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MICE HARBORING A HAJDU CHENEY SYNDROME MUTATION ARE SENSITIZED TO OSTEOARTHRITIS

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Abstract

Osteoarthritis is a joint disease characterized by cartilage degradation, altered gene expression and inflammation. NOTCH1 and NOTCH2 receptors and the JAGGED1 ligand regulate chondrocyte biology; however, the contribution of Notch signaling to osteoarthritis is controversial. Hajdu Cheney Syndrome (HCS) is a rare genetic disorder affecting the skeleton and associated with *NOTCH2* mutations that lead to NOTCH2 gain-of-function. A murine model of the disease (*Notch2^{tm1.1Ecan}*) was used to test whether the HCS mutation increases the susceptibility to osteoarthritis. The knee of three-month-old *Notch2^{tm1.1Ecan}* male mice and control sex-matched littermates was destabilized by resection of the medial meniscotibial ligament, and changes in the joint analyzed two months thereafter. Expression of Notch target genes was increased in the femoral heads of *Notch2^{tm1.1Ecan}* mice, documenting Notch signal activation. Periarticular bone and cartilage structures were unaffected in *Notch2^{tm1.1Ecan}* mutants subjected to sham surgery, indicating that NOTCH2 gain-of-function had no discernible impact on joint structure under basal conditions. However, destabilization of the medial meniscus increased osteophyte volume and thickened subchondral bone in *Notch2^{tm1.1Ecan}* mice compared to wild type littermates. Moreover, destabilized *Notch2^{tm1.1Ecan}* mutants exhibited histological signs of moderate to severe cartilage degeneration, demonstrating joint sensitization to the development of osteoarthritis. Chondrocyte cultures from *Notch2^{tm1.1Ecan}* mutants expressed increased *Il6* mRNA levels following exposure to JAGGED1, possibly explaining the susceptibility of *Notch2^{tm1.1Ecan}* mice to osteoarthritis. In conclusion, *Notch2^{tm1.1Ecan}* mutants are sensitized to the development of osteoarthritis in destabilized joints and NOTCH2 activation may play a role in the pathogenesis of the disease.

Keywords

Osteoarthritis; Hajdu Cheney Syndrome; Notch2; Chondrocytes

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1. INTRODUCTION

The four Notch receptors are determinants of cell fate and function [1, 2]. Notch interactions with Jagged (JAG) or Delta-like ligands lead to the cleavage of the receptor and the release of the Notch intracellular domain (NICD) from the cellular membrane [3]. The NICD translocates to the nucleus and activates the transcription of Notch target genes, such as *Hes1*, *Hey1*, *Hey2* and *HeyL*, following interactions with recombination signal binding protein for immunoglobulin kappa J region (RBPJ) and mastermind-like (MAML) [4, 5]. Although all Notch receptors signal through this pathway, each receptor carries out distinct functions. This is because of different patterns of cellular expression and structural differences between Notch receptors as well as distinct interactions between individual NICDs and RBPJ [6–8].

Notch signaling suppresses chondrogenesis [9, 10]. Work in mice suggests that Notch inhibits the maturation of growth plate chondrocytes and prevents the acquisition of a hypertrophic phenotype, although under selected conditions Notch is capable of inducing hypertrophic differentiation [9–12]. Activation of NOTCH1 and NOTCH2 and overexpression of JAG1 in articular chondrocytes are associated with osteoarthritis [13, 14]. However, studies on models of NOTCH1 misexpression in mice have reported controversial results on the role of Notch in osteoarthritis [14, 15]. Similarly, experiments on the inactivation of *Rbpj* in chondrocytes revealed both protective and detrimental effects of RBPJ on joint integrity [12, 16, 17].

Hajdu Cheney Syndrome (HCS) is a rare genetic disease presenting with a variety of developmental and skeletal abnormalities, including osteoporosis with fractures and acro-osteolysis [18–20]. HCS is associated with *NOTCH2* mutations that create a premature stop codon immediately upstream of the sequences coding for the proline- (P), glutamic acid- (E), serine- (S) and threonine- (T)- rich (PEST) domain [21–25]. The PEST domain is required for the proteasomal degradation of NOTCH2 and the mutations are predicted to lead to the expression of a stable NOTCH2 and gain-of-function. Recently, we established a murine model of HCS (*Notch2^{tm1.1Ecan}*) that harbors a 6955C>T substitution in the *Notch2* gene creating a stop codon and leading to the premature termination of the protein product upstream of the PEST domain. Heterozygous *Notch2^{tm1.1Ecan}* mutants exhibit NOTCH2 gain-of-function and generalized osteopenia secondary to enhanced bone resorption [26].

Since HCS is associated with inflammatory lesions that lead to the lysis of distal phalanges, we asked the question as to whether *Notch2^{tm1.1Ecan}* are susceptible to the development of conditions with an inflammatory component, such as osteoarthritis. For this purpose, the knee of *Notch2^{tm1.1Ecan}* and control mice was destabilized by sectioning the medial meniscus tibial ligament. Phenotypic changes were analyzed by microcomputed tomography (μ CT) and histological assessment of the medial tibial plateau. Potential mechanisms of NOTCH2 activation were explored in chondrocyte cultures.

2. MATERIALS AND METHODS

2.1 Hajdu Cheney Mutant Mice

Notch2^{tm1.1Ecan} mice harboring a 6955C>T substitution in the *Notch2* locus were generated by homologous recombination in 129SvJ;C57BL/6J embryonic stem cells and characterized in previous studies [26–28]. Genotyping was conducted in tail DNA extracts by polymerase chain reaction (PCR) using forward primer *Nch2Lox* gF 5'-CCCTTCTCTCTGTGCGGTAG-3' and reverse primer *Nch2Lox* gR 5'-CTCAGAGCCAAAGCCTCACTG-3' (Integrated DNA Technologies; IDT, Coralville, IA).

2.2 Destabilization of the Medial Meniscus (DMM) Surgery

Notch2^{tm1.1Ecan} mice were backcrossed into a C57BL/6J background for 5 to 8 generations except for mutant mice used to analyze the epiphyseal growth plate which were in a 129SvJ/C57BL/6 background. DMM surgeries were carried out in three-month-old *Notch2^{tm1.1Ecan}* male mice and wild type sex-matched littermates, as described [29]. Surgeries were conducted under a dissecting microscope to facilitate identification of the structures of the mouse knee. A longitudinal incision was made over the distal patella to the proximal tibial plateau. The joint capsule immediately medial to the patellar tendon was incised and opened to allow dissection of the fat pad over the cranial horn of the medial meniscus. The medial meniscotibial ligament was identified as the structure running from the cranial horn of the medial meniscus laterally onto the anterior tibial plateau and subsequently dissected. Joints from mice subjected to sham surgery in which the meniscotibial ligament was visualized but not transected served as sham controls. In both DMM and sham interventions, incisions of the joint capsule and subcutaneous layers were sutured. Studies were approved by the Institutional Animal and Care Use Committees of UConn Health.

2.3 Microcomputed Tomography

Osteophyte formation and microarchitecture of subchondral bone were determined with a Scanco μ CT 40 microcomputed tomography instrument (Scanco Medical AG, Bassersdorf, Switzerland), which is calibrated periodically using a phantom provided by the manufacturer [30]. Knees were scanned in 70 % ethanol at high resolution, energy level of 55 kVp, intensity of 145 μ A, and integration time of 200 ms at a spatial resolution of 6 μ m resulting in isotropic voxels of 216 μ m³ volume. Osteophytes were identified as mineralized tissue protruding from periarticular bone. Contours were drawn manually to define the region of interest for analysis and bone volume measured to determine osteophyte volume. Subchondral bone thickness of the medial aspect of the tibial plateau is the anatomical location most susceptible to degenerative changes in response to DMM surgery, was measured as described [31, 32]. A Gaussian filter ($\sigma = 0.8$) and operator-defined thresholds were used for analysis.

2.4 Histomorphometric Analysis and Scoring of Knee Sections

Dissected knees were fixed in 70 % ethanol, demineralized in Immunocal (Decal Chemical Corp, Tallman, NY) for 10 days and embedded in paraffin. Histomorphometry of the medial aspect of the tibial plateau was carried out in five μ m-thick coronal knee sections stained

