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Hairy and Enhancer of Split-related with YRPW Motif-Like (HeyL) is Dispensable for Bone Remodeling in Mice

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Abstract

Notch induces Hairy Enhancer of Split (*Hes*)1 and Hes-related with YRPW motif (*Hey*) *Hey1*, *Hey2* and *Hey-like* (*HeyL*) expression in osteoblasts, but it is not known whether any of these target genes mediates the effect of Notch in the skeleton. We demonstrated that Notch1 activation in osteoblasts/osteocytes induces *Hes1*, *Hey1*, *Hey2* and *HeyL*, but *HeyL* was induced to a greater extent than other target genes. To characterize *HeyL* null mice for their skeletal phenotype, microcomputed tomography (μ CT) and histomorphometric analysis of *HeyL* null and sex-matched littermate controls was performed. μ CT demonstrated modest cancellous bone osteopenia in 1 month old male mice and normal microarchitecture in 3 month old male *HeyL* null mice. Female *HeyL* null mice were not different from controls at either 1 or 3 months of age. Bone histomorphometry did not demonstrate differences between *HeyL* null mice of either sex and littermate controls. In conclusion, *HeyL* null mice do not exhibit an obvious skeletal phenotype demonstrating that *HeyL* is dispensable for skeletal homeostasis.

Keywords

Notch; HeyL; bone remodeling; osteoblasts; osteocytes

INTRODUCTION

Notch (1 to 4) are transmembrane receptors that play a central role in skeletal development and homeostasis, and in osteoblast and osteoclast differentiation [Fortini, 2009; Sahlgren and Lendahl, 2006; Zanolli and Canalis, 2010; Zanolli and Canalis, 2016]. Notch signaling

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²The abbreviations used are: ATCC, American type culture collection; bHLH, basic helix-loop-helix; Col1a1 collagen type I α 1; FBS, fetal bovine serum; Hes, Hairy Enhancer of Split; Hey, Hes-related with YRPW motif; HeyL, Hey-like; IDT, integrated DNA technologies; μ CT, microcomputed tomography; NICD, Notch intracellular domain; PCR, polymerase chain reaction; Psen, Presenilin, qRT-PCR, quantitative reverse transcription-PCR; Rpl38, ribosomal protein L38; SMI, structure model index.

³The authors declare that they have no conflicts of interest with the contents of this article.

⁴The *in vivo* animal work was conducted while the investigators worked at St Francis Hospital and Medical Center (Hartford, CT) and the analysis of the samples was completed at UConn Health (Farmington, CT).

is activated following interactions of Notch receptors with ligands of the Jagged and Delta-like families. This results in the proteolytic cleavage of the receptor and the release of the Notch intracellular domain (NICD) [Zanotti and Canalis, 2010]. The NICD translocates to the nucleus, where it forms a complex with recombination signal binding protein for immunoglobulin kappa J region and mastermind-like to induce the transcription of Notch target genes [Kovall, 2008; Nam et al., 2006; Schroeter et al., 1998; Wilson and Kovall, 2006]. Classic canonical Notch target genes include Hairy Enhancer of Split (*Hes*) and Hes-related with YRPW motif (*Hey*) [Iso et al., 2003; Kageyama et al., 2007]. Activation of Notch1 in skeletal cells leads to the induction of *Hes1*, *Hey1*, *Hey2* and Hey-like (*HeyL*), but it is not known which, if any, of these target genes mediates the effects of Notch in the skeleton [Canalis et al., 2013b].

Hey genes encode for 3 basic helix-loop-helix (bHLH) transcription factors termed Hey1, Hey2 and Hey-like (HeyL) [Iso et al., 2003]. Hey proteins are structurally similar to Hes, and Hes and Heys, can form homodimers and heterodimers that bind to DNA to regulate transcription [Iso et al., 2003]. Heys play an important role in cardiovascular development. Inactivation of *Hey2* results in embryonic lethality due to cardiovascular defects, and the dual inactivation of *Hey1* and *Hey2* phenocopies the global deletion of *Notch1* [Fischer et al., 2004; Kokubo et al., 2005; Kokubo et al., 2004; Xin et al., 2007]. Similarly, the deletion of *Hey1* and *HeyL* in mice impairs cardiovascular development [Fischer et al., 2007].

Notch1 and Notch2 inhibit the maturation of osteoblasts and their activation causes osteopenia [Canalis et al., 2013b; Canalis et al., 2016; Yorgan et al., 2016; Zanotti and Canalis, 2014; Zanotti et al., 2008]. However, the effects of Notch are cell-context dependent; and when activated in osteocytes, Notch1 induces osteoprotegerin, inhibits bone resorption and causes osteopetrosis [Canalis et al., 2013a]. The effects of Notch in the skeleton appear to be mediated by the canonical signaling pathway, but the genes responsible for the actions of Notch in bone have not been defined [Canalis et al., 2015; Dong et al., 2010; Tao et al., 2010]. *Hes1*, *Hey1*, *Hey2* and *HeyL* are responsive to Notch canonical signaling and may be responsible, singly or in combination, for the effects of Notch in the skeleton [Zanotti et al., 2011b; Zanotti et al., 2013]. Previously, we reported that transgenic expression of *Hes1* under the control of the collagen type-I $\alpha 1$ (*Col1a1*) promoter causes osteopenia by inhibiting bone formation and inducing bone resorption [Zanotti et al., 2011a]. These effects of *Hes1* phenocopied only partially the skeletal phenotype of mice harboring a gain-of-Notch1 function, suggesting that other genes might be downstream effectors of Notch signaling in the skeleton [Engin et al., 2008; Zanotti et al., 2011a; Zanotti et al., 2008]. Transgenic expression of *Hey1* under the control of the β -actin promoter as well as the global inactivation of *Hey1* cause modest osteopenia [Salie et al., 2010]. We demonstrated that the conditional inactivation of *Hey2* in cells of the osteoblastic lineage cause a modest and apparently transient increase in cancellous bone, whereas the transgenic expression of *Hey2* in *Col1a1*-expressing cells causes osteopenia [Zanotti and Canalis, 2013]. These observations would suggest that Hes and Hey genes play a negative regulatory role in bone homeostasis. *HeyL* inactivation was reported to cause an increase in trabecular bone volume, but the effect was mostly observed in the context of a heterozygous inactivation of *Hey1* [Tu et al., 2012].

