from RIKEN (RBRC06002) in an ICR background (26). In Rosa<sup>STOP</sup>/Hes1 mice, Hes1 coding sequences are cloned into the Rosa-26 locus downstream of a Neo-STOP cassette flanked by loxP sequences, so that HES1-RES-GFP is expressed following the excision of the cassette by Cre recombination. To induce or delete Hes1 in differentiated cells of the osteoclast lineage, mice harboring sequences coding for the Cre recombinase knocked-in into the Ctsk locus (Ctsk<sup>Cre</sup>) were used in a C57BL/6 background (61, 70). Genotyping was conducted in tail DNA extracts by PCR using specific primers from Integrated DNA Technologies (IDT) (Table S1).

For the deletion of Hes1, Hes1loxP alleles were introduced into Ctsk<sup>Cre</sup> mice to create Ctsk<sup>Cre/WT</sup>;Hes1<sup>loxP/loxP</sup> mice, and these were crossed with Hes1loxP/loxP to generate Hes1<sup>Δ/Δ</sup> deleted and Hes1<sup>loxP/loxP</sup> control litters. For the induction of HES1, Ctsk<sup>Cre/WT</sup> mice were crossed with homozygous Rosa<sup>STOP</sup>/Hes1<sup>loxP/loxP</sup> mice to generate ~50% Hes1-induced and ~50% Rosa<sup>STOP</sup>/Hes1<sup>loxP/loxP</sup> control litters. For the deletion of Hes1 in the context of the Notch2<sup>tm1.1Ecan</sup> mutation, Ctsk<sup>Cre/WT</sup>;Hes1<sup>loxP/loxP</sup> mice were crossed with Notch2<sup>tm1.1Ecan</sup>;Hes1<sup>loxP/loxP</sup> mice to create Notch2<sup>tm1.1Ecan</sup>;Hes1<sup>Δ/Δ</sup> and Notch2<sup>tm1.1Ecan</sup>;Hes1<sup>loxP/loxP</sup> controls. Recombination of loxP flanked sequences was documented in extracts from tibiae using specific primers (Table S1). All animal experiments were approved by the Institutional Animal Care and Use Committee of UConn Health.

**BMM cultures, osteoclast formation, and adenovirus-Cre-mediated recombination**

To obtain BMMs, the marrow from experimental and control sex-matched littermate mice was removed by flushing with a 26-gauge needle, and erythrocytes were lysed in 150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA (pH 7.4), as described previously (73). Cells were centrifuged, and the sediment suspended in α-minimum essential medium (α-MEM) in the presence of 10% fetal bovine serum (FBS; both from Thermo Fisher Scientific) and recombinant human M-CSF at 30 ng/ml. M-CSF complementary DNA (cDNA) and expression vector were obtained from D. Fremont, and M-CSF was purified as previously reported (34). Cells were seeded on uncoated plastic petri dishes at a density of 300,000 cells/cm² and cultured for 3 days. For osteoclast formation, cells were collected following treatment with 0.25% trypsin/EDTA for 5 min and seeded on tissue culture plates at a density of 62,500 cells/cm² in α-MEM with 10% FBS, M-CSF at 30 ng/ml, and 0.1 mM EDTA for 3 days. BMMs were then treated with 10 ng/ml RANKL for 3 days, and cultures were stained for tartrate-resistant acid phosphatase (TRAP).
and recombinant murine RANKL at 10 ng/ml. Tnfsf11, encoding RANKL, cDNA expression vector was obtained from M. Glogauer, and glutathione-S-transferase–tagged RANKL was expressed and purified as described (75). Cultures were carried out until multinucleated tartrate resistant acid phosphatase (TRAP)–positive cells were formed. TRAP enzyme histochemistry was conducted using a commercial kit (Sigma–Aldrich), in accordance with the manufacturer’s instructions. TRAP–positive cells containing more than three nuclei were considered osteoclasts.

For actin structure staining and bone resorption assay of osteoclasts in vitro, BMMs were seeded at a density of 62,500 cells/cm² on bovine cortical bone slices and cultured in α-MEM with 10% FBS, M-CSF at 30 ng/ml, and RANKL at 10 ng/ml. To visualize the sealing zone of osteoclasts on bone slices, cells were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.3% Triton X-100 for 5 min. To block nonspecific background staining, cells on bone discs were incubated with 2% bovine serum albumin for 1 h and stained with Alexa Fluor 594 Phalloidin (Thermo Fisher Scientific) at a 1:40 dilution for 20 min. The sealing zone was viewed on a Leica fluorescence microscope (model DMi6000B), and collected images were processed using the Leica Application Suite X 1.5.1.1387 (Leica Microsystems). After visualizing the sealing zone, cells were stained for TRAP to assess cellular morphology. To visualize bone resorption pits, bone slices were sonicated to remove osteoclasts and stained with 1% toluidine blue in 1% sodium borate. To assess the ability of osteoclasts to resorb bone, the total resorption area/total bone sealing zone, cells were stained for TRAP to assess cellular