with fast green/Safranin-O. Joint degeneration was scored in stained sections on a zero to six scale, where six indicates a > 75 % loss of articular cartilage, following guidelines proposed by the Osteoarthritis Research Society International [33]. Cartilage thickness and number of chondrocytes were measured at a magnification of 100x using an OsteoMeasure morphometry system (Osteometries, Atlanta, GA). Analysis was limited to the medial tibial plateau of operated knees as this is the most susceptible to degeneration following DMM surgery [32]. To obtain histological images of femoral epiphyseal growth plates, femurs were dissected, fixed in 70% ethanol and embedded in methyl methacrylate. Bones were sectioned at a thickness of 5 μ m along the sagittal plane on a Microm microtome (Richards-Allan Scientific, Kalamazoo, MI), and stained with either toluidine blue or fast green/Safranin-O. Growth plate width, border and area were measured at a magnification of 100x using an OsteoMeasure morphometry system (OsteoMetrics, Atlanta, GA).

2.5 Chondrocyte Cultures

Chondrocyte-enriched cells were isolated from the epiphyses of long bones of the hind and forelimb from three- to four-day-old *Notch2^{tm1.1Ecan}* mice in a 129SvJ;C57BL/6J mixed genetic background. Surrounding tissues were dissected under a Unitron Z850 stereo microscope (Unitron, Commack, NY), and epiphyseal cartilage collected in high glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies), as described [34]. The tissue was exposed to 0.25 % trypsin 0.9 mM EDTA (Thermo Fisher) for 40 min, and subsequently incubated with 200 U/ml of collagenase type II (Worthington Biochemical Corporation, Lakewood, NJ) for 2 h at 37°C with continuous mixing. Digested cartilage was strained through a 70 μ m membrane and cells collected by centrifugation and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) and 100 μ g/ml ascorbic acid (Sigma-Aldrich) at 37°C in a humidified 5 % CO₂ incubator [35]. To activate Notch receptors, chondrocyte-enriched cells were seeded on culture plates coated with a human JAG1-IgG₁ fragment crystallizable (Fc) recombinant fusion protein 240 ng/cm² (R&D Systems, Minneapolis, MN), and cultured for 24 h. Parallel cultures on an equimolar amount of bovine serum albumin (BSA) served as controls and to establish basal levels of Notch signaling [36].

2.6 RNA Integrity and Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from chondrocyte-enriched cells with the RNeasy kit (Qiagen, Valencia, CA) and from homogenized femoral heads with the micro RNeasy kit (Qiagen), in accordance with manufacturer's instructions. The integrity of the RNA was assessed by microfluidic electrophoresis on an Experion system (Bio-Rad, Hercules, CA), and only 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; Table 1) with the iQ SYBR Green Supermix (Bio-Rad) at 60°C for 35 cycles [37, 38]. Transcript copy number was estimated by comparison with a serial dilution of cDNA for *Il6* (Thermo Scientific), *Hes1* and *Rpl38* (from American Type Culture Collection; ATCC, Manassas, VA), *Hey1* and *Hey2* (T. Iso, Los Angeles, CA) and *HeyL* (D. Srivastava, Dallas, TX) [39, 40]. Amplification reactions were conducted in CFX96 qRT-PCR detection systems (Bio-Rad), and fluorescence was monitored during every PCR cycle at the annealing step. Data are

expressed as copy number corrected for *Rpl38* expression estimated by comparison with a serial dilution of *Rpl38*.

2.7 Transient Transfections

Chondrocytes were transfected with a plasmid containing six dimeric CSL or RBPJ consensus sequences upstream of the β -globin basal promoter (12xCSL-Luc; L.J. Strobl, Munich, Germany) cloned upstream of *Luciferase* [41]. Transfections were conducted in cells cultured to 70 % confluence using X-tremeGENE 9 (1.5 μ l X-tremeGENE 9/1 DNA), according to manufacturer's instructions (Roche, Indianapolis, IN). A construct where the cytomegalovirus (CMV) promoter directs the expression β -galactosidase (CMV/ β -galactosidase; Clontech, Mountain View, CA) was used to correct for transfection efficiency. Cells were exposed to the X-tremeGENE 9-DNA mix for 16 h and the medium replaced after 24 h. Subsequently, cells were harvested in reporter lysis buffer (Promega, Madison, WI) and lysed by freezing at -80°C and thawing at 37°C . Luciferase and β -galactosidase activity were determined with the Luciferase Assay System kit (Promega) and galacton plus (Life Technologies) in accordance with manufacturer's instructions on an Optocomp luminometer (MGM Instruments, Hamden, CT).

