HHS Public Access

Author manuscript

*Birth Defects Res*. Author manuscript; available in PMC 2025 January 01.

Published in final edited form as:

*Birth Defects Res*. 2024 January ; 116(1): e2266. doi:10.1002/bdr2.2266.

# *Hdac1* and *Hdac2* positively regulate Notch1 gain-of-function pathogenic signaling in committed osteoblasts of male mice

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## Abstract

Skeletal development requires precise extrinsic and intrinsic signals to regulate processes that form and maintain bone and cartilage. Notch1 is a highly conserved signaling receptor that regulates cell fate decisions by controlling the duration of transcriptional bursts. Epigenetic molecular events reversibly modify DNA and histone tails by influencing the spatial organization of chromatin and can fine-tune the outcome of a Notch1 transcriptional response. Histone deacetylase 1 and 2 (HDAC1 and HDAC2) are chromatin modifying enzymes that mediate osteoblast differentiation. While an HDAC1-Notch interaction has been studied *in vitro* and in *Drosophila*, its role in mammalian skeletal development and disorders is unclear. Osteosclerosis is a bone disorder with an abnormal increase in the number of osteoblasts and excessive bone formation. Here, we tested whether *Hdac1* and *Hdac2 (Hdac1/2)* contribute to the pathogenesis of osteosclerosis in a murine model of the disease owing to conditionally cre-activated expression of the Notch1 intracellular domain in immature osteoblasts. Importantly, selective homozygous deletions of *Hdac1*/2 in osteoblasts partially alleviate osteosclerotic phenotypes (Col2.3kb-Cre;TG$^{RosaN1ICD/+}$;*Hdac1*$^{flox/flox}$;*Hdac2*$^{flox/flox}$) with a 40% decrease in bone volume (BV/TV) and a 22% decrease in trabecular thickness in 4 weeks old when compared to male mice with heterozygous deletions of *Hdac1/2* (Col2.3kb-Cre;TG$^{RosaN1ICD/+}$;*Hdac1*$^{flox/+}$;*Hdac2*$^{flox/+}$). Osteoblast-specific deletion of *Hdac1/2* in male and female mice results in no overt bone phenotype in the absence of the Notch1 gain-of-function (GOF) allele. These results provide evidence that *Hdac1* and *Hdac2* contribute to Notch1 pathogenic signaling in the mammalian skeleton. Our study on epigenetic regulation of Notch1 gain-of-function-induced osteosclerosis

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Conflict of interest statement

The authors declare no conflicts of interest.

may facilitate further mechanistic studies of skeletal birth defects caused by Notch-related gain-of-function mutations in human patients, such as Adams-Oliver disease, congenital heart disease, and lateral meningocele syndrome.

**Keywords**

Notch1; Epigenetics; HDAC1; HDAC2; Osteoblast; Osteosclerosis

## Introduction

The developmental consequences for dysregulation of Notch receptors (NOTCH1-NOTCH4) in human congenital disease with severe birth defects are well documented, for example, Alagille syndrome, congenital heart disease, Hajdu-Cheney syndrome, and lateral meningocele syndrome (Masek & Andersson, 2017). Loss of function in the *Notch1* gene leads to embryonic death around the mid-gestation (Conlon et al., 1995; Swiatek et al., 1994). In the skeletal system, genetic studies in mice with tissue-specific loss or gain of Notch function have demonstrated regulatory roles for Notch signaling in osteoblast specification, proliferation and differentiation, and osteoclast activity (Bai et al., 2008; Engin et al., 2008; Hilton et al., 2008; Zanotti et al., 2008). These studies have also linked the homeostatic function of Notch to adult bone diseases, including osteopenia, osteoporosis, and osteosclerosis. Osteosclerosis is a bone disease characterized by abnormal thickening and progressive increase in skeletal bone mass due to an increased number of osteoblasts (Whyte, 2008). Notch gain-of-function in committed osteoblasts within a developing skeleton increases osteoblast proliferation and inhibits terminal osteoblast differentiation, resulting in osteosclerotic phenotypes in mice of both sexes (Engin et al., 2008). Moreover, Notch signaling in committed osteoblasts has been shown to be canonical in nature because it is completely dependent on RBPJ, a Notch nuclear effector (i.e. Recombination Signal Binding Protein For Immunoglobulin Kappa J Region, CBF1, RBPJK) (Tao, Chen, Yang, et al., 2010). These genetic studies in mice indicate that Notch1 plays a critical role not only in early development but also in promoting birth defects in the postnatal skeleton. However, how epigenetic factors contribute to the Notch1-induced pathophysiological processes in skeletal birth defects remains largely unknown.