To inactivate or induce Hes1 in osteoclast precursors in vitro, BMMs from homozygous Hes1loxP/loxP or Rosa[STOP]C mice were cultured in the presence of M-CSF at 30 ng/ml and RANKL at 10 ng/ml for 2 days, prior to being transduced with Ad-Cre or CMV-GFP (Ad-GFP [Vector Biolabs]) as control, at multiplicity of infection of 100 and cultured with M-CSF and RANKL for two additional days until the formation of multinucleated TRAP-positive cells. To inactivate Hes1 in the context of the Notch26955C>T mutation, Hes1loxP/loxP alleles were introduced into Notch26955-1.1Emut, Hes1loxP/loxP mice to create Notch26955-1.1Emut, Hes1loxP/loxP mice, and BMMs were cultured and transduced with Ad-Cre or Ad-GFP.

**qRT-PCR**

Total RNA was extracted from osteoclasts with the RNeasy Mini kit (Qiagen) and homogenized bones with the RNeasy Micro kit (Qiagen), in accordance with the manufacturer’s instructions. The integrity of the RNA extracted from bones was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad), and RNA with a quality indicator number equal to or higher than 7.0 was used for subsequent analysis. Equal amounts of RNA were reverse transcribed using the iScript RT-PCR kit (Bio-Rad) and amplified in the presence of specific primers (all from IDT) with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) at 60 °C for 40 cycles. Transcription copy number was estimated by comparison with a serial dilution of cDNA for Acp5 and Notch2 (all from Thermo Fisher Scientific), Hes1 (American Type Culture Collection), Nfatc1 (Addgene; plasmid 11793; created by A. Rao), Bcl6, Mafb, Atp6v0d2, and Ocstimp (all from Dharamco).

The level of Notch26955C>T mutant transcript was measured as described previously (17). Total RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase in the presence of reverse primers for Notch2 (5’-GGATCTGGTACATAGAG-3’) and Rpl38 (Table S2). Notch2 cDNA was amplified by qPCR in the presence of TaqMan gene expression assay mix, including specific primers (5’-CATCGTGACTTTCCA-3’ and 5’-GGATCTGGTACATAGAG-3’) and a 6-carboxyfluorescein-labeled DNA probe of sequence 5’-CAATTGGCTAGGCACG-3’ covalently attached to a 3’-minor groove binder quencher (Thermo Fisher Scientific), and SsoAdvanced Universal Probes Supermix (Bio-Rad) at 60 °C for 45 cycles (76). Notch26955C>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(-) (Thermo Fisher Scientific) by isothermal single reaction assembly using commercially available reagents (New England Biolabs) (77).

Amplification reactions were conducted in CFX96 qRT–PCR detection systems (Bio-Rad), and fluorescence was monitored at the end of the elongation step during every PCR cycle. Data are expressed as copy number correlated for Rpl38 and fluorescence was monitored at the end of the elongation step during every PCR cycle. Data are expressed as copy number corrected for Rpl38 with a serial dilution of cDNA for Rpl38 (American Type Culture Collection) (78).

**Illumina transcriptome library preparation and sequencing**

Total RNA was quantified, and purity ratios were determined for each sample using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). To assess RNA quality, total RNA was analyzed on the Agilent TapeStation 4200 (Agilent Technologies) using the RNA High Sensitivity assay. Ribosomal integrity numbers were recorded for each sample. Total RNA was reverse transcribed using the Illumina TruSeq Stranded mRNA Sample Preparation kit (Illumina). Libraries were validated for length and adapter dimer removal using the Agilent TapeStation 4200 (Agilent Technologies) and then they were quantified and normalized using the dsDNA High Sensitivity Assay for Qubit 3.0 (Thermo Fisher Scientific). Sample libraries were prepared for Illumina sequencing by denaturing and diluting the libraries per manufacturer’s protocol (Illumina). All samples were pooled into one sequencing pool, equally normalized, and run as one sample pool across the Illumina NextSeq 500 using
version 2.5 chemistry. Target read depth was achieved for each sample with paired end 75 bp reads. Raw reads were trimmed with Sickle (version 1.33), with a quality threshold of 30 and length threshold of 45, following that the trimmed reads were mapped to Homo Sapiens genome (GRCh38 ensembl release 99) with HISAT2 (version 2.1.0) (79). The resulting SAM files were then converted into BAM format using samtools (version 1.9) (80), and the PCR duplicates were removed using PICARD (http://broadinstitute.github.io/picard/). The counts were generated against the features with HTSeq-count (81). The differential expression of genes between conditions was evaluated using DESeq2 (82). Covariates were introduced in the DESeq2 analysis to increase the accuracy of results, and genes showing less than ten counts across the compared samples were excluded from the analysis. Genes with a false discovery rate <0.05 were considered significant and used in the downstream analysis. The processed RNA-Seq results were further analyzed by using IPA (Qiagen).

**Immunoblotting**

Cells from control and experimental mice were extracted in buffer containing 25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenyl methyl sulfonyl fluoride, and a protease inhibitor cocktail (all from Sigma–Aldrich). Total cell lysates (40 μg of total protein) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 8 or 12% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore). The blots were probed with anti-HES1 (11988) and β-actin (3700) antibodies from Cell Signaling Technology or anti-NFATc1 antibody (556602) from BD Biosciences. The blots were exposed to anti-rabbit, anti-antirat, or antimouse IgG conjugated to horseradish peroxidase (Sigma–Aldrich) and incubated with a chemiluminescence detection reagent (Bio-Rad). Chemiluminescence was detected by ChemiDoc XR+ molecular imager (Bio-Rad) with Image Lab software (version 5.2.1), and the amount of protein present in individual bands was quantified (25).

**μCT**

Femoral microarchitecture was determined using a μCT instrument (Scanco μCT 40; Scanco Medical AG), which was calibrated periodically using a phantom provided by the manufacturer (83, 84). Femurs were scanned in 70% ethanol at a total of 160 consecutive 6 μm thick slices were cut using a Microm microtome (Richards-Allan Scientific). Mineralization surface per bone surface and mineral apposition rate were measured on unstained sections visualized under UV light and a triple diamidino-2-phenylindole/fluorescein/Texas red set long-pass filter, and bone formation rate was calculated (85).

**Bone histomorphometry**

Bone histomorphometry was carried out in CtskCre/WT; Notch2tm1.1Ecan;Hes1Δ/Δ, CtskCre/WT;Hes1Δ/Δ, and CtskCre/WT; RosaHes1 mice, and sex-matched controls were injected with calcein 20 mg/kg and demeclocycline 50 mg/kg at a 5 or 7 days of interval and sacrificed 2 days after demeclocycline administration. For static cancellous bone histomorphometry and to assess for the presence of TRAP-positive multinucleated cells, bones were decalcified in 14% EDTA for 14 days and embedded in paraffin, and 7 μm sections were stained for the presence of TRAP and counterstained with hematoxylin and analyzed at a 100× magnification using OsteoMeasureXP software (Osteometrics). Stained sections were used to draw bone tissue and measure trabecular separation, number and thickness, and eroded surface, as well as to count osteoblast and osteoclast number. To assess dynamic parameters of bone histomorphometry, undecalcified femurs were embedded in methyl methacrylate, and 5 μm sections were cut using Microm microtome (Richards-Allan Scientific). Mineralizing surface per bone surface and mineral apposition rate were measured on unstained sections visualized under UV light and a triple diamidino-2-phenylindole/fluorescein/Texas red set long-pass filter, and bone formation rate was calculated (85).

**Statistics**

Data are expressed as means ± SD and presented as biological replicates except for experiments where BMMs were transduced with adenoviruses or cells were extracted for immunoblotting, and these are presented as technical replicates representative of two or more experiments. Statistical differences were determined by Student’s t test or two-way analysis of variance with Tukey analysis for multiple comparisons, respectively.

**Data availability**

Data not shown will be shared upon request to Ernesto Canalis at canalis@uchc.edu.

**Supporting information**—This article contains supporting information.

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and E. C. writing—original draft; J. Y. and E. C. writing—review and editing; J. Y. visualization; E. C. project administration; E. C. funding acquisition.

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Abbreviations—The abbreviations used are: α-MEM, α-minimum essential medium; μCT, microcomputed tomography; Ad-Cre, adenovirus carrying cytomegalovirus-Cre; Ad-GFP, adenoviruses carrying GFP; Bmpl1, B lymphocyte–induced maturation protein 1; BMM, bone marrow–derived macrophage; BV/TV, bone volume/total volume; cDNA, complementary DNA; FBS, fetal bovine serum; HCS, Hajdu–Cheney syndrome; HES1, hairy and enhancer of split 1; IL, interleukin; IPA, ingenuity pathway analysis; M-CSF, macrophage colony-stimulating factor; NFATc1, nuclear factor of activated T cells, cytoplasmic 1; RANKL, receptor activator of NF-κB ligand; SMI, structure model index; TNFα, tumor necrosis factor α; TRAP, tartrate resistant acid phosphatase.

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