2.8 Statistics

Data are expressed as medians $\pm$ S.D. Statistical differences were determined by Mann-Whitney rank sum test or two-way analysis of variance with Holm-Šídák post-hoc analysis for pairwise or multiple comparisons, respectively. Correlation coefficients were calculated by Spearman analysis.

3. RESULTS

3.1 General Appearance of *Notch2*^{tm1.1Ecan} Mice

Heterozygous *Notch2*^{tm1.1Ecan} mutants were crossed with wild type mice to generate heterozygous *Notch2*^{tm1.1Ecan} mutants and wild type littermate controls. All mice except those used to analyze the epiphyseal growth plate were in a C57BL/6J genetic background. Because female mice are protected from the deleterious effects of the DMM surgery on joint integrity, the study was carried out only in male mice [42]. The general appearance of mutant and control mice was not substantially different between control and *Notch2*^{tm1.1Ecan} mice (Figure 1). However, at one year of age the weight of wild type mice was greater than that of *Notch2*^{tm1.1Ecan} mutants. Although femoral length was not significantly decreased in *Notch2*^{tm1.1Ecan} mice, there was a moderate correlation between weight and femoral length in *Notch2*^{tm1.1Ecan} and control mice ($r = 0.55$, $p < 0.05$) (Figure 1). In accordance with the unaffected femoral length, analysis of the epiphyseal growth plate of 1- and 3-month-old mice (both in a 129SvJ/C57BL/6 background) revealed no differences between *Notch2*^{tm1.1Ecan} and control mice (Table 2, Figure 2).

3.2 Notch Signaling is Induced in Joints from *Notch2*^{tm1.1Ecan} Mice

To validate *Notch2*^{tm1.1Ecan} mutant mice as a model of NOTCH2 gain-of-function in the joint, total RNA was extracted from the femoral heads of five-month-old *Notch2*^{tm1.1Ecan} C57BL/6J male mice and sex-matched littermate wild type controls. Analysis of gene

expression by qRT-PCR revealed that *Notch2^{tm1.1Ecan}* mice exhibited increased levels of the Notch target genes *Hey1*, *Hey2* and *HeyL*, an effect indicative of NOTCH2 activation in synovial joints (Figure 3). *Hes1* transcript levels were not affected.

3.3 Knees of *Notch2^{tm1.1Ecan}* Mutant Mice are Sensitized to Osteoarthritis

To determine the impact of the *Notch2^{tm1.1Ecan}* mutation on joint integrity, three-month-old *Notch2^{tm1.1Ecan}* male mice and wild type littermates of the same sex were subjected to DMM, as described [29]. Sham-operated male *Notch2^{tm1.1Ecan}* mice and sex-matched wild type littermates served as controls to monitor the effects of the *Notch2* mutation on joint integrity. μ CT and histomorphometric analysis of the medial tibial plateau revealed that *Notch2^{tm1.1Ecan}* mice subjected to sham surgeries displayed normal joint architecture and cartilage structure, demonstrating that *Notch2^{tm1.1Ecan}* mice have no appreciable joint abnormalities at five months of age. In addition, intact contralateral knees from sham- or DMM-operated *Notch2^{tm1.1Ecan}* mice displayed no evidence of degenerative joint changes (data not shown).

In agreement with previous reports, μ CT analysis confirmed that DMM leads to osteophyte formation in wild type mice (Figure 4A) [29]. *Notch2^{tm1.1Ecan}* mice exhibited a greater osteophyte volume and more pronounced tibial subchondral bone thickening following DMM than wild type littermates (Figure 4B). These observations were substantiated by histological assessments, which identified moderate to severe cartilage degeneration in *Notch2^{tm1.1Ecan}* mutants and only mild degeneration in wild type littermates (Figure 5). Histomorphometric analysis of *Notch2^{tm1.1Ecan}* mice revealed a tendency toward reduced cartilage area and as a consequence a reduction in total chondrocyte number both indicative of cartilage degradation (Table 3). However, the number of chondrocytes/area was not statistically different between control and *Notch2^{tm1.1Ecan}* mutant mice (Table 3).