Notch receptors are activated through contact with Jagged or Delta-like ligands on neighboring cells and their interaction triggers a series of regulated successive intramembranous proteolytic cleavages of the receptors that leads to the nuclear translocation of the N1ICD (Tao, Chen, & Lee, 2010). The activated N1ICD recruits DNA-binding protein RBPJ from repressor complexes and forms the nuclear transcription complex (NTC) together with co-activator Mastermind-like (MAML). The NTC then binds to Notch regulatory elements, including super-enhancers, which leads to the subsequent recruitment of other transcriptional co-regulators to induce expression of target genes (Wang et al., 2014). Components of the NTC physically interact with p300/CBP, a histone acetyltransferase (HAT) that acetylates H3K18 and H3K27 (Jin et al., 2011), as well as other chromatin–associated complexes containing epigenetic regulators such as polycomb

repressive complex 1 (PRC1), lysine-specific histone demethylase 1 (LSD1), and histone deacetylase 1 (HDAC1) (Yatim et al., 2012).

HDAC1 and HDAC2 (HDAC1/2) are class I HDACs that are highly expressed in many tissues, including bone and osteoblast cells (Bradley et al., 2015; Torres et al., 2021). They are enzymatic subunits in several corepressor complexes (NuRD, Sin3A, CoREST, NCoR/SMRT and ES-specific NODE) and catalyze the removal of the acetyl moiety from the ε-amino group of lysines in histone and non-histone proteins (Bradley et al., 2015). Global HDAC1 deficiency in mice causes embryonic death before mid-gestation (Lagger et al., 2002), whereas mice lacking HDAC2 develop cardiac defects while surviving perinatally (Montgomery et al., 2007). Redundant and compensatory roles of HDAC1 and HDAC2 have been described during myocardial growth, development of B-cell precursors, smooth muscle differentiation, and glomerular disease (Inoue et al., 2019; Montgomery et al., 2007; Tang et al., 2012; Yamaguchi et al., 2010). HDACs influence global chromatin acetylation and structure. As acetylation is an epigenetic mark for a transcriptionally “on” state, HDACs are traditionally identified as transcriptional corepressors for contributing to a more condensed chromatin structure. Studies have also shown that there is a positive correlation for HATs and HDACs (HDAC1, HDAC2, HDAC3, HDAC6) with gene expression, Pol II binding, and acetylation levels (Wang et al., 2009). HDACs are recruited to active genes in order to repress them (i.e. remove the acetyl groups from histones after the activities of Pol lI and HATs) and prevent the binding of Pol II by maintaining a low level of acetylation on genes that are marked by H3K4 methylation. In this way, HDACs keep promoters in a primed state for future activation (Wang et al., 2009). It has been suggested that dynamic NOTCH1-RBPJ binding sites associated with the activation of Notch target genes are sensitive to gamma-secretase-inhibitor treatment and are located mainly in distal enhancers (i.e. regions with high levels of “activating” H3K27ac and H3K4me marks) (Wang et al., 2014). Whether HDAC1/2 bind to these dynamic active enhancer sites to participate in regulation of Notch signaling *in vivo* is still unclear.

Genetic evidence from *Drosophila* indicates that *HDAC1* positively regulates Notch signaling during wing development and depletion of *HDAC1* causes wing notches on the margin of the adult wing by affecting Notch and the transcription of Notch target genes (Zehua Wang et al., 2018). Mechanistic studies using HDAC inhibitors in human vascular cells suggest that HDAC activity is required for Notch signaling-meditated cell differentiation (Tang et al., 2012). To dissect the relative contributions of HDAC1/2 owing to Notch1 gain-of-function, we leveraged an established model of the disease, called Notch1 gain-of-function (GOF) bigenic mice, in which the expression of the Notch1 intracellular domain (N1ICD) was conditionally activated in immature osteoblasts expressing Col1a1 2.3kb-Cre (Tao, Chen, Yang, et al., 2010). Expression of N1ICD cells at early stages of mesenchymal/osteoblast differentiation in mice was not tolerated and led to embryonic lethality, likely due to suppression of mesenchymal stem cell and osteoblast progenitor differentiation (Tao et al., 2014). In this study, we bred the GOF mice with floxed alleles of *Hdac1/2* to study the effects of conditional *Hdac1/2* deletion in pathological osteoblasts. We hypothesized that if Notch1 gain-of-function mediates the skeletal defect, and if the defect is via the HDAC1/2 pathway, then deletion of *Hdac1/2* within osteoblasts should rescue most, if not all, of the osteosclerotic phenotype. Here we report that osteosclerosis

owing to Notch1 GOF is partially HDAC1/2-dependent suggesting that HDAC1 and HDAC2 cooperatively and positively regulate pathological Notch1 gain-of-function in mice.

## Methods

### Animals and associated experimental procedures

*Hdac1*$^{flox/flox}$ and *Hdac2*$^{flox/flox}$ (*Hdac1/2*$^{flox/flox}$ or *Hdac1*$^{flox/flox}$;*Hdac2*$^{flox/flox}$) mice were a gift from Dr. Eric Olson (University of Texas Southwestern Medical Center, Dallas, Texas, USA) (Montgomery et al., 2007). The *Col1a1 2.3kb-Cre* (Dacquin et al., 2002), *Rosa261*$^{N1ICD}$ mice (Murtaugh et al., 2003), which allows for bone tissue-restricted activation of a conditional Notch1 intracellular domain were crossed to *Hdac1/2*$^{flox/flox}$ to drive *Hdac1*- and *Hdac2*-KO to committed osteoblasts. The mice generated for this study were maintained on a hybrid 129XC57BL/6 background. Mice were housed at 22.7 °C with humidity of 30–70% and a 14 h light/10 h dark cycle. Genotyping was performed by PCR using previously described protocols (Montgomery et al., 2007; Tao, Chen, Yang, et al., 2010). These studies were approved by the Sanford Research Institutional Animal Care and Use Committee.

### MicroCT analysis

Femoral microarchitecture of whole skeletons was determined using a μCT scanner (μCT 50, Scanco Medical, Basserdorf, Switzerland). Femurs were scanned in 70% ethanol at medium resolution, energy level of 90 peak kilovoltage, intensity of 66 μA, and integration time of 1500 ms. A total of 240 slices from the growth plate at distal metaphysis were acquired at a default isotropic voxel size (10.2 μm). Contours were manually drawn every 10 to 20 slices, whereas the remaining slice contours were iterated automatically. Total volume, bone volume, bone volume fraction, trabecular thickness, trabecular number, connectivity density, structure model index, and material density were measured in trabecular regions using a Gaussian filter ($\sigma = 0.5$) and a threshold of 130. 3D renderings were generated on the Scanco μCT.

### Statistical Analysis

Data were analyzed using a Student's t-test. P-values less than or equal to 0.05 were marked and denoted in the following way: * ($p<0.05$), ** ($p<0.01$), ***($p<0.001$). All data unless otherwise specified are expressed as mean ± standard deviation.

## Results

### Normal skeletal microarchitecture after osteoblast-specific deletion of Hdac1 and Hdac2

We first investigated the role of *Hdac1 and Hdac2 (Hdac1/2)* in committed osteoblasts by crossing mice expressing Cre under the control of a 2.3kb fragment of the promoter for *Col1a1* with *Hdac1/2* flox/flox (i.e., Col2.3-Cre; *Hdac1*$^{flox/flox}$; *Hdac2*$^{flox/flox}$). We found mice with conditional double knockout (cdKO) of *Hdac1/2* did not have any obvious gross phenotypes. Transgenic cdKO mice had a healthy appearance and were comparable with those of control littermate mice, which included *Hdac1/2*$^{flox/flox}$ or Cre transgenic mice with normal Hdac1/2 alleles. Femoral microarchitecture, determined by microcomputed

tomography (μCT), of 1-month-old male or female *Hdac1/2* cdKO showed no significant differences in trabecular bone when compared with control sex-matched littermates (Table 1). In the male distal femur, the bone volume/total volume (BV/TV; 8.8 for cdKO vs. 9.63% for controls), trabecular separation (404.0 for cdKO vs. 338.7 μm for controls), trabecular number (2.6 for cdKO vs. 3.2 1/mm for controls), trabecular thickness (40.3 for cdKO vs. 40.1 μm for controls), and connectivity density (177.1 for cdKO vs. 181.8 $1/mm^3$ for controls) were all comparable (cdKO mice (n=7) versus littermate controls (n=8)).

## Osteoblast-specific deletion of Hdac1 and Hdac2 rescues skeletal microarchitecture of mice in which N1ICD is constitutively expressed.

We next investigated whether deletion of *Hdac1/2* affected the skeletal phenotype of mice harboring a pathological Notch gain-of-function mutation. We previously reported that osteoblastic activity and trabecular bone volume were massively increased in 4-week-old GOF mice (i.e. Col2.3kb-Cre;$TG^{RosaN1ICD/+}$, TG indicates Notch1 GOF transgene) and that aged mice developed osteosarcoma with a high incidence (Tao, Chen, Yang, et al., 2010; Tao et al., 2014). In agreement with these previously reported bone histomorphometric data, we found that GOF male mice had a 76% increase in BV/TV (40.0 for GOF vs 9.63% for controls, $p<0.0001$), a 70% increase in trabecular number (11.0 for GOF vs 3.2 1/mm in controls $p<0.00001$), and a 297% reduction in trabecular separation (85.3 for GOF vs 338.7 μm in controls, $p<0.01$) when compared to wildtype mice of the same age. We did not obtain an adequate number of female GOF mice for statistical analyses, so we report results from male mice.

In Table 2, we show that when compared with male mice harboring heterozygous deletions of *Hdac1*/2 (Col2.3kb-Cre;$TG^{RosaN1ICD/+}$;$Hdac1^{flox/+};Hdac2^{flox/+}$), the GOF mice with homozygous deletions of *Hdac1/2* (Col2.3kb-Cre;$TG^{RosaN1ICD/+}$;$Hdac1^{flox/flox};Hdac2^{flox/flox}$, "rescue mice") had a 40% decrease in BV/TV (27.5% BV/TV for homozygous in the vs 45.7% for heterozygous deletion of *Hdac1/2* in a GOF background) and a 22% decrease in trabecular thickness (38.3 μm thick trabeculae for homozygous vs 49.1 μm for heterozygous deletion of *Hdac1/2* in a GOF background). When compared with the GOF mice, which have normal *Hdac1/2* expression, the GOF mice with homozygous deletion of *Hdac1/2* had a 31% decrease in BV/TV (27.5% for homozygous in a GOF background vs 40.0% for GOF mice) and an 11% decrease in trabecular thickness (38.3 for homozygous in a GOF background vs 43.1 for GOF mice) (Table 2 and Figure 1). In addition, the GOF mice with homozygous deletion of *Hdac1/2* had a 44% increase of tail length compared with the GOF mice (i.e. an average tail length of 5.3 cm for homozygous deletion of *Hdac1/2* in a GOF background vs 3.7 cm for GOF mice) whereas there were no significant change of whole-body weights between two groups at 4 weeks old (Figure 1). Moreover, we found no significant changes in trabecular microarchitecture between the GOF mice with normal expression of *Hdac1/2* and the GOF mice with a heterozygous deletion of *Hdac1/2* (i.e., Col2.3kb-Cre; $TG^{RosaN1ICD/+}$ vs. Col2.3kb-Cre; $TG^{RosaN1ICD/+}$; $Hdac1^{flox/+}$; $Hdac2^{flox/+}$). All aforementioned mice exhibit growth retardation and osteosclerotic phenotypes when compared to cdKO mice (i.e., Col2.3kb-Cre; $Hdac1^{flox/flox}$; $Hdac2^{flox/flox}$).

## Discussion

Our results indicate that Hdac1 and Hdac2 contribute to pathological features arising from Notch1 gain-of-function signaling in osteoblasts. Previous studies showed that GOF mice of both sexes exhibit an osteosclerotic phenotype associated with progressive growth retardation and excessive bone formation (i.e. thickened skulls, rib cages, tail vertebrae and limb long bones) (Tao, Chen, Yang, et al., 2010). Data from 4-week-old male mice in this study agrees with these previously reported bone morphometric analyses with substantial increases in trabecular bone volume/total volume (BV/TV) and trabecular number (Table 2 and Figure 1). Importantly, the osteosclerotic phenotype and abnormal microarchitecture of the GOF male mice is partially rescued by deletion of *Hdac1* and *Hdac2* genes.

*Hdac1* and *Hdac2* (*Hdac1/2)* are critical for survival and skeletal homeostasis (Bradley et al., 2015; Wang et al., 2021). Tissue-specific KO of *Hdac1/2* has been reported in several cell types including cardiomyocytes, B-cells, and podocytes (Inoue et al., 2019; Montgomery et al., 2007; Yamaguchi et al., 2010). To date, an osteoblast-specific KO of *Hdac1/2* has not been reported. Using a pharmacological approach, our previous study showed pro-osteoblastic differentiation effects of a pan-Hdac inhibitor, suberoylanilide hydroxamic acid (SAHA; a.k.a. vorinostat or Zolinza™) on MC3T3 cells but not primary osteoblasts (McGee-Lawrence et al., 2011; Schroeder & Westendorf, 2005). *In vivo*, SAHA decreased the number of immature osteoblasts, but promoted the activity of mature osteoblasts, resulting in a net reduction in trabecular bone volume fraction and trabecular number (McGee-Lawrence et al., 2011). In this study, we find no significant phenotypes or changes to trabecular bone microarchitecture at 4-weeks-old when *Hdac1*/2 is deleted from Col2.3kb-Cre positive osteoblasts. This suggests that *Hdac1/2* function is dispensable in maturing osteoblasts. However, in the context of Notch1 gain-of-function signaling *in vivo*, we find that deletion of *Hdac1/2* reduces trabecular BV/TV and thickness, but not trabecular number. While only a partial rescue, this suggests that Hdac1 and Hdac2 are required to positively regulate Notch1 gain-of-function signaling in maintaining activities of pathological osteoblasts.

One limitation of the present experimental design is that the mechanism behind the partial rescue phenotype in osteoblasts from Col2.3kb-Cre;$TG^{RosaN1ICD/+}$; *Hdac1f/f; Hdac2f/f* mice needs to be further elucidated. One of the possible mechanisms of action on osteoblast activity is that HDAC1/2 work cooperatively with Notch1 to form activation complexes and/or repression complexes to regulate distinct sets of downstream target genes, such as those involved in osteoblast proliferation and differentiation, which needs to be experimentally explored. Another possible mechanism is that in addition to modifying histones, HDAC1/2 may regulate N1ICD protein directly, since previous work has shown that HDACs regulate NOTCH receptor stability, promoting their direct deacetylation, and their regulation by the ubiquitin-proteasome pathway (Ferrante et al., 2020; Palermo et al., 2012; Pinazza et al., 2018). Results from our genetic rescue study are consistent with previous studies that show removal of HDAC1/2 may impair Notch function by either instability of the N1ICD protein or reduction of a certain set of Notch target genes in osteoblasts (Tang et al., 2012; Z. Wang et al., 2018). An additional limitation of the present experimental design is that our results do not necessarily apply to female mice since male mice were studied. It has been suggested that dynamic NOTCH1-RBPJ binding

sites associated with activation of Notch target genes are sensitive to gamma-secretase-inhibitor treatment and are located mainly in distal enhancers (i.e. regions with high levels of H3K27ac and "activating" H3K4me marks), which also overlap with Runx1 binding sites (Wang et al., 2014). Runx1 is a transcription factor that has been shown to enhance osteoblast lineage commitment and promotes bone formation in mice in part by regulating BMP and WNT/β-catenin signaling pathway (Tang et al., 2021). Whether *Hdac1* and *Hdac2* bind to these dynamic active sites or play functional roles different from acute regulation of direct target genes needs to be determined. Recently, a puzzling phenomenon has emerged. Both loss-of-function and gain-of-function mutations in NOTCH1 can cause Adams-Oliver syndrome (AOS), a rare disorder characterized by limb defects, scalp hypoplasia, and cranial defects (Stittrich et al., 2014). The patient's abnormal skull phenotype may overlap with the skull abnormalities observed in Notch1 GOF mice. In conclusion, our "proof-of-principle" study on a Hdac1/2 epigenetic regulation of Notch1-induced osteosclerosis in male mice may help our understanding and aid future investigation on other human skeletal birth defects caused by Notch gain of function mutations, such as Adams-Oliver disease, congenital heart disease, and lateral meningocele syndrome.

## Acknowledgments:

We would like to thank Dr. Shuta Ishibe for his help in obtaining the *Hdac1*$^{fl/fl}$ *and Hdac2*$^{fl/fl}$ mice from Dr. Eric Olson used in this study.

### Funding information

Sanford Center for Pediatric Research from National Institute of General Medical Sciences, Grant/Award Number: P20GM103620; Mayo Clinic T32 Training Program from National Institute of Diabetes and Digestive and Kidney Diseases, Grant/Award Number: T32DK007352; National Science Foundation, Grant/Award Number: 1725656.

## Data availability statement

The data that support the findings of this study are available from the corresponding author, [JT], upon reasonable request.

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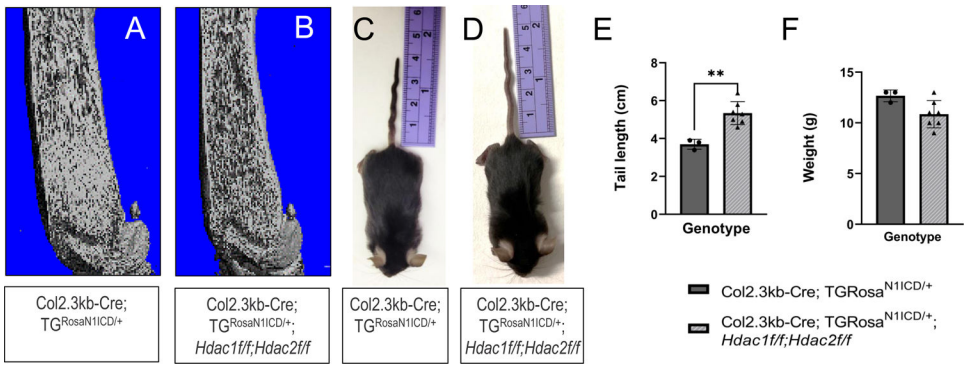


**Figure 1: Hdac1 and Hdac2 positively regulate N1ICD induced pathological skeletal disease.**

Transgenic male mice expressing a gain-of-function Notch mutation develop osteosclerosis (A). Homozygous deletion of Hdac1/2 partially ameliorates pathogenic bone density in Notch1 gain-of-function mice (B). Tails of 4-week-old mice with a Notch1 gain-of-function mutation in committed osteoblasts (C, n=3) are significantly shorter than tails of mice with conditional deletion of *Hdac1* and *Hdac2* (D, E, n=7). (F) Body weight is not different in these cohorts of mice. Significantly difference between groups measured by student's t-test (**$p<0.01$).

**Table 1:**

MicroCT analyses of femoral bone microarchitecture for Hdac1/2 cdKO and control mice.

| | Male | | Female | |
|---|---|---|---|---|
| | **Control** | ***Col2.3kb-Cre; Hdac1*** $^{flox/flox}$ ***; Hdac2*** $^{flox/flox}$ | **Control** | ***Col2.3kb-Cre; Hdac1*** $^{flox/flox}$ ***Hdac2*** $^{flox/flox}$ |
| | **n = 8** | **n = 7** | **n = 7** | **n = 10** |
| **Trabecular bone** | | | | |
| Distal femur trabecular bone | | | | |
| Bone volume/total volume (%) | 9.63 ± 2.8 | 8.8 ± 0.7 | 8.13 ± 0.9 | 8.23 ± 1.2 |
| Trabecular separation (μm) | 338.7 ± 94.6 | 404.0 ± 95.9 | 396.8 ± 59.0 | 437.9 ± 108.2 |
| Trabecular number (1/mm) | 3.2 ± 1.0 | 2.6 ± 0.6 | 2.66 ± 0.4 | 2.5 ± 0.6 |
| Trabecular thickness (μm) | 40.1 ± 2.5 | 40.3 ± 2.2 | 40.1 ± 2.0 | 40.7 ± 1.7 |
| Connectivity density (1/$mm^3$) | 181.8 ± 30.3 | 177.1 ± 22.9 | 172.9 ± 30.6 | 165.0 ± 29.0 |

Microcomputed tomography was performed in distal femurs for trabecular bone from 4-week-old mice and sex-matched littermate controls. Values means +/ SD.

There is no significantly difference between Col2.3kb-Cre; $Hdac1^{flox/flox}$;$Hdac2^{flox/flox}$ (cdKO) and control mice by student's t-test).

**Table 2.**

MicroCT analyses of femoral bone microarchitecture for Notch1 GOF male mice with or without *Hdac1/2* homozygous or heterozygous loss of function.

| | Male | | |
|---|---|---|---|
| | **Col2.3kb-Cre; TG$^{RosaN1ICD/+}$; *Hdac1*$^{flox/flox;}$ *Hdac2*$^{flox/flox}$** | **Col2.3kb-Cre; TG$^{RosaN1ICD/+}$; *Hdac1*$^{flox/+}$; *Hdac2*$^{flox/+}$** | **Col2.3kb-Cre; TG$^{RosaN1ICD/+}$** |
| | **n = 11** | **n = 10** | **n = 3** |
| **Trabecular bone** | | | |
| Distal femur trabecular bone | | | |
| Bone volume/total volume (%) | 27.5 ± 6.2 | 45.7 ± 13.7*** | 40.0 ± 9.9* |
| Trabecular separation (μm) | 117.1 ± 32.8 | 96.3 ± 27.8 | 85.4 ± 10.8 |
| Trabecular number (1/mm) | 8.9 ± 2.2 | 10.3 ± 1.7 | 11.0 ± 1.3 |
| Trabecular thickness (μm) | 38.3 ± 2.46 | 49.1 ± 9.9** | 43.1 ± 5.9* |
| Connectivity density (1/mm$^3$) | 1096.2 ± 386.7 | 1043.5 ± 158.0 | 1260.3 ± 117.2 |

Microcomputed tomography was performed in distal femurs for trabecular bone from 4-week-old mice and sex-matched littermate controls. Values means +/ SD.

Significantly difference between sample and Col2.3kb-Cre; TG$^{RosaN1ICD/+}$; *Hdac1*$^{flox/flox}$ *Hdac2*$^{flox/flox}$ mice by student's t-test *p<0.05, **p<0.01, ***p<0.001.