HeyL is particularly responsive to Notch1 activation in skeletal cells, suggesting that it could be an important mediator of Notch action in bone. Because of these reasons, we wished to reassess the role of HeyL in bone remodeling. The purpose of the present work was to define the skeletal phenotype of *HeyL* null mice and answer the question as to whether the phenotype of Notch1 activation could be explained by direct effects of HeyL in bone cells. To this end, *HeyL* null and control sex-matched littermate mice were characterized by bone microarchitectural and histomorphometric analyses.

MATERIALS AND METHODS

Rosa^{Notch} Mice

Rosa^{Notch} mice were obtained from the Jackson Laboratory in a C57BL/6J genetic background [Murtaugh et al., 2003; Stanger et al., 2005]. In these mice, the *Rosa26* locus is targeted with a DNA construct encoding for the Notch1 intracellular domain (NICD), preceded by a STOP cassette flanked by *loxP* sites, cloned downstream of the *Rosa26* promoter. Expression of the NICD from the targeted *Rosa26* locus occurs following the excision of the STOP cassette by Cre recombination of *loxP* sequences [Buchholz et al., 1996; Sauer and Henderson, 1988]. To activate Notch1 in mature cells of the osteoblastic lineage and osteocytes, transgenic mice expressing the Cre recombinase under the control of a ~10 kilobase fragment of the dentin matrix protein (*Dmp*) 1 promoter (*Dmp1-Cre*) in a C57BL/6J genetic background were obtained from J. Feng (Dallas, TX) [Lu et al., 2007]. Homozygous *Rosa*^{Notch} mice were mated with hemizygous *Dmp1-Cre*^{+/-} mice to create 50% *Dmp1-Cre*^{+/-}; *Rosa*^{Notch/WT} experimental and 50% *Rosa*^{Notch/WT} littermate controls. Genotyping was carried out by polymerase chain reaction (PCR) in tail DNA extracts using specific primers (all from Integrated DNA Technologies (IDT), Coralville, IA; Table 1A). Deletion of the *loxP*-flanked STOP cassette by the Cre recombinase was documented by PCR in DNA extracted from tibiae, as previously published [Canalis et al., 2015; Canalis et al., 2013b].

HeyL Null Mice

To study the skeletal consequences of the global *HeyL* inactivation, we used mice where exons 2 to 4, encoding for the bHLH domain, were replaced with an in-frame *LacZ-neo* cassette [Fischer et al., 2007]. *HeyL* null mice were backcrossed into a C57BL/6J background for >9 generations and were kindly provided by M. Gessler (Wuerzburg, Germany) following the removal of the *neo* selection cassette by Cre recombination. *HeyL* null mice were previously shown to be grossly normal and fertile [Fischer et al., 2007]. Genotyping of *HeyL* null mice was conducted in tail DNA extracts by PCR using specific primers (all from IDT; Table 1A). In this study, mice homozygous for the *HeyL* deletion and control mice were obtained by intermating heterozygous *HeyL* null mice. One and 3 month old male and female null and control sex-matched littermate mice were analyzed.

Animal studies were approved by the Institutional Animal Care and Use Committee of St. Francis Hospital and Medical Center.

Microcomputed Tomography (μ CT)

Bone microarchitecture of femurs from experimental and control mice was determined using a microcomputed tomography instrument (μ CT 40; Scanco Medical AG, Bassersdorf, Switzerland), which was calibrated periodically using a phantom provided by the manufacturer [Bouxsein et al., 2010; Glatt et al., 2007]. Femurs were scanned in 70% ethanol at high resolution, energy level of 55 kVp, intensity of 145 μ A and integration time of 200 ms. A total of 100 slices at midshaft and 160 slices at the distal metaphysis were acquired at an isotropic voxel size of 216 μm^3 and a slice thickness of 6 μm , and chosen for analysis. Trabecular bone volume fraction and microarchitecture were evaluated starting approximately 1.0 mm proximal from the femoral condyles. Contours were manually drawn every 10 slices a few voxels away from the endocortical boundary to define the region of interest for analysis. The remaining slice contours were iterated automatically. Trabecular regions were assessed for total volume, bone volume, bone volume fraction (bone volume/total volume), trabecular thickness, trabecular number, trabecular separation, connectivity density and structure model index (SMI), using a Gaussian filter ($\sigma = 0.8$), and user defined thresholds [Bouxsein et al., 2010; Glatt et al., 2007]. For analysis of femoral cortical bone, contours were iterated across 100 slices along the cortical shell of the femoral mid shaft, excluding the marrow cavity. Analysis of bone volume/total volume, porosity, cortical thickness, total cross sectional and cortical bone area, periosteal perimeter, endosteal perimeter and material density were performed using a Gaussian filter ($\sigma = 0.8$, support = 1), and user defined thresholds.

Bone Histomorphometric Analysis

Static and dynamic cancellous bone histomorphometry was carried out on experimental and control mice after they were injected with calcein, 20 mg/kg, and demeclocycline, 50 mg/kg, both intraperitoneally, at an interval of 2 to 5 days, and sacrificed 2 days following demeclocycline injections. Five micron longitudinal sections of undecalcified femurs embedded in methyl methacrylate were cut on a microtome (Microm, Richards-Allan Scientific, Kalamazoo, MI), and stained with 0.1% toluidine blue. Static parameters of bone formation and resorption were measured in a defined area between 360 μm and 2160 μm from the growth plate, using an OsteoMeasure morphometry system (OsteoMetrics, Atlanta, GA). For dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured on unstained sections under ultraviolet light, using a triple diamidino-2-phenylindole/fluorescein/Texas red set long pass filter, and bone formation rate was calculated. The terminology and units used for cancellous bone are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [Dempster et al., 2013; Parfitt et al., 1987].

Osteoblast/Osteocyte-enriched Cultures

Osteoblast/osteocyte-enriched cells were obtained following a modification of a previously described method [Halleux et al., 2012]. Femurs were removed aseptically from 1 month old *Dmp1-Cre^{+/-}; Rosa^{Notch/WT}* and *Rosa^{Notch/WT}* control mice; the surrounding tissues were dissected, the proximal epiphyseal end was excised, and the bone marrow was removed by centrifugation. The distal epiphysis was excised, and femurs were digested for 20 min at

37°C with type II collagenase from *Clostridium histolyticum* (Worthington Biochemical Corp., Lakewood, NJ) pretreated with N- α -tosyl-L-lysyl-chloromethylketone hydrochloride) (Calbiochem, La Jolla, CA) and subsequently exposed to 5mM EDTA (Life Technologies, Grand Island, NY) for 20 min at 37°C. The resulting terminally differentiated osteoblast/osteocyte-enriched cortical femurs were cultured in DMEM supplemented with non-essential amino acids (both from Life Technologies), 100 μ g/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO), and heat-inactivated 10% fetal bovine serum (FBS, Atlanta Biologicals, Inc., Atlanta, GA) for 4 days in a humidified 5% CO₂ incubator at 37°C.

Quantitative Reverse Transcription (qRT)-PCR

Total RNA was extracted by chloroform/phenol extraction from homogenized osteoblast/osteocyte-enriched cell cultures and from tibiae of 3 month old mice with the micro RNeasy kit (Qiagen, Valencia, CA), in accordance with manufacturer's instructions. The integrity of the RNA was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad, Hercules, CA) and mRNA levels were determined by qRT-PCR [Nazarenko et al., 2002a; Nazarenko et al., 2002b]. For this purpose, equal amounts of RNA were reverse-transcribed using iScript RT-PCR kit (Bio-Rad), according to manufacturer's instructions, and amplified in the presence of specific primers (all from IDT; Table 1B), and iQ SYBR Green Supermix (BioRad), at 60°C for 35 cycles. Transcript copy number was estimated by comparison with a serial dilution of cDNA for *Notch1* *Nicd* (from Thermo Fischer Scientific, Waltham, MA), *Hes1* (from American Type Tissue Collection (ATCC), Manassas, VA), *Hey1* and *Hey2* (both from T. Iso, Los Angeles, CA) and *HeyL* (from D. Srivastava, Dallas, TX) [Iso et al., 2001a; Nakagawa et al., 1999]. Amplification reactions were conducted in a CFX96 qRT-PCR detection system (BioRad), and fluorescence was monitored during every PCR cycle at the annealing step. Data are expressed as copy number corrected for ribosomal protein L38 (*Rpl38*) copy number, estimated by comparison with a serial dilution of *Rpl38* (from ATCC), and controls normalized to 1 [Kouadjo et al., 2007].

Statistics

Data are expressed as means $\pm$ SEM. Statistical differences were determined by unpaired Student's *t*-test.

RESULTS

Induction of Notch Target Genes in Osteoblasts/Osteocytes

To explore for differential gene expression in response to Notch activation in mature cells of the osteoblastic lineage, femurs enriched in osteoblasts/osteocytes from *Dmp1-Cre^{+/-};Rosa^{Notch/WT}* mice, were analyzed for changes in Notch target gene expression. Transcript levels for the *Notch1* *Nicd* and its target genes *Hey1*, *Hey2*, *HeyL* and *Hes1* were increased in femurs from *Dmp1-Cre^{+/-};Rosa^{Notch/WT}* mice compared to controls (Figure 1). Although transcripts for all Notch canonical target genes were increased, Notch1 activation caused a greater induction of *HeyL* transcripts, suggesting that HeyL could mediate effects of Notch1 in osteoblasts. As an initial step to understand the role of HeyL in skeletal homeostasis, we analyzed the skeletal phenotype of *HeyL* null mice.

General Characteristics of HeyL Null Mice

Homozygous *HeyL* null mice were obtained following heterozygous intercrossings and compared to sex-matched littermate controls, all in a C57BL/6J genetic background. Homozygous *HeyL* null mice did not express *HeyL* mRNA, appeared normal, were fertile and their weight and femoral length were not different from that of littermate wild type controls (Figure 2).

Femoral Microarchitecture

μ CT of the distal femur revealed that 1 month old male *HeyL* null mice had a modest decrease in trabecular bone volume associated with reduced trabecular thickness and higher SMI, indicative of a higher prevalence of rod-like trabeculae (Table 2). The modest cancellous bone osteopenia was transient and 3 month old *HeyL* null male mice had bone volume/total volume, connectivity and SMI that were not different from values obtained in wild type littermate sex-matched mice (Table 2, Figure 3). μ CT of the distal femur of female 1 and 3 month old mice revealed no differences in either cancellous or cortical bone between *HeyL* null mice and littermate female control mice (Table 3, Figure 3).

Bone Histomorphometric Analysis

Cancellous bone histomorphometric analysis of femurs from 1 and 3 month old male mice confirmed that the skeleton of *HeyL* null mice was essentially normal and not different from wild type littermate controls, and *HeyL* null mice exhibited normal bone volume/tissue volume, osteoblast and osteoclast numbers, and parameters of bone formation and bone resorption (Table 4).

Cancellous bone histomorphometry of 3 month old female mice confirmed that *HeyL* null mice were not different from wild type sex-matched littermates (Table 5).

DISCUSSION

In the present work, we confirmed *Hes1*, *Hey1*, *Hey2* and *HeyL* induction following the activation of Notch1 in preparations enriched in osteoblasts/osteocytes. We demonstrate that even though all these Notch target genes were induced, the greatest response was observed on the expression of *HeyL* mRNA, suggesting that HeyL could be a mediator of Notch actions in bone. To this end, we explored the general and skeletal phenotypic characteristics of *HeyL* null mice. Confirming prior observations, the global inactivation of *HeyL* had no obvious phenotypic impact and mice appeared healthy and of normal weight [Fischer et al., 2007]. In addition, the *HeyL* inactivation caused only a minimal phenotypic impact in the skeleton.

The skeletal phenotype of *HeyL* null mice was mild, transient and observed only in 1 month old male mice, indicating that *HeyL* does not play a significant role in skeletal homeostasis. Global *HeyL* null mice, particularly when carrying a heterozygous null mutation of *Hey1* were reported to have increased bone mass, but we were unable to reproduce these results [Tu et al., 2012]. The reason for the discrepancy in the results between the two studies is not immediately apparent. Since the deletion of one *Hey1* allele accentuated the phenotype of

HeyL null mice it is possible that *Hey1* played a compensatory role preventing the detection of a *HeyL* null phenotype in the present study. It is of interest that we detected a modest decrease in bone mass instead of the increase reported in the previous study [Tu et al., 2012]. Moreover, the decrease in cancellous bone volume was transient, and observed only in 1 month old male mice, and the skeletal phenotype of adult *HeyL* null mice was not different from wild type mice. The genetic background of the mice studied in the previous and present report was C57BL/6, so that it should have not influenced the phenotypic manifestations [Tu et al., 2012]. It is of interest that the previous report shows data only for female mice, although this should not explain the differences observed since we did not detect a skeletal phenotype in either sex.

The differences between the skeletal phenotype of *HeyL* male and female mice were slight, although sexually dimorphic phenotypes have been shown for other members of the *Hes* and *Hey* families. For example, *Hes1* overexpression under the control of the 3.6 kb fragment of the *Col1a1* promoter induces osteoclastogenesis in male mice and suppresses osteoblastogenesis in female mice, confirming the need to report the skeletal consequences of genetic manipulations in animals of both sexes [Canalis et al., 2017; Glatt et al., 2007; Zanotti et al., 2014; Zanotti et al., 2011a].

The absence of an obvious skeletal phenotype in *HeyL* null mice is likely because of genetic compensation by other members of the *Hey* family of genes, since *Hey1* can compensate for *HeyL*, and *Hey2* can compensate for *Hey1* function [Fischer et al., 2004; Fischer et al., 2007]. The reason is because members of the *Hey* family of proteins can form homodimers as well as heterodimers with other *Hes* or *Hey* proteins to bind to DNA consensus sequences [Fischer et al., 2007; Iso et al., 2001b]. Our studies are in line with the modest skeletal phenotype exhibited by *Hey1* null mice and by mice harboring a conditional inactivation of *Hey2* in cells of the osteoblast lineage [Salie et al., 2010; Zanotti and Canalis, 2013]. This would suggest that each independent member of the *Hey* family of genes plays only a modest role in skeletal homeostasis. It is conceivable that the simultaneous inactivation of *Hey1*, *2* and *L* would obviate genetic compensation and result in a pronounced skeletal phenotype, or one resembling the phenotype caused by the dual deletion of *Notch1* and *Notch2* or of Presenilin (*Psen1* and *Psen2*) in skeletal cells [Hilton et al., 2008; Zanotti and Canalis, 2014]. Since canonical Notch signaling induces *Hes1*, *Hey1*, *Hey2* and *HeyL* in an indiscriminate fashion, it is not possible to define which of these Notch targets plays a greater role mediating the actions of Notch in a given cellular context. Moreover, the local environment likely plays a role in modifying the actions of Notch and its target genes in a tissue specific manner.

In conclusion, *HeyL* plays a minimal role in the regulation of skeletal remodeling and is dispensable for skeletal homeostasis.

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References

- Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res.* 2010; 25:1468–1486. [PubMed: 20533309]
- Buchholz F, Ringrose L, Angrand PO, Rossi F, Stewart AF. Different thermostabilities of FLP and Cre recombinases: implications for applied site-specific recombination. *Nucleic Acids Res.* 1996; 24:4256–4262. [PubMed: 8932381]
- Canalis E, Adams DJ, Boskey A, Parker K, Kranz L, Zanotti S. Notch Signaling in Osteocytes Differentially Regulates Cancellous and Cortical Bone Remodeling. *J Biol Chem.* 2013a; 288:25614–25625. [PubMed: 23884415]
- Canalis E, Bridgewater D, Schilling L, Zanotti S. Canonical Notch Activation in Osteocytes Causes Osteopetrosis. *Am J Physiol Endocrinol Metab.* 2015; 310:E171–E182. [PubMed: 26578715]
- Canalis E, Parker K, Feng JQ, Zanotti S. Osteoblast Lineage-specific Effects of Notch Activation in the Skeleton. *Endocrinology.* 2013b; 154:623–634. [PubMed: 23275471]
- Canalis E, Schilling L, Yee SP, Lee SK, Zanotti S. Hajdu Cheney Mouse Mutants Exhibit Osteopenia, Increased Osteoclastogenesis and Bone Resorption. *J Biol Chem.* 2016; 291:1538–1551. [PubMed: 26627824]
- Canalis E, Schilling L, Zanotti S. Effects of Sex and Notch Signaling on the Osteocyte Cell Pool. *J Cell Physiol.* 2017; 232:363–370. [PubMed: 27192486]
- Dempster DW, Compston JE, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR, Parfitt AM. Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res.* 2013; 28:2–17. [PubMed: 23197339]
- Dong Y, Jesse AM, Kohn A, Gunnell LM, Honjo T, Zuscik MJ, O'Keefe RJ, Hilton MJ. RBPjkappa-dependent Notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Development.* 2010; 137:1461–1471. [PubMed: 20335360]
- Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang MM, Chen Y, Wang L, Zheng H, Sutton RE, Boyce BF, Lee B. Dimorphic effects of Notch signaling in bone homeostasis. *Nat Med.* 2008; 14:299–305. [PubMed: 18297084]
- Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The Notch target genes *Hey1* and *Hey2* are required for embryonic vascular development. *Genes Dev.* 2004; 18:901–911. [PubMed: 15107403]
- Fischer A, Steidl C, Wagner TU, Lang E, Jakob PM, Friedl P, Knobloch KP, Gessler M. Combined loss of *Hey1* and *HeyL* causes congenital heart defects because of impaired epithelial to mesenchymal transition. *Circ Res.* 2007; 100:856–863. [PubMed: 17303760]
- Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. *Dev Cell.* 2009; 16:633–647. [PubMed: 19460341]
- Glatt V, Canalis E, Stadmeier L, Bouxsein ML. Age-Related Changes in Trabecular Architecture Differ in Female and Male C57BL/6J Mice. *J Bone Miner Res.* 2007; 22:1197–1207. [PubMed: 17488199]
- Halleux C, Kramer I, Allard C, Kneissel M. Isolation of mouse osteocytes using cell fractionation for gene expression analysis. *Methods Mol Biol.* 2012; 816:55–66. [PubMed: 22130922]
- Hilton MJ, Tu X, Wu X, Bai S, Zhao H, Kobayashi T, Kronenberg HM, Teitelbaum SL, Ross FP, Kopan R, Long F. Notch signaling maintains bone marrow mesenchymal progenitors by suppressing osteoblast differentiation. *Nat Med.* 2008; 14:306–314. [PubMed: 18297083]
- Iso T, Kedes L, Hamamori Y. HES and HERP families: multiple effectors of the Notch signaling pathway. *J Cell Physiol.* 2003; 194:237–255. [PubMed: 12548545]
- Iso T, Sartorelli V, Chung G, Shichinohe T, Kedes L, Hamamori Y. HERP, a new primary target of Notch regulated by ligand binding. *Mol Cell Biol.* 2001a; 21:6071–6079. [PubMed: 11486044]
- Iso T, Sartorelli V, Poizat C, Iezzi S, Wu HY, Chung G, Kedes L, Hamamori Y. HERP, a novel heterodimer partner of HES/E(spl) in Notch signaling. *Mol Cell Biol.* 2001b; 21:6080–6089. [PubMed: 11486045]

- Kageyama R, Ohtsuka T, Kobayashi T. The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development*. 2007; 134:1243–1251. [PubMed: 17329370]
- Kokubo H, Miyagawa-Tomita S, Nakazawa M, Saga Y, Johnson RL. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev Biol*. 2005; 278:301–309. [PubMed: 15680351]
- Kokubo H, Miyagawa-Tomita S, Tomimatsu H, Nakashima Y, Nakazawa M, Saga Y, Johnson RL. Targeted disruption of hesr2 results in atrioventricular valve anomalies that lead to heart dysfunction. *Circ Res*. 2004; 95:540–547. [PubMed: 15297376]
- Kouadjo KE, Nishida Y, Cadrin-Girard JF, Yoshioka M, St-Amand J. Housekeeping and tissue-specific genes in mouse tissues. *BMC Genomics*. 2007; 8:127. [PubMed: 17519037]
- Kovall RA. More complicated than it looks: assembly of Notch pathway transcription complexes. *Oncogene*. 2008; 27:5099–5109. [PubMed: 18758478]
- Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res*. 2007; 86:320–325. [PubMed: 17384025]
- Murtaugh LC, Stanger BZ, Kwan KM, Melton DA. Notch signaling controls multiple steps of pancreatic differentiation. *Proc Natl Acad Sci U S A*. 2003; 100:14920–14925. [PubMed: 14657333]
- Nakagawa O, Nakagawa M, Richardson JA, Olson EN, Srivastava D. HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. *Dev Biol*. 1999; 216:72–84. [PubMed: 10588864]
- Nam Y, Sliz P, Song L, Aster JC, Blacklow SC. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell*. 2006; 124:973–983. [PubMed: 16530044]
- Nazarenko I, Lowe B, Darfler M, Ikonomi P, Schuster D, Rashtchian A. Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. *Nucleic Acids Res*. 2002a; 30:e37. [PubMed: 11972352]
- Nazarenko I, Pires R, Lowe B, Obaidy M, Rashtchian A. Effect of primary and secondary structure of oligodeoxyribonucleotides on the fluorescent properties of conjugated dyes. *Nucleic Acids Res*. 2002b; 30:2089–2195. [PubMed: 11972350]
- Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res*. 1987; 2:595–610. [PubMed: 3455637]
- Sahlgren C, Lendahl U. Notch signaling and its integration with other signaling mechanisms. *Regen Med*. 2006; 1:195–205. [PubMed: 17465803]
- Salie R, Kneissel M, Vukevic M, Zamurovic N, Kramer I, Evans G, Gerwin N, Mueller M, Kinzel B, Susa M. Ubiquitous overexpression of Hey1 transcription factor leads to osteopenia and chondrocyte hypertrophy in bone. *Bone*. 2010; 46:680–694. [PubMed: 19857617]
- Sauer B, Henderson N. Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1. *Proc Natl Acad Sci U S A*. 1988; 85:5166–5170. [PubMed: 2839833]
- Schroeter EH, Kisslinger JA, Kopan R. Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature*. 1998; 393:382–386. [PubMed: 9620803]
- Stanger BZ, Datar R, Murtaugh LC, Melton DA. Direct regulation of intestinal fate by Notch. *Proc Natl Acad Sci U S A*. 2005; 102:12443–12448. [PubMed: 16107537]
- Tao J, Chen S, Yang T, Dawson B, Munivez E, Bertin T, Lee B. Osteosclerosis owing to Notch gain of function is solely Rbpj-dependent. *J Bone Miner Res*. 2010; 25:2175–2183. [PubMed: 20499347]
- Tu X, Chen J, Lim J, Karner CM, Lee SY, Heisig J, Wiese C, Surendran K, Kopan R, Gessler M, Long F. Physiological notch signaling maintains bone homeostasis via RBPjk and Hey upstream of NFATc1. *PLoS Genet*. 2012; 8:e1002577. [PubMed: 22457635]
- Wilson JJ, Kovall RA. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. *Cell*. 2006; 124:985–996. [PubMed: 16530045]
- Xin M, Small EM, van RE, Qi X, Richardson JA, Srivastava D, Nakagawa O, Olson EN. Essential roles of the bHLH transcription factor Hrt2 in repression of atrial gene expression and

maintenance of postnatal cardiac function. *Proc Natl Acad Sci U S A*. 2007; 104:7975–7980. [PubMed: 17468400]

Yorgan T, Vollersen N, Riedel C, Jeschke A, Peters S, Busse B, Amling M, Schinke T. Osteoblast-specific Notch2 inactivation causes increased trabecular bone mass at specific sites of the appendicular skeleton. *Bone*. 2016; 87:136–146. [PubMed: 27102824]

Zanotti S, Canalis E. Notch and the Skeleton. *Mol Cell Biol*. 2010; 30:886–896. [PubMed: 19995916]

Zanotti S, Canalis E. Hairy and Enhancer of Split-related with YRPW Motif (HEY)2 Regulates Bone Remodeling in Mice. *J Biol Chem*. 2013; 288:21547–21557. [PubMed: 23782701]

Zanotti S, Canalis E. Notch1 and Notch2 expression in osteoblast precursors regulates femoral microarchitecture. *Bone*. 2014; 62:22–28. [PubMed: 24508387]

Zanotti S, Canalis E. Notch Signaling and the Skeleton. *Endocr Rev*. 2016; 37:223–53. [PubMed: 27074349]

Zanotti S, Kalajzic I, Aguila HL, Canalis E. Sex and genetic factors determine osteoblastic differentiation potential of murine bone marrow stromal cells. *PLoS One*. 2014; 9:e86757. [PubMed: 24489784]

Zanotti S, Smerdel-Ramoya A, Canalis E. Hairy and enhancer of split (HES)1 is a determinant of bone mass. *J Biol Chem*. 2011a; 286:2648–2657. [PubMed: 21084301]

Zanotti S, Smerdel-Ramoya A, Canalis E. Reciprocal regulation of notch and nuclear factor of activated T-cells (NFAT)c1 transactivation in osteoblasts. *J Biol Chem*. 2011b; 286:4576–4588. [PubMed: 21131365]

Zanotti S, Smerdel-Ramoya A, Canalis E. Nuclear Factor of Activated T-cells (Nfat)c2 Inhibits Notch Signaling in Osteoblasts. *J Biol Chem*. 2013; 288:624–632. [PubMed: 23166323]

Zanotti S, Smerdel-Ramoya A, Stadmeier L, Durant D, Radtke F, Canalis E. Notch Inhibits Osteoblast Differentiation And Causes Osteopenia. *Endocrinology*. 2008; 149:3890–3899. [PubMed: 18420737]

Figure 1.

Notch1 *Nicd*, *Hey1*, *Hey2*, *HeyL* and *Hes1* mRNA levels in osteoblast/osteocyte-rich cell extracts from 1 month old *Dmp1-Cre^{+/-};Rosa^{Notch/WT}* male mice (black bars) and control *Rosa^{Notch/WT}* littermates (white bars) cultured for 4 days. mRNA levels are expressed as copy number corrected for *Rpl38* and controls normalized to 1. Values are means $\pm$ SEM; n = 4. *Significantly different from control mice, $p < 0.05$ by unpaired *t*-test.

Figure 2.

HeyL expression, weight and femoral length of *HeyL* null mice (black bars and dots) and littermate sex-matched controls (white bars, open circles). Total RNA was obtained from tibiae of 3 month old mice and *HeyL* mRNA levels are shown in A, whereas weight and femoral length were obtained at 1 and 3 months of age and are shown in B. Transcript levels are expressed as *HeyL* copy number corrected for *Rp138* expression. Values are means $\pm$ SEM; n = 4 to 9.

Figure 3.
Representative skeletal microarchitecture assessed by μ CT of 3 month old male and female *HeyL* null mice and littermate sex-matched controls.

Table 1

Primers used for allele identification by PCR or for qRT-PCR determination.

A. Genotyping			
Allele	Strand	Sequence 5'-3'	Amplicon Size (base pairs)
<i>Dmp1-Cre</i> transgene	Forward Reverse	CCCGCAGAACCTGAAGATG GACCCGGCAAAACAGGTAG	534
<i>HeyL</i>	Wild type Forward Null Forward Reverse	GGATCCTTCAGCTCTGAGAAA CGCCAGGGTTTTCCCAGTCACGAC TGTTGCACACTCACCCCTCT	Wild type = 123 Null = 210
<i>Rosa^{Notch}</i>	Forward Wild type Reverse <i>Rosa^{Notch}</i> Reverse	GGAGCGGGAGAAATGGATATG AAAGTCGCTCTGAGTTGTTATTG GCGAAGAGTTTGCCTCAACC	Wild type = 600 <i>Rosa^{Notch}</i> = 250

B. qRT-PCR			
Gene	Strand	Sequence 5'-3'	GenBank Accession Number
<i>Hes1</i>	Forward Reverse	ACCAAAGACGGCCTCTGAGCACAGAAAGT ATTCTTGCCCTTCGCCTCTT	NM_008235
<i>Hey1</i>	Forward Reverse	ATCTCAACAACACTACGCATCCCAGC GTGTGGGTGATGTCCGAAGG	NM_010423
<i>Hey2</i>	Forward Reverse	AGCGAGAACAAATTACCCTGGGCAC GGTAGTTGTGGTGAATTGGACCT	NM_013904
<i>HeyL</i>	Forward Reverse	CAGTAGCCTTTCTGAATTGCGAC AGCTTGGAGGAGCCCTGTTTC	NM_013905
<i>Notch1</i>	Forward Reverse	GTGCTCTGATGGACGACAAT GCTCCTCAAACCGGAACCTC	NM_008714
<i>Rpl38</i>	Forward Reverse	AGAACAAGGATAATGTGAAGTTCAAGGTTT CTGCTTCAGCTTCTCTGCCTTT	NM_001048057; NM_001048058; NM_023372

Table 2

Femoral microarchitecture assessed by μ CT of 1 and 3 month old *HeyL* null male mice and littermate controls.

	1 Month		3 Months	
	Control	<i>HeyL</i> null	Control	<i>HeyL</i> null
<i>Distal Femur Trabecular Bone</i>				
Bone Volume/Total Volume (%)	6.6 $\pm$ 0.7	5.1 $\pm$ 0.3 *	8.4 $\pm$ 1.6	8.0 $\pm$ 0.5
Trabecular Separation (μ m)	213 $\pm$ 8	208 $\pm$ 6	203 $\pm$ 10	197 $\pm$ 5
Trabecular Number (1/mm)	4.8 $\pm$ 0.2	4.9 $\pm$ 0.1	4.9 $\pm$ 0.2	5.0 $\pm$ 0.1
Trabecular Thickness (μ m)	24 $\pm$ 1	22 $\pm$ 1 *	33 $\pm$ 2	31 $\pm$ 1
Connectivity Density (1/mm ³)	379 $\pm$ 51	288 $\pm$ 34	240 $\pm$ 42	260 $\pm$ 20
Structure Model Index	2.5 $\pm$ 0.1	2.8 $\pm$ 0.1 *	2.3 $\pm$ 0.2	2.4 $\pm$ 0.1
Density of Material (mg HA/cm ³)	853 $\pm$ 4	891 $\pm$ 8 *	966 $\pm$ 9	963 $\pm$ 5
<i>Femoral Midshaft Cortical Bone</i>				
Bone Volume/Total Volume (%)	89.2 $\pm$ 0.3	89.3 $\pm$ 0.4	88.6 $\pm$ 0.2	89.0 $\pm$ 0.3
Porosity (%)	10.8 $\pm$ 0.3	10.7 $\pm$ 0.4	11.4 $\pm$ 0.2	11.0 $\pm$ 0.3
Cortical Thickness (μ m)	93 $\pm$ 1	94 $\pm$ 2	150 $\pm$ 4	152 $\pm$ 4
Total Area (mm ²)	1.46 $\pm$ 0.08	1.53 $\pm$ 0.05	1.59 $\pm$ 0.08	1.58 $\pm$ 0.05
Bone Area (mm ²)	0.45 $\pm$ 0.03	0.45 $\pm$ 0.02	0.73 $\pm$ 0.04	0.72 $\pm$ 0.02
Periosteal Perimeter (mm)	4.3 $\pm$ 0.1	4.4 $\pm$ 0.1	4.5 $\pm$ 0.1	4.4 $\pm$ 0.1
Endocortical Perimeter (mm)	3.6 $\pm$ 0.1	3.7 $\pm$ 0.1	3.3 $\pm$ 0.1	3.3 $\pm$ 0.1
Density of Material (mg HA/cm ³)	935 $\pm$ 5	937 $\pm$ 7	1159 $\pm$ 4	1165 $\pm$ 8

μ CT was performed on distal femurs for trabecular bone and midshaft for cortical bone from 1 and 3 month old male *HeyL* null mice and control male littermates (Control). Values are means $\pm$ SEM; n = 4 to 9.

* Significantly different between *HeyL* null and control, $p < 0.05$.

Table 3

Femoral microarchitecture assessed by μ CT of 1 and 3 month old *HeyL* null female mice and littermate controls.

	1 Month		3 Months	
	Control	<i>HeyL</i> null	Control	<i>HeyL</i> null
<i>Distal Femur Trabecular Bone</i>				
Bone Volume/Total Volume (%)	3.6 $\pm$ 0.4	3.6 $\pm$ 0.4	4.5 $\pm$ 0.5	4.7 $\pm$ 0.7
Trabecular Separation (μ m)	265 $\pm$ 16	247 $\pm$ 14	278 $\pm$ 11	301 $\pm$ 10
Trabecular Number (1/mm)	3.9 $\pm$ 0.3	4.1 $\pm$ 0.2	3.6 $\pm$ 0.1	3.4 $\pm$ 0.1
Trabecular Thickness (μ m)	22 $\pm$ 1	21 $\pm$ 1	34 $\pm$ 0	38 $\pm$ 2
Connectivity Density (1/mm ³)	148 $\pm$ 3	98 $\pm$ 30	98 $\pm$ 16	89 $\pm$ 21
Structure Model Index	2.9 $\pm$ 0.1	3.0 $\pm$ 0.1	3.0 $\pm$ 0.1	2.9 $\pm$ 0.1
Density of Material (mg HA/cm ³)	868 $\pm$ 8	894 $\pm$ 10	979 $\pm$ 8	995 $\pm$ 6
<i>Femoral Midshaft Cortical Bone</i>				
Bone Volume/Total Volume (%)	88.7 $\pm$ 0.2	89.0 $\pm$ 0.6	88.9 $\pm$ 0.2	88.4 $\pm$ 0.3
Porosity (%)	11.3 $\pm$ 0.2	11.0 $\pm$ 0.6	11.1 $\pm$ 0.2	11.6 $\pm$ 0.3
Cortical Thickness (μ m)	90 $\pm$ 2	93 $\pm$ 2	156 $\pm$ 2	151 $\pm$ 3
Total Area (mm ²)	1.42 $\pm$ 0.10	1.47 $\pm$ 0.08	1.54 $\pm$ 0.04	1.57 $\pm$ 0.03
Bone Area (mm ²)	0.42 $\pm$ 0.02	0.43 $\pm$ 0.02	0.73 $\pm$ 0.02	0.74 $\pm$ 0.01
Periosteal Perimeter (mm)	4.2 $\pm$ 0.1	4.3 $\pm$ 0.1	4.4 $\pm$ 0.1	4.4 $\pm$ 0.1
Endocortical Perimeter (mm)	3.5 $\pm$ 0.1	3.6 $\pm$ 0.1	3.1 $\pm$ 0.1	3.2 $\pm$ 0.1
Density of Material (mg HA/cm ³)	921 $\pm$ 7	919 $\pm$ 10	1188 $\pm$ 3	1178 $\pm$ 7

μ CT was performed on distal femurs for trabecular bone and midshaft for cortical bone from 1 and 3 month old female *HeyL* null mice and control female littermates (Control). Values are means $\pm$ SEM; n = 4 to 6.

Table 4

Cancellous bone histomorphometry of 1 and 3 month old *HeyL* null male mice and littermate controls.

	1 Month		3 Months	
	Control	<i>HeyL</i> null	Control	<i>HeyL</i> null
<i>Distal Femur Trabecular Bone</i>				
Bone Volume/Tissue Volume (%)	7.6 ± 1.6	9.6 ± 1.1	11.3 ± 2.2	9.4 ± 0.2
Trabecular Number (1/mm)	2.5 ± 0.3	3.4 ± 0.2 *	2.9 ± 0.2	3.1 ± 0.5
Trabecular Thickness (µm)	30 ± 3	28 ± 2	38 ± 5	31 ± 1
Osteoblasts/Bone Perimeter (1/mm)	19.9 ± 3.6	20.1 ± 2.3	10.1 ± 1.6	10.2 ± 1.7
Osteoid Surface/Bone Surface (%)	1.4 ± 0.2	1.2 ± 0.4	2.7 ± 0.9	1.2 ± 0.2
Osteoclasts/Bone Perimeter (1/mm)	6.8 ± 0.6	6.6 ± 0.5	4.9 ± 0.3	5.6 ± 0.5
Eroded Surface/Bone Surface (%)	16.6 ± 1.6	17.4 ± 1.2	8.9 ± 0.7	9.3 ± 0.7
Mineral Apposition Rate (µm/day)	3.3 ± 0.1	3.7 ± 0.4	0.9 ± 0.1	1.1 ± 0.1
Mineralizing Surface/Bone Surface (%)	7.8 ± 2.0	4.1 ± 0.3	3.1 ± 0.6	3.0 ± 1.0
Bone Formation Rate (µm ³ /µm ² /day)	0.26 ± 0.08	0.15 ± 0.01	0.03 ± 0.01	0.03 ± 0.01

Bone histomorphometry was performed on distal femurs from 1 and 3 month old male *HeyL* null mice and control male littermates (Control). Values are means ± SEM; n = 3 to 6.

* Significantly different between *HeyL* null and control, $p < 0.05$.

Table 5

Cancellous bone histomorphometry of 3 month old *HeyL* null female mice and littermate controls.

	Control	<i>HeyL</i> null
<i>Distal Femur Trabecular Bone</i>		
Bone Volume/Tissue Volume (%)	3.1 ± 0.7	4.1 ± 0.2
Trabecular Number (1/mm)	1.3 ± 0.3	1.6 ± 0.1
Trabecular Thickness (µm)	23 ± 1	25 ± 1
Osteoblasts/Bone Perimeter (1/mm)	21.9 ± 3.3	24.3 ± 0.7
Osteoid Surface/Bone Surface (%)	1.7 ± 0.9	2.8 ± 0.8
Osteoclasts/Bone Perimeter (1/mm)	7.6 ± 0.8	8.0 ± 0.5
Eroded Surface/Bone Surface (%)	18.1 ± 2.2	18.7 ± 1.0
Mineral Apposition Rate (µm/day)	1.9 ± 0.1	2.1 ± 0.2
Mineralizing Surface/Bone Surface (%)	11.7 ± 3.2	7.7 ± 2.2
Bone Formation Rate (µm ³ /µm ² /day)	0.22 ± 0.06	0.15 ± 0.03

Bone histomorphometry was performed on distal femurs from 3 month old female *HeyL* null mice and control female littermates (Control). Values are means ± SEM; n = 3.