3.4 Chondrocytes from *Notch2^{tm1.1Ecan}* Mice Display Enhanced Canonical Notch Activation

To investigate potential mechanisms of NOTCH2 action, primary chondrocyte-enriched cells were harvested from the epiphyseal cartilage of long bones from three- to four-day-old *Notch2^{tm1.1Ecan}* mice and littermate controls. To prevent chondrocyte de-differentiation in culture, primary cells were maintained in a monolayer without further expansion [43, 44]. Under these conditions, the differentiated chondrocyte phenotype is retained and we refer to these cells as chondrocytes [35, 45].

To stimulate Notch signaling, cells were seeded on substrates coated with JAG1, whereas cells cultured on BSA served as controls. To measure the activation of RBPJ-mediated Notch signaling, chondrocytes were transiently transfected with the 12xCSL-Luc reporter. Under basal conditions, 12xCSL-Luc activity was not different between cells from wild type and *Notch2^{tm1.1Ecan}* mice. JAG1 induced the transactivation of the 12xCSL-Luc reporter in cells from *Notch2^{tm1.1Ecan}* mice to a greater extent than in control chondrocytes (Figure 6A). To confirm these results, gene expression was analyzed by qRT-PCR in parallel chondrocyte cultures. JAG1 caused a modest non-significant induction of *Hey1*, and a significant increase in *Hey2* and *HeyL* in cells from *Notch2^{tm1.1Ecan}* mice compared to controls, indicating

greater Notch activation in *Notch2^{tm1.1Ecan}* cells. Similarly to the results *in vivo*, *Hes1* expression was not affected (Figure 6B).

3.5 JAG1 Induces Il6 Expression in Chondrocytes from *Notch2^{tm1.1Ecan}* Mice

Inflammation is a common finding in osteoarthritis, and articular chondrocytes contribute to this process by releasing cytokines that induce inflammation [46–48]. We tested whether *Notch2^{tm1.1Ecan}* chondrocytes had enhanced expression of gene markers associated with inflammation and osteoarthritis. Analysis of gene expression by qRT-PCR revealed that JAG1 induced *Il6* transcripts to a greater extent in chondrocytes from *Notch2^{tm1.1Ecan}* mice than in control cells, suggesting that the NOTCH2 gain-of- function might exacerbate osteoarthritis by enhancing *Il6* expression (Figure 7).

4. DISCUSSION

In this study, we report that the *Notch2*HCS mutation exacerbates osteoarthritis progression following DMM. Both calcified and cartilaginous tissues of the joint were affected, and the mechanism is likely related to a NOTCH2 gain-of-function and induction of *Il6* expression in chondrocytes. Absence of changes in chondrocyte density *in vivo* and in the expression of cyclin e1 and cyclin d1 *in vitro* (data not shown) suggest that NOTCH2 does not act by inducing chondrocyte proliferation. *Notch2^{tm1.1Ecan}* mice were not appreciably different from wild type littermates although they weighed less than controls as they aged. NOTCH2 gain-of-function was documented in femoral heads of *Notch2^{tm1.1Ecan}* mice, and the mutation did not affect joint integrity under basal conditions. The knees from mice subjected to sham intervention and knees contralateral to those undergoing DMM did not display degenerative changes. This is consistent with previous observations indicating a small and transient effect of the *Notch2^{tm1.1Ecan}* mutation on femoral elongation, suggesting a modest effect on cartilage tissue [26]. The present findings are consistent with the observation that individuals afflicted by HCS are not reported to present with osteoarthritis [49]. However, this simply may be related to the shorter life span of individuals affected by HCS.

Recently, we demonstrated that *Notch2^{tm1.1Ecan}* mice display splenomegaly and reallocation of marginal zone B-cells, but no evidence of T-cell abnormalities or of a generalized inflammatory process [28, 50]. These findings, coupled with the absence of macrophage infiltration in the synovium of sham- or DMM-operated *Notch2^{tm1.1Ecan}* mutants, suggest that NOTCH2 activation in joint tissues, and not in immune cells, drives osteoarthritis progression in *Notch2^{tm1.1Ecan}* mice.

The reported *in vitro* experiments demonstrated enhanced RBPJ-mediated Notch signaling in chondrocytes from *Notch2^{tm1.1Ecan}* mice. However, previous work demonstrated that deletion of *Rbpj* in the limb bud during development or in articular chondrocytes in post-natal life causes severe osteoarthritis [16, 17]. Although this would imply a protective function of Notch signaling on the integrity of the joint, it is important to note that the deletion of *Rbpj* prevents the activation of all Notch receptors and not exclusively NOTCH2. Moreover, RBPJ is a transcriptional repressor that induces gene expression only following interactions with the NICD, so that its downregulation could result in the induction of Notch target genes and lead to cellular changes analogous to those associated with the activation of

Notch signaling [51]. It is possible that the joint degeneration in the context of the *Rbpj* deletion in chondrocytes is secondary to functions of RBPJ that are not related to Notch signaling.

NOTCH2 gain-of-function in chondrocyte cultures was associated with an induction of *Il6* transcripts. This is in agreement with the stimulatory effects of NOTCH1 on *Il6* expression in chondrocytes and supports the notion that activation of Notch in cartilage contributes to osteoarthritis progression by inducing inflammatory cytokines [14, 45, 52]. Stimulation of chondrocyte cultures with JAG1 was required to observe increased *Il6* expression in *Notch2^{tmL1Ecan}* chondrocytes. It is of interest that JAG1 is induced in human osteoarthritic chondrocytes and in the articular cartilage of DMM- operated mice, so that the induction of JAG1 may be important to sensitize destabilized joints to the effects of Notch on osteoarthritis [12, 13]. Previous work revealed a critical function of HES1 in osteoarthritis progression and HES1 can induce *Il6* expression in chondrocytes [53]. However, HES1 expression was not increased in femoral heads or chondrocyte cultures from *Notch2^{tmL1Ecan}* mutants, suggesting that the enhanced *Il6* expression in *Notch2^{tmL1Ecan}* mutants is secondary to a direct effect of NOTCH2 [52].

This is the first report describing the detrimental consequences of NOTCH2 activation on joint integrity. However, it is not clear whether these findings can be extrapolated to the function of other Notch receptors in the joint. This is because previous studies have used models of NOTCH1 misexpression and have reported conflicting results. NOTCH1 activation under the control of the *Agc1* promoter leads to joint degeneration. In contrast, NOTCH1 induction in *Col2a1*-expressing cells protects the joint from injury, and its downregulation exacerbates experimental osteoarthritis through a HES1- dependent mechanism [14, 15, 54]. The reason for the discrepant results is not entirely clear and might be secondary to differences in the timing and pattern of expression of the *Agc1-Cre* and *Col2a1-Cre* alleles [55–57]. In addition, studies on NOTCH1 overexpression were conducted using a *Rosa^{Notch}* mouse model, where levels of *Notch1* expression are determined by the *Rosa26* promoter and not by the native regulatory regions of the *Notch1* gene [58]. The *Notch2^{tmL1Ecan}* model has the advantage of expressing a *Notch2* mutant gene governed by the native regulatory sequences of the *Notch2* locus, resulting in physiologically controlled levels of gene expression [26].

5. CONCLUSIONS

In conclusion, *Notch2^{tmL1Ecan}* mutants are sensitized to osteoarthritis in destabilized joints and NOTCH2 activation may play a role in the pathogenesis of the disease.

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ABBREVIATIONS

6.

ATCC	American type culture collection
bp	base pair
BSA	bovine serum albumin
CMV	cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
DMM	destabilization of the medial meniscus
FBS	fetal bovine serum
Fc	fragment crystallizable
HCS	Hajdu Cheney Syndrome
JAG	Jagged
kb	kilobase
IDT	Integrated DNA Technologies
MAML	mastermind-like
μCT	microcomputed tomography
NICD	Notch intracellular domain
PCR	polymerase chain reaction
PEST	proline- (P), glutamic acid- (E), serine- (S) and threonine- (T)-rich
qRT-PCR	quantitative reverse transcription-PCR
RBPJ	recombination signal binding protein for immunoglobulin kappa J region.

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Highlights

- *Notch2^{tm1.1Ecan}* mice are a model of Hajdu Cheney Syndrome.
- *Notch2^{tm1.1Ecan}* mice exhibit NOTCH2 gain-of-function.
- *Notch2^{tm1.1Ecan}* mice are sensitized to the development of osteoarthritis.
- IL-6 expression is enhanced in *Notch2^{tm1.1Ecan}* chondrocytes.

Figure 1. *Notch2^{tm1.1Ecan}* mice have no appreciable changes in general appearance.

Weight and femoral length of male *Notch2^{tm1.1Ecan}* mice (grey boxes) and sex-matched wild type littermate controls (white boxes). Horizontal continuous lines represent the median and interrupted lines represent the mean; upper and lower limits of the box represent the 75th and 25th percentile. Whiskers (bars) indicate the maximum and minimum values. On the right panel, the correlation between femoral length and weight is shown ($r = 0.55$, $p < 0.05$) by Spearman analysis. Number of observations for control mice: $n = 2$ at 6 months and $n = 6$ at 12 months; number of observations for *Notch2^{tm1.1Ecan}* mice: $n = 5$ at 6 at 12 months.* Significantly different between control and *Notch2^{tm1.1Ecan}*, $p < 0.05$ by two-way ANOVA with Holm-Šidák post-hoc analysis.

Figure 2. Epiphyseal growth plates of *Notch2^{tm1.1Ecan}* mice are not affected. Representative histological sections of epiphyses from 1- and 3-month old *Notch2^{tm1.1Ecan}* male mice and control sex- matched littermates in a 129SvJ/C57BL/6 background stained with fast green/Safranin-O. Full data set in Table 2.

Figure 3. Notch signaling is induced in joints from *Notch2^{tm1.1Ecan}* mice.

Total RNA was extracted from femoral heads of five-month-old *Notch2^{tm1.1Ecan}* male mice (grey bars) and sex-matched wild type littermate controls (white bars). *Hes1*, *Hey1*, *Hey2* and *HeyL* mRNA levels were measured by qRT-PCR and data are expressed as copy number, corrected for *Rpl38*. Values are medians $\pm$ SD; n = 6. * Significantly different between control and *Notch2^{tm1.1Ecan}*, $p < 0.05$ by Mann-Whitney rank sum test.

Figure 4. *Notch2^{tm1.1Ecan}* mice are sensitized to the effects of DMM surgery on mineralized tissues of the joint.

Three-month-old *Notch2^{tm1.1Ecan}* male mice (grey bars) and sex-matched wild type littermate controls (Control; white bars) were subjected to DMM, and knees collected at 5 months of age and analyzed by μCT . In panel A, three-dimensional reconstructions of the mineralized tissues of knees from representative control and *Notch2^{tm1.1Ecan}* mice and osteophyte volume. Arrows indicate the presence of osteophytes. In panel B, thickness maps of the subchondral bone in femurs from representative control and *Notch2^{tm1.1Ecan}* mice and average subchondral bone thickness. Values are medians $\pm$ SD; $n = 4$ for control; $n = 7$ for *Notch2^{tm1.1Ecan}*. * Significantly different between control and *Notch2^{tm1.1Ecan}*, $p < 0.05$ by Mann-Whitney rank sum test.

Figure 5. *Notch2^{tm1.1Ecan}* mice display greater cartilage degeneration than wild type mice following DMM surgery.

Three-month-old *Notch2^{tm1.1Ecan}* male mice (grey bars) and sex-matched wild type littermate controls (Control; white bars) were subjected to DMM, and knees collected at 5 months of age, dissected and demineralized. Histological analysis was conducted in fast green/Safranin-O stained sections from paraffin-embedded knees. Images are from representative control and *Notch2^{tm1.1Ecan}* mice. Cartilage degeneration was scored by two observers blinded to the identity of the mouse under investigation and average scores for each section were used. Values are medians ± SD; n = 3 for control; n = 6 for *Notch2^{tm1.1Ecan}*.

Figure 6. Notch signaling is induced in chondrocytes from *Notch2^{tm1.1Ecan}* mice.

Chondrocyte-enriched cells were isolated from epiphyses of long bones from three- to four-day-old *Notch2^{tm1.1Ecan}* mice (grey bars) and age-matched wild type (white bars) littermate controls. Cells were cultured on substrates coated with JAG1, to induce Notch signaling, or BSA control. In panel A, the 12xCSL-Luc reporter construct was transiently co-transfected with a CMV β-galactosidase construct; data are expressed as luciferase/β-galactosidase activity. In panel B, gene expression measured by qRT-PCR in total RNA extracts. Data are expressed as *Hes1*, *Hey1*, *Hey2* and *HeyL* copy number, corrected for *Rpl38*. Values for panels A & B are medians ± SD; n = 4 for all data sets with the exception of *Hey2* expression in control cells seeded on BSA, where n = 3. * Significantly different between control and *Notch2^{tm1.1Ecan}*, $p < 0.05$; #significantly different between BSA and JAG1, $p < 0.05$ by two-way ANOVA with Holm-Šidák post-hoc analysis.

Figure 7. *Il6* expression is induced by JAG1 in chondrocytes from *Notch2*^{tm1.1Ecan} mice. Chondrocyte-enriched cells were isolated from epiphyses of long bones from three- to four-day-old *Notch2*^{tm1.1Ecan} mice (grey bars) and wild type (white bars) littermate controls. Cells were cultured on substrates coated with JAG1, to induce Notch signaling, or BSA as a control. *Il6* expression was measured by qRT-PCR in total RNA extracts and data are expressed as *Il6* copy number corrected for *Rpl38*. Values are medians $\pm$ SD; n = 4 for all data sets. * Significantly different between control and *Notch2*^{tm1.1Ecan}, $p < 0.05$; #

significantly different between BSA and JAG1, $p < 0.05$ by two-way ANOVA with Holm-Šídák post-hoc analysis.

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Table 1.

Primers used for qRT-PCR determinations. GenBank accession numbers identify the transcripts recognized by primer pairs.

Gene	Strand	Sequence 5'-3'	GenBank Accession Number
<i>Hes1</i>	Forward	5'-ACCAAAGACGGCCTCTGAGCACAGAAAGT-3'	NM_008235
	Reverse	5'-ATTCTTGCCCTTCGCCTCTT-3'	
<i>Hey1</i>	Forward	5'-ATCTCAACAACACGCATCCCAGC-3'	NM_010423
	Reverse	5'-GTGTGGGTGATGTCCGAAGG-3'	
<i>Hey2</i>	Forward	5'-AGCGAGAACAATTACCCTGGGCAC-3'	NM_013904
	Reverse	5'-GGTAGTTGTCGGTGAATTGGACCT-3'	
<i>HeyL</i>	Forward	5'-CAGTAGCCTTTCTGAATTGCGAC-3'	NM_013905
	Reverse	5'-AGCTTGGAGGAGCCCTGTTTC-3'	
<i>Il6</i>	Forward	5'-CGGCCTTCCCTACTTCACAAGTCCG-3'	NM_031168
	Reverse	5'-CAGGTCTGTTGGGAGTGGTATCC-3'	
<i>Rpl38</i>	Forward	5'-AGAACAAAGGATAATGTGAAGTTCAAGGTTTC-3''	NM_001048057; NM_001048058; NM_023372
	Reverse	5'-CTGCTTCAGCTTCTCTGCCTTT-3'	

Table 2.

Analysis of epiphyseal growth plates of 1- and 3-month old *Notch2^{tm1.1Ecan}* mice and sex- matched littermate controls.

Growth Plate	1 Month		3 Month	
	Control n = 5	<i>Notch2^{tm1.1Ecan}</i> n = 5	Control n = 4	<i>Notch2^{tm1.1Ecan}</i> n = 3
Area (mm ²)	0.47 ± 0.17	0.35 ± 0.15	0.22 ± 0.01	0.23 ± 0.06
Border (mm)	2.43 ± 0.53	2.12 ± 0.30	2.80 ± 0.11	2.83 ± 0.30
Width (mm)	143.1 ± 22.3	143.8 ± 32.1	63.9 ± 5.9	64.3 ± 7.2

Femurs from *Notch2^{tm1.1Ecan}* and control littermates were sectioned sagittally and stained with toluidine blue or fast green/Safranin-O. Values are medians ± SD.

Table 3.

Cartilage histomorphometry in five-month-old male *Notch2^{tm1.1Ecan}* mice and sex-matched littermate controls subjected to DMM.

	Control	<i>Notch2^{tm1.1Ecan}</i>
	n = 3	n = 7
Cartilage Area (mm ²)	0.07 ± 0.02	0.04 ± 0.02
Number of Chondrocytes	202 ± 26	117 ± 47 *
Number of Chondrocytes/Area (mm ⁻²)	2917 ± 642	3618 ± 2026

Knees from *Notch2^{tm1.1Ecan}* and control littermates subjected to DMM were sectioned coronally at a thickness of five µm and stained with fast green/Safranin-O. Histomorphometric indices were measured in one section from each animal at the medial side of the tibial plateau. Values are medians ± S.D.

*Significantly different between Control and *Notch2^{tm1.1Ecan}*, $p < 0.05$ by Mann-Whitney rank sum test.