

Cartilage-specific RBPj κ -dependent and -independent Notch signals regulate cartilage and bone development

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SUMMARY

The Notch signaling pathway has emerged as an important regulator of endochondral bone formation. Although recent studies have examined the role of Notch in mesenchymal and chondro-osteoprogenitor cell populations, there has yet to be a true examination of Notch signaling specifically within developing and committed chondrocytes, or a determination of whether cartilage and bone formation are regulated via RBPj κ -dependent or -independent Notch signaling mechanisms. To develop a complete understanding of Notch signaling during cartilage and bone development we generated and compared general Notch gain-of-function (*Rosa-NICD^{fl/+}*), RBPj κ -deficient (*Rbpj κ ^{fl/fl}*), and RBPj κ -deficient Notch gain-of-function (*Rosa-NICD^{fl/+};Rbpj κ ^{fl/fl}*) conditional mutant mice, where activation or deletion of floxed alleles were specifically targeted to mesenchymal progenitors (*Prx1Cre*) or committed chondrocytes (inducible *Col2Cre^{ERT2}*). These data demonstrate, for the first time, that Notch regulation of chondrocyte maturation is solely mediated via the RBPj κ -dependent pathway, and that the perichondrium or osteogenic lineage probably influences chondrocyte terminal maturation and turnover of the cartilage matrix. Our study further identifies the cartilage-specific RBPj κ -independent pathway as crucial for the proper regulation of chondrocyte proliferation, survival and columnar chondrocyte organization. Unexpectedly, the RBPj κ -independent Notch pathway was also identified as an important long-range cell non-autonomous regulator of perichondral bone formation and an important cartilage-derived signal required for coordinating chondrocyte and osteoblast differentiation during endochondral bone development. Finally, cartilage-specific RBPj κ -independent Notch signaling likely regulates *Ihh* responsiveness during cartilage and bone development.

KEY WORDS: RBPj κ , Notch, Chondrocyte, Cartilage, Perichondrium, Endochondral, Mouse

INTRODUCTION

The limb skeleton is derived via the process of endochondral ossification. The process is initiated when lateral plate mesoderm-derived mesenchymal progenitor cells (MPCs) form condensations within the limb field that give rise to cartilaginous anlagen, ultimately providing a template for bone formation. Multipotent MPCs within the anlagen give rise to lineage-restricted chondrocytes and osteoblasts after transitioning through a bipotential chondro-osteoprogenitor (COP) phase. Centrally localized COPs undergo chondrogenesis, forming the cartilaginous tissue, whereas COPs near the periphery of condensations undergo osteoblastogenesis to form the perichondrial bone (Kronenberg, 2003). Once committed, chondrocytes proliferate rapidly until cells nearest the center of the anlagen exit the cell cycle and begin the process of maturation or hypertrophic differentiation. During the maturation process committed chondrocytes transition from proliferative cells to pre-hypertrophic, hypertrophic, and finally terminally hypertrophic chondrocytes, sequentially activating the genes *Ihh*, *Col10a1* and *Mmp13* (Hilton et al., 2005). Concurrently, osteoprogenitors in the perichondrium undergo osteoblastic

differentiation on the surface of the anlagen adjacent to prehypertrophic and hypertrophic chondrocytes. The development of the cartilage and perichondrium are tightly linked, as signals from both populations of cells affect the growth and maturation of the other in a coordinated manner (Colnot et al., 2004; Long and Linsenmayer, 1998). As the skeletal rudiments expand and develop, vasculature from the surrounding tissue infiltrates the center of each element, leading to the formation of a marrow cavity that requires apoptosis of terminally hypertrophic chondrocytes and the subsequent ossification of the cartilaginous matrix by invading osteoblasts. It is this process of chondrocyte proliferation, maturation and apoptosis along with communication between cartilage and perichondrium that drives normal longitudinal growth and development of the endochondral bones (Kronenberg, 2003).

The Notch signaling pathway is an important regulator of a diverse set of cellular processes, such as cell proliferation, differentiation, fate determination, stem/progenitor cell self-renewal and cell-cell communication in both embryonic and adult organs (Artavanis-Tsakonas et al., 1999; Chiba, 2006; Lai, 2004). In mammals, Notch signaling is primarily initiated during cell-cell interactions when one of the 11 Notch ligands [JAG1-2, DLL1,3,4, DLK1-2, MAGP1-2 (MFAP2 – Mouse Genome Informatics), DNER and NB3 (CNTN6 – Mouse Genome Informatics)] activates a single-pass transmembrane cell surface Notch receptor (NOTCH1-4) leading to a series of receptor cleavage events, mediated by ADAM protease and proteins of the gamma-secretase complex. Cleavage results in the release of the Notch intra-cellular domain (NICD) and its translocation to the nucleus, where it binds the transcriptional regulators RBPj κ and MAML, creating a transcriptionally active complex. NICD-RBPj κ -MAML ternary

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complexes drive expression of downstream Notch target genes, such as the *Hes/Hey* family of basic helix-loop-helix (bHLH) transcription factors (Kopan and Ilagan, 2009). This axis of Notch signaling is often referred to as the RBPjk-dependent Notch pathway. Recent studies in *Drosophila* have demonstrated that the NICD can function independently of RBPjk (Matsuno et al., 1997; Romain et al., 2001). While the exact nature of this RBPjk-independent Notch pathway remains elusive in mammalian systems, the NICD has been shown to interact with members of the BMP (Dahlqvist et al., 2003; Itoh et al., 2004), TGF β (Blokzijl et al., 2003; Ross and Kadesch, 2001), WNT/ β -CATENIN (Axelrod et al., 1996; Hayward et al., 2005) and NF- κ B (Vacca et al., 2006; Vilimas et al., 2007; Wang et al., 2001) protein families and affect various aspects of signaling.

Several signaling pathways including IHH/PTHrP, BMP, TGF β , and WNT/ β -CATENIN have been well studied in their regulation of cartilage and bone development (Baffi et al., 2004; Chung et al., 2001; Retting et al., 2009; Song et al., 2009; Vortkamp et al., 1996; Wu et al., 2008); however, the Notch pathway has only recently been implicated in a few aspects of these processes. The first in vivo genetic evidence of a role for Notch in regulating cartilage development came from a study by Hilton et al. (Hilton et al., 2008) in which the 'upstream' Notch signaling components (PSEN1 and PSEN2 or NOTCH1 and NOTCH2) were conditionally removed from MPCs of the mouse limb using the *Prx1Cre* transgene. These data revealed that loss of Notch signaling in MPCs led to a delay in the onset of chondrocyte maturation at embryonic day 14.5 (E14.5), as well as a delay in the progression of chondrocyte terminal maturation at E18.5. A more recent study found that loss of the canonical Notch effector RBPjk in bi-potentiated COP cells using the *Col2Cre* transgene also led to the delayed progression of chondrocytes to terminal maturation at E18.5, although accelerated chondrocyte maturation was observed in E14.5 cartilage rudiments (Mead and Yutzey, 2009). Interestingly, the Notch loss-of-function (LOF) mutant mice developed by Hilton et al. (Hilton et al., 2008) displayed a reduction in chondrocyte proliferation, whereas the RBPjk LOF mutant mice produced by Mead and Yutzey (Mead and Yutzey, 2009) exhibited an increase in chondrocyte proliferation. Previous studies have also demonstrated that Notch gain-of-function (GOF) alleles activated in early limb progenitor cells, either MPCs or COPs, leads to an inhibition of differentiation and a maintenance of the progenitor cell pool (Dong et al., 2010; Mead and Yutzey, 2009), whereas loss of Notch signaling in progenitors enhances chondrogenesis and osteogenesis (Dong et al., 2010; Hilton et al., 2008). Collectively, these data suggest that RBPjk-dependent and -independent Notch signals may regulate different aspects of chondrocyte proliferation and maturation or that the timing at which Notch signals are removed or activated in the skeletal lineages differentially affects cartilage development. To determine the precise role of Notch signaling during cartilage development and maturation we have developed and analyzed general Notch GOF (*Rosa-NICD^{f/f}*), RBPjk-deficient (*Rbpjk^{f/f}*) and RBPjk-deficient Notch GOF (*Rosa-NICD^{f/f};Rbpjk^{f/f}*) mutants using both the *Prx1Cre* line to target recombination to MPCs and the *Col2Cre^{ERT2}* to target recombination to committed chondrocytes, as well as a primary chondrocyte culture model.

MATERIALS AND METHODS

Mouse strains

All mouse strains, including *Rosa-NICD^{f/f}*, *Rbpjk^{f/f}*, *Prx1Cre* and *Col2Cre^{ERT2}*, are as previously described (Chen et al., 2007; Han et al., 2002; Logan et al., 2002; Murtaugh et al., 2003). *Prx1Cre* mice were

obtained from the Jackson Laboratory (Bar Harbor, ME, USA); *Rosa-NICD^{f/f}*, *Rbpjk^{f/f}* and *Col2Cre^{ERT2}* mice were generous gifts from Drs Douglas Melton (Harvard University, MA, USA), Tasuku Honjo (Kyoto Graduate School of Medicine, Japan) and Di Chen (University of Rochester, NY, USA), respectively. Embryos of the genotypes *Prx1Cre;Rbpjk^{f/f}*, *Prx1Cre;Rosa-NICD^{f/f};Rbpjk^{f/f}*, *Col2Cre^{ERT2};Rbpjk^{f/f}*, *Col2Cre^{ERT2};Rosa-NICD^{f/f}*, *Col2Cre^{ERT2};Rosa-NICD^{f/f};Rbpjk^{f/f}* and Cre-negative littermate controls were produced in Mendelian ratios to be analyzed from E14.5 to E18.5. *Prx1Cre;Rosa-NICD^{f/f}* mutant mice have been previously analyzed (Dong et al., 2010). *Prx1Cre;Rbpjk^{f/f}* and *Prx1Cre;Rosa-NICD^{f/f};Rbpjk^{f/f}* mutant mice survived to adulthood and were viable and fertile, whereas all *Col2Cre^{ERT2}* mutant genotypes died at or just before birth.

Analyses of mouse embryos

Embryonic tissues were harvested at E14.5-E18.5, fixed in 10% neutral buffered formalin, decalcified in 14% EDTA, processed, and embedded in paraffin before sectioning at 6 μ m. Hematoxylin and Eosin (H&E), Alcian Blue/Orange G (AB/OG), Masson's trichrome (TriCh) and TRAP staining was performed according to standard methodologies. In situ hybridization was performed as described previously (Dong et al., 2010; Hilton et al., 2005; Hilton et al., 2007; Hilton et al., 2008), using ³⁵S-labeled riboprobes. BrdU immunostaining analyses were performed by injecting pregnant females with 0.1 mg/g body weight of BrdU 2 hours before embryo harvest. BrdU detection was performed on paraffin sections using the Invitrogen BrdU Staining Kit per the manufacturer's instructions. Analyses of apoptotic chondrocytes were performed using TUNEL staining (In Situ Cell Death Detection Kit, Roche) on limb sections according to the manufacturer's instructions.

Primary chondrocyte cultures

Primary chondrocytes were isolated from the ribcages of 3-day-old mouse pups of the indicated genotypes (*C56BL6/J*, *Rbpjk^{f/f}* and *Rosa-NICD^{f/f}*). Chondrocytes were isolated and initially cultured as previously described (Gunnell et al., 2010). Cells were treated 1 day after plating with either *N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT), Ad5-CMV-GFP or Ad5-CMV-Cre adenoviruses in complete media and allowed to mature for up to 10 days, as indicated. Adenoviral vectors were purchased from Baylor College of Medicine and used at a 1:1000 dilution of the supplied stock. Alkaline phosphatase (AP) staining and real-time RT-PCR were performed as previously described (Dong et al., 2005; Dong et al., 2010). Primer sequences for *Col2a1*, *Col10a1*, *Mmp13*, *Hes1* and *Actb* are listed in supplementary material Table S1.

RESULTS

Loss of RBPjk in chondrocytes and perichondrial cells delays chondrocyte maturation

To begin to understand the role of RBPjk-dependent and -independent Notch signaling in cartilage and bone development, we analyzed embryonic mouse hind limbs from RBPjk-deficient (*Rbpjk^{f/f}*) and RBPjk-deficient Notch GOF (*Rbpjk^{f/f};Rosa-NICD^{f/f}*) mice. Floxed alleles were targeted for recombination using *Prx1Cre*, which is expressed specifically in limb-bud MPCs that give rise to chondrocytes, osteoblasts and connective tissue cells of the limb skeleton (Logan et al., 2002) (supplementary material Fig. S1A,C). Forelimbs and hind limbs were harvested from embryos at E14.5 (Fig. 1A and data not shown) and E18.5 (Fig. 1B, supplementary material Fig. S2). E14.5 humerus sections were analyzed by H&E staining and in situ hybridization. Analyses of *Rbpjk^{f/f}* LOF humerus sections revealed reduced *Ihh* and *Col10a1* expression domains (Fig. 1Ab,e,h) compared with Cre-negative littermates (Fig. 1Aa,d,g). RBPjk-deficient Notch GOF mutant sections also revealed smaller zones of *Ihh*, *Col10a1* and *Mmp13* (Fig. 1Ac,f,i,l). Furthermore, in both the *Rbpjk^{f/f}* LOF and *Rbpjk^{f/f};Rosa-NICD^{f/f}* GOF mutants the percentage of hypertrophic zone length compared with total limb length was significantly decreased compared with littermate controls,

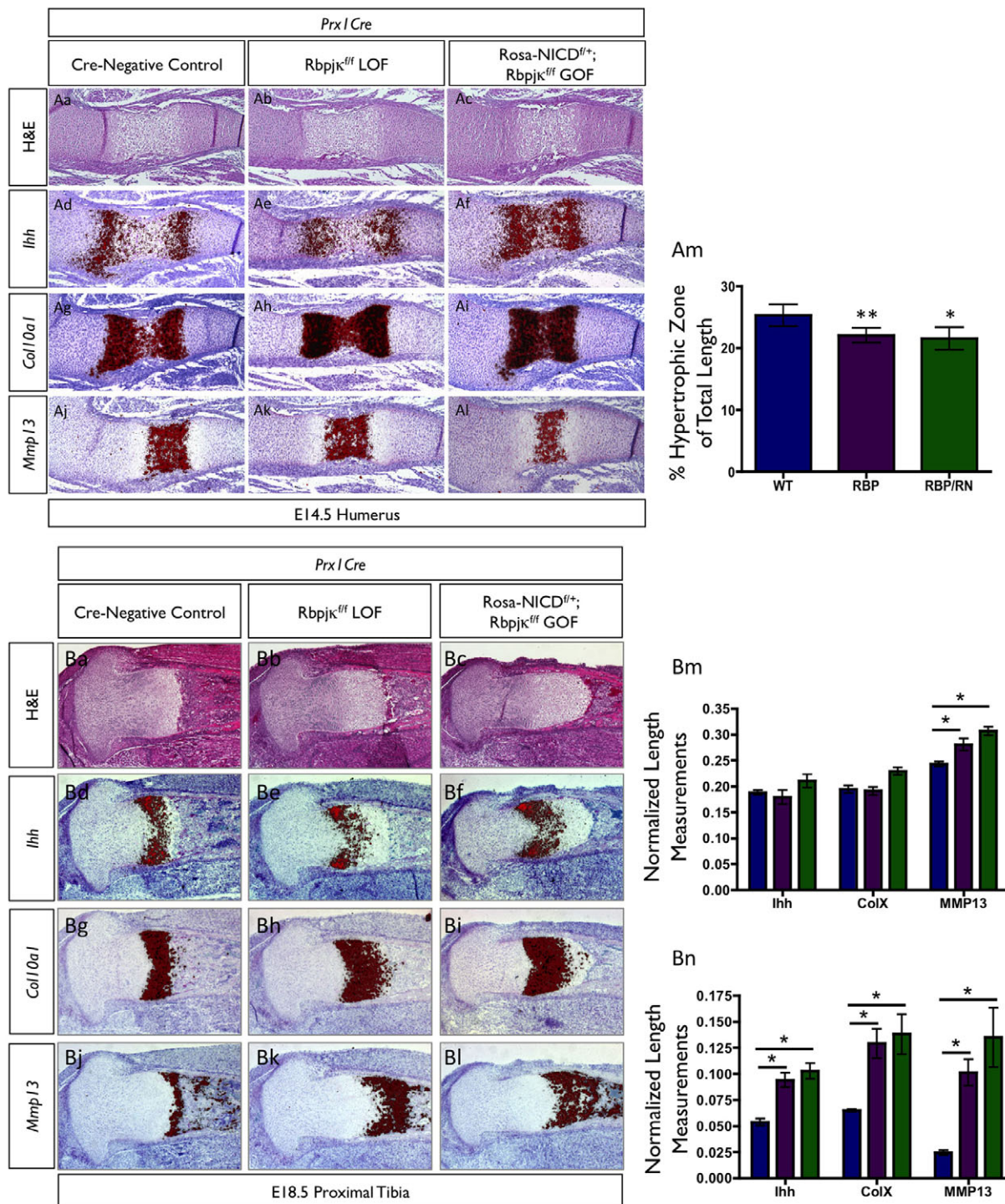


Fig. 1. Loss of RBPjk-dependent Notch signaling in both chondrogenic and osteogenic lineages results in delayed onset and progression of chondrocyte maturation. (A) (a-c) H&E staining of wild-type, *Prx1Cre;Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) and *Prx1Cre;Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) embryonic humerus sections at E14.5. (d-l) In situ hybridization for *Ihh* (d-f), *Col10a1* (g-i) and *Mmp13* (j-l). Medial longitudinal sections were compared and the percentage of hypertrophic zone to total cartilage length was determined. (m) Summary of results. (B) (a-c) H&E staining of wild-type, *Rbpjk^{fl/fl}* LOF and *Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF embryonic tibia sections at E18.5. (d-l) In situ hybridization for *Ihh* (d-f), *Col10a1* (g-i) and *Mmp13* (j-l). Medial longitudinal sections were compared and lengths from the epiphysis to the start of *Ihh*, *Col10a1* and *Mmp13* domains (m) and the lengths of the *Ihh*, *Col10a1* and *Mmp13* domains (n) were determined and analyzed. Lengths were normalized to the full length of the corresponding element and averaged across three groups. *, $P < 0.05$.

although not different from one another (Fig. 1Am). Collectively these data indicate that RBPjk-dependent Notch signaling promotes the onset of chondrocyte hypertrophy at E14.5.

We next analyzed hind limbs and forelimbs from E18.5 embryos in a similar manner, including in situ hybridization on E18.5 tibia sections for markers of chondrocyte maturation, *Ihh*, *Col10a1* and

Mmp13. The *Ihh* and *Col10a1* expression domains were significantly expanded in both mutants (Fig. 1Bd-i, quantified in Fig. 1Bn). Importantly, the terminal hypertrophic domain, marked by *Mmp13* expression and normally restricted to the deepest layers of terminal hypertrophic chondrocytes (Fig. 1Bj), was expanded to numerous cell layers in both the RBPjk-deficient and RBPjk-deficient Notch GOF mutant elements (Fig. 1Bk,l). More detailed analysis of the Cre-negative control, RBPjk-deficient and RBPjk-deficient Notch GOF mutants revealed no difference in length from the epiphysis to the start of the *Ihh* and *Col10a1* domains (Fig. 1Bm). However, when the lengths of the *Ihh*, *Col10a1* and *Mmp13* domains were analyzed (Fig. 1Bn) a significant increase was identified in both the RBPjk-deficient and RBPjk-deficient Notch GOF mutants. The increase in the lengths of the *Ihh* and *Col10a1* domains explains why the length from the epiphysis to the start of the *Mmp13* domain is increased (Fig. 1Bm). Together these data indicate that at E18.5 loss of RBPjk-dependent Notch signaling primarily leads to a delay in the progression of chondrocyte hypertrophy, from pre-hypertrophy to terminal maturation, as evidenced by the cells remaining at each stage of hypertrophy longer (Fig. 1Bn).

TRAP staining for osteoclasts and in situ hybridization for markers of vascular development [*Mmp9* and *Vegf* (*Vegfa* – *Mouse Genome Informatics*)] were also assessed to determine if any dysregulation in these processes could be part of the underlying cause for the expanded mutant hypertrophic domains. In fact, TRAP staining (supplementary material Fig. S3A) revealed an increase in osteoclasts at the chondro-osseous junction, whereas in situ hybridization for markers of vascular development, *Mmp9* and *Vegf* (supplementary material Fig. S4) revealed normal or even enhanced expression, indicating that cartilage matrix removal and cartilage vascularization are not impaired in these mutant mice at E18.5, although at E15.5 the RBPjk-deficient and RBPjk-deficient Notch GOF mutants exhibit a delayed marrow cavity establishment and transition to bone due to the delayed progression of terminal chondrocyte maturation (data not shown). These data demonstrate that loss of Notch signaling in both the chondrogenic and osteogenic lineages inhibits the maturation of chondrocytes in an RBPjk-dependent manner, whereas the RBPjk-independent pathway appears to have little effect on chondrocyte maturation when activated in these lineages.

Notch signaling promotes chondrocyte maturation in vitro

In order to study the effects of Notch signaling specifically in the chondrocyte lineage, we cultured primary chondrocytes from wild-type mice treated with DAPT, a small molecule inhibitor of the gamma-secretase complex that blocks the final Notch cleavage event and subsequent release of the NICD, resulting in the loss of all Notch signaling. Primary chondrocytes were plated in monolayer and cultured for a 7-day timecourse to assess maturation in the presence or absence of DAPT. Staining for AP at 5 and 7 days post-culture revealed a decrease in activity within DAPT-treated cultures (Fig. 2Aa). Real-time RT-PCR analysis for chondrocyte maturation genes *Col2a1*, *Col10a1* and *Mmp13* were conducted to confirm the AP staining results (Fig. 2Ab-d). At both 5 and 7 days post-culture, the immature chondrocyte marker gene, *Col2a1*, was significantly increased in DAPT cultures relative to control levels (Fig. 2Ab). This inversely correlates with the hypertrophic marker, *Col10a1*, which was significantly decreased relative to control cells (Fig. 2Ac) at the same time points. Furthermore, expression levels for the terminally hypertrophic marker gene, *Mmp13*, in DAPT-treated cells was approximately threefold lower at 7 days compared with control cells (Fig. 2Ad).

We next isolated primary chondrocytes from *Rosa-NICD^{f/f}* pups and treated with either control adenovirus expressing GFP (Ad5-CMV-GFP) or adenovirus expressing Cre recombinase (Ad5-CMV-Cre) in order to activate Notch signaling (Fig. 2B). Over the course of a 10-day maturation assay we observed accelerated chondrocyte maturation in Cre-recombinase-infected cells, based on levels of AP staining (Fig. 2Ba). Expression of *Col2a1* was significantly decreased in NICD overexpressing cultures relative to controls at 6 days post-culture (Fig. 2Bb). This is in agreement with the *Col10a1* (Fig. 2Bc) data, which showed significant increases in the NICD overexpressing cultures at both 6 and 10 days. *Mmp13* (Fig. 2Bd) was also significantly increased at the indicated time points. These data indicate that Notch signaling induces chondrocyte maturation in vitro.

Finally, to understand the role of RBPjk-dependent Notch signaling in chondrocyte maturation, we harvested primary chondrocytes from *Rbpjk^{f/f}* pups and infected the cells with Ad5-CMV-GFP or Ad5-CMV-Cre adenoviruses (Fig. 2C). Over the course of a 7-day maturation assay the Cre-infected cells exhibited reduced levels of AP staining (Fig. 2Ca). At 5 days post-culture, *Col2a1* levels (Fig. 2Cb) were significantly increased over GFP-controls, which is in agreement with the significantly reduced levels of *Col10a1* (Fig. 2Cc) at this time point. Levels of *Mmp13* (Fig. 2Cd) are significantly reduced at both time points tested. These data are consistent with those obtained by blocking all Notch signaling with DAPT in wild-type cells, and therefore provide the first in vitro evidence that Notch signaling promotes chondrocyte maturation, likely via the RBPjk-dependent pathway. In all cases, based on RT-PCR, the levels of expression for the RBPjk-dependent Notch target gene, *Hes1*, was altered in a manner consistent with those expected from the aforementioned Notch perturbations (Fig. 2Ae,Be,Ce).

RBPjk-dependent Notch signaling promotes the onset of chondrocyte maturation, and in part, the terminal maturation of chondrocytes in a cartilage-specific manner

To determine the cartilage-specific role of Notch signaling during endochondral bone development in vivo, we used the inducible *Col2Cre^{ERT2}* transgenic mouse line to target recombination of the general Notch GOF (*Rosa-NICD^{f/f}*), RBPjk-deficient (*Rbpjk^{f/f}*) and RBPjk-deficient Notch GOF (*Rosa-NICD^{f/f};Rbpjk^{f/f}*) floxed alleles specifically to the committed chondrocyte population by injecting tamoxifen after E12.5 (Fig. 3, supplementary material Fig. S1). Histological examination of the humeri of cartilage-specific *Rosa-NICD^{f/f}* GOF mutants at E15.0 revealed the formation of an early rudimentary marrow cavity – which is not evident in controls (Fig. 3Aa,b). Further, in situ hybridization for *Ihh* expression indicated a wider spacing between the primary zones of expression, as well as expanded domains of expression in the cartilage-specific *Rosa-NICD^{f/f}* GOF mutants compared with controls (yellow arrowheads, Fig. 3Ac,d). The expression domain of *Col10a1* was separated in the cartilage-specific *Rosa-NICD^{f/f}* GOF mutants highlighting the formation of the marrow cavity (orange bracket, Fig. 3Af), whereas control sections maintained a continuous domain of *Col10a1* expression (Fig. 3Ae). Finally, the *Mmp13* expression domain of cartilage-specific *Rosa-NICD^{f/f}* GOF mutants is wider than that of the controls at E15.0, indicating an expanded domain of terminally differentiated chondrocytes and an early infiltration of osteogenic cells (yellow arrows, Fig. 3Ag,h). Examination of the humeri of E15.5 cartilage-specific RBPjk-deficient and RBPjk-deficient Notch GOF mutant mice revealed a significantly smaller marrow

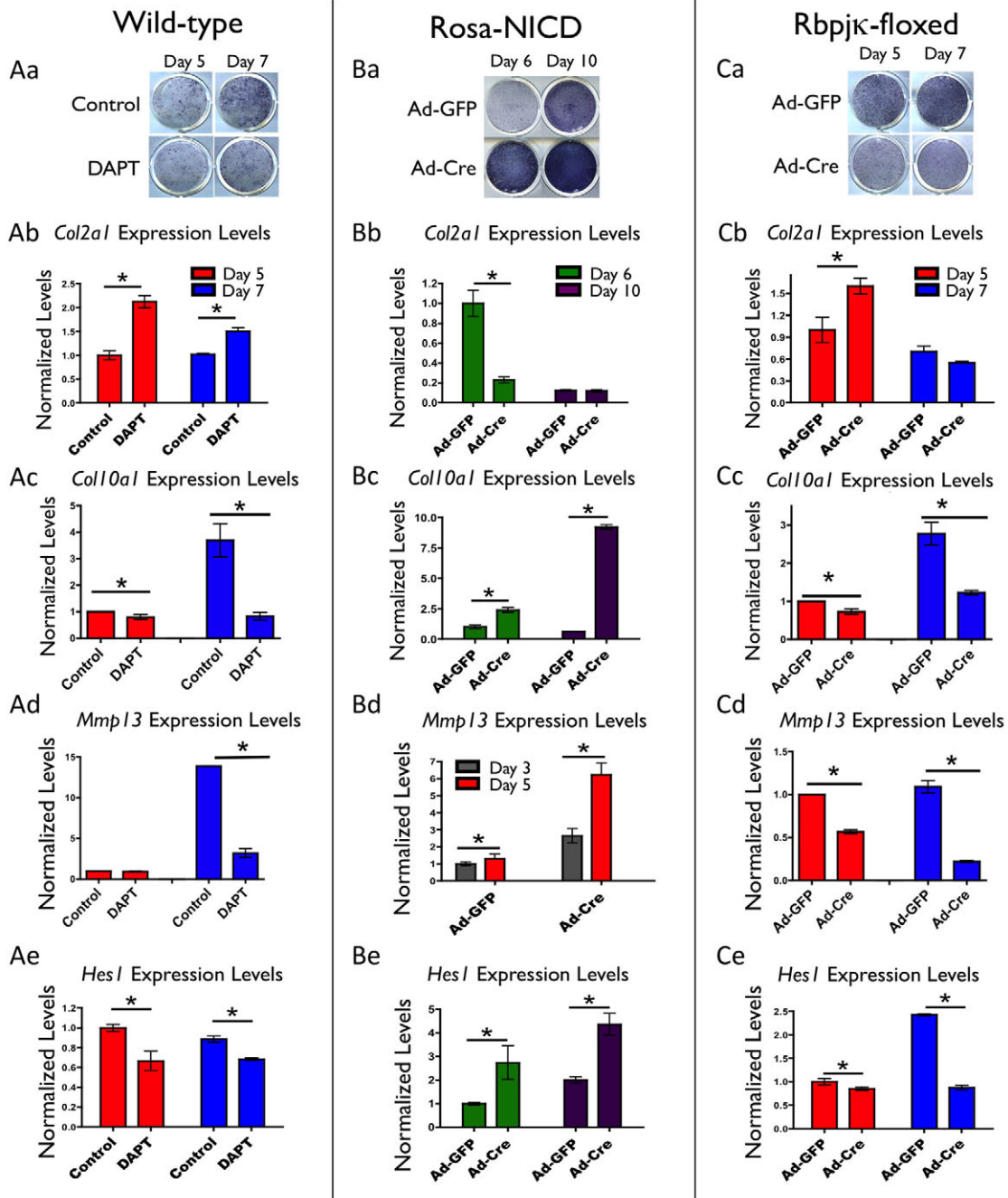


Fig. 2. Notch-mediated effects on chondrocyte maturation occur in a chondrocyte-specific manner in vitro. (A) (a) AP staining of control treated or DAPT-treated wild-type primary chondrocytes. (b,c,d,e) Real-time RT-PCR results for *Col2a1* (b), *Col10a1* (c), *Mmp13* (d) and *Hes1* (e). (B,C) (Ba,Ca) AP staining of control infected or Ad-CMV-Cre infected *Rosa-NICD^{f/+}* (Ba) or *Rbpjk^{fl/fl}* (Ca) primary chondrocytes. (Bb-e,Cb-e) Real-time RT-PCR results for *Col2a1* (Bb,Cb), *Col10a1* (Bc,Cc), *Mmp13* (Bd,Cd) and *Hes1* (Be,Ce). The y-axis of graphs is relative gene expression normalized to *Actb* and to the control at the earliest time point indicated. *, $P < 0.05$.

cavity by H&E staining (Fig. 3Ba-c, red dotted lines delineate the marrow cavity) compared with controls. Further molecular analyses revealed that the major domains of *Ihh* expression were localized closer together in both the cartilage-specific RBPjk-deficient and the RBPjk-deficient Notch GOF mutants (yellow arrowheads, Fig. 3Bd-f), as were the *Col10a1* expression domains (orange brackets, Fig. 3Bg-i). The *Mmp13* expression domain was reduced in each of the two mutants compared with controls (yellow arrows, Fig. 3Bj-l). Together, these data indicate that

overexpression of NICD in the presence of RBPjk can accelerate chondrocyte maturation. Furthermore, we determined that the Notch-mediated maturational effects are governed solely via the RBPjk-dependent pathway, as the RBPjk-deficient Notch GOF mutant phenocopies the RBPjk-deficient mutant.

When examining the cartilage-specific Notch mutants at E18.5 (Fig. 3C,D) we found that the *Rosa-NICD^{f/+}* GOF mutants (Fig. 3Cb,f,j,n) had a decrease in the overall length of the hypertrophic zone compared with controls (Fig. 3Ca,e,i,m), suggesting an

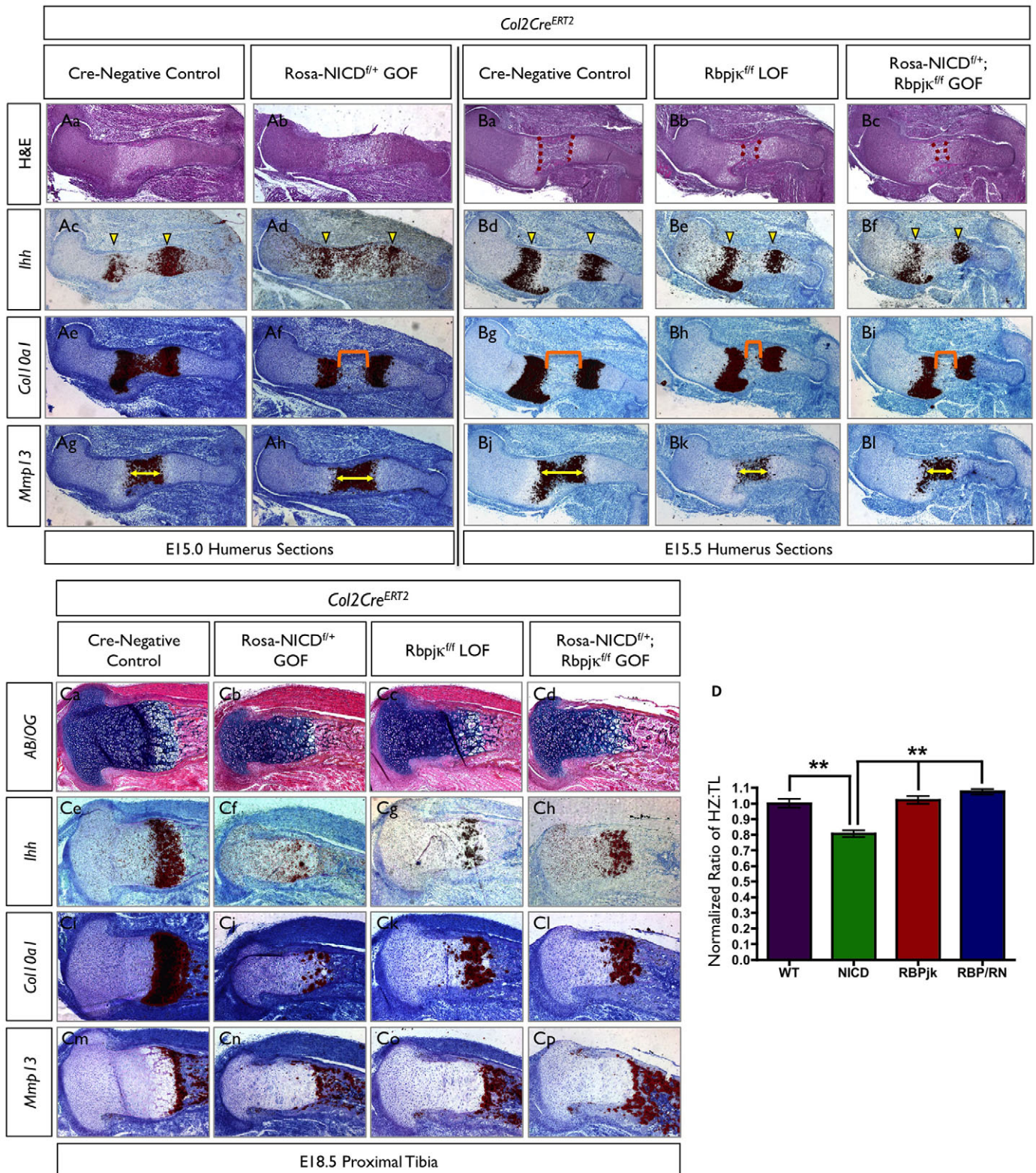


Fig. 3. Cartilage-specific RBPjk-dependent Notch signaling promotes chondrocyte maturation. (A,B) (Aa,b,Ba-c) H&E staining of control, *Col2Cre^{ERT2};Rosa-NICD^{fl/+}* (*Rosa-NICD^{fl/+}* GOF), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) and *Col2Cre^{ERT2};Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) embryonic humerus sections at E15.5. (Ac-h,Bd-l) In situ hybridization for *Ihh* (Ac,d,Bd-f), *Col10a1* (Ae,f,Bg-i) and *Mmp13* (Ag,h,Bj-l). (C) (a-d) H&E staining of control, *Rosa-NICD^{fl/+}* GOF, *Rbpjk^{fl/fl}* LOF and *Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF embryonic tibia sections at E18.5. (e-p) In situ hybridization for *Ihh* (e-h), *Col10a1* (i-l) and *Mmp13* (m-p). (D) The ratio of the hypertrophic zone to total growth plate length of E18.5 tibia sections. Yellow arrowheads: *Ihh*-expressing domains. Yellow double-headed arrows: *Mmp13* expression domains. Orange brackets: distance between *Col10a1* domains. Dashed red contours: chondro-osseous junction. **, $P < 0.01$.

acceleration in the progression to terminal chondrocyte hypertrophy. When quantified (Fig. 3D) we identified a 20% decrease in the ratio of hypertrophic zone to total length (HZ:TL) in the Rosa-NICD^{f/+} GOF mutant samples. Surprisingly, when we examined the cartilage-specific RBPjk-deficient (Fig. 3Cc,g,k,o) and RBPjk-deficient Notch GOF (Fig. 3Cd,h,l,p) E18.5 mutants we did not observe an expansion of the hypertrophic zone, which was predicted based on the delayed onset of maturation seen in the E14.5 embryos (Fig. 3B) and the expanded hypertrophic zones identified at E18.5 in mutants where the Notch pathway mutations targeted both the chondrocyte and osteoblast lineages (Fig. 1B,C) (Hilton et al., 2008; Mead and Yutzey, 2009). The fact that the HZ:TL ratio of the RBPjk-deficient Notch GOF mice were similar to RBPjk-deficient and wild-type mice, and not similar to the Rosa-NICD^{f/+} GOF mice, further supports the data that the ability of NICD to induce accelerated maturation is contingent on RBPjk-dependent Notch signaling. These data demonstrate that terminal chondrocyte maturation and turnover of the hypertrophic cartilage matrix is mediated at least in part by RBPjk-dependent Notch signals in chondrocytes, but likely also requires active Notch signals in the developing perichondrium. Interestingly, we observed an increase in *Mmp13* expression by in situ hybridization within the osteogenic lineage of cartilage-specific RBPjk-deficient (Fig. 3Co) and RBPjk-deficient Notch GOF mutants (Fig. 3Cp) compared with controls and Rosa-NICD^{f/+} GOF (Fig. 3Cm,n) mutants; suggesting a possible compensatory mechanism leading to increase turnover of the cartilaginous matrix at the chondro-osseous junction.

Cartilage-specific RBPjk-independent Notch signaling affects chondrocyte morphology and enhances perichondrial bone formation

Detailed examination of Masson's-trichrome-stained E18.5 tibia sections from the *Col2Cre*^{ERT2} mutants revealed aberrant chondrocyte morphology in all levels of the growth plate (Fig. 4A) as well as enhanced perichondrial bone formation (Figs 4, 5), which were not observed in *Prx1Cre* mutant mice (Fig. 4B). Inspection of the resting zone of all three cartilage-specific mutants revealed chondrocytes that were larger and more compacted or disorganized compared with controls (orange boxes, Fig. 4Aa-d). The columnar zones (black boxes, Fig. 4Aa-d) were highly disorganized and lacking continuous normal stacks of proliferating chondrocytes. In place of traditional columns, groups of clonally proliferating chondrocytes interspersed with larger chondrocytes of aberrant morphology were observed. While the growth plate was highly disorganized, the clones of proliferating cells did not appear to lose their polarity, as indicated by the long axis of the cells maintaining a perpendicular orientation with respect to the growth axis. In the hypertrophic zone (green boxes, Fig. 4Aa-d) there appeared to be a decrease in the cellularity of all three mutants compared with controls, whereas the hypertrophic cells present were larger and morphologically aberrant compared with normal hypertrophic zone chondrocytes. Interestingly, when these same genetic perturbations in Notch signaling were induced in MPCs (*Prx1Cre*), we did not observe any significant change in chondrocyte morphology, polarity or size throughout any zone of the growth plate at E18.5. The only morphologically distinct feature of the *Prx1Cre* mutant mice at this stage was the persistence of the hypertrophic zone and a slightly thickened bone collar surrounding the most distal regions of the persistent hypertrophic cartilage (Fig. 4B).

In addition to the cartilage phenotypes observed in the E18.5 tibia sections from the *Col2Cre*^{ERT2} mutants, we observed the presence of enhanced perichondrial bone formation in cartilage-

specific Rosa-NICD^{f/+} GOF, RBPjk-deficient and RBPjk-deficient Notch GOF mutant sections, which was not observed in the controls (yellow box, Fig. 4Aa-d). Masson's trichrome staining revealed a significantly wider region of perichondrial bone and a greater number of cuboidal osteoprogenitor cells in the cartilage-specific Rosa-NICD^{f/+} GOF (Fig. 5B), RBPjk-deficient (Fig. 5C) and RBPjk-deficient Notch GOF (Fig. 5D) mutant sections compared with controls (Fig. 5A). The perichondrial bone in these embryos also extends significantly above the hypertrophic zone of chondrocytes, the point at which perichondrial bone formation is normally observed. In situ hybridization revealed increased and advanced domains of *Coll1a1* (Fig. 5E-H) and osteocalcin (*Oc*; *Bglap* – Mouse Genome Informatics) (Fig. 5I-L) expression in the three mutants. These analyses are consistent with the morphological changes associated with the perichondrial cells as they transition from a flat and fibroblastic appearance (inactive lining cell morphology) to the more cuboidal shape associated with active and functionally mature osteoblasts. Based on these histological and molecular analyses, we were able to determine that the cartilage-specific RBPjk-deficient Notch GOF mutant (Fig. 5D,H,L) mice have the greatest and most consistent increase in perichondrial bone formation. The significant advances and robust thickening in perichondrial bone formation were not observed in our *Prx1Cre* Notch mutants, although both RBPjk-deficient and RBPjk-deficient Notch GOF mutants show slight thickening of the bone collar surrounding the persistent terminal hypertrophic cartilage (Fig. 4Ba-c). Additionally, TRAP staining was performed to determine whether a decrease in osteoclasts could be responsible for the excessive amounts of perichondrial bone (supplementary material Fig. S3B). Interestingly, our results indicated a significant increase in the number of osteoclasts within the perichondrial and trabecular bone regions. Therefore, these data indicate that alterations in Notch signaling specifically in chondrocytes can lead to impaired communication between the chondrocytes and perichondrium, resulting in the inability to maintain normal organization and morphology of the growth plate chondrocytes and a cell-non-autonomous enhancement in perichondrial osteoblast differentiation and bone formation. Finally, our data suggest that the Notch signal(s) that coordinate perichondrial bone development with growth plate development is/are likely to be RBPjk-independent in nature, as the RBPjk-deficient Notch GOF mutant had the most significant and consistent enhancement in perichondrial bone formation.

Although the mechanisms for this Notch cell-non-autonomous regulation of perichondrial bone formation is unclear, we did assess alterations to IHH signaling, because it is the preeminent cartilage-derived mediator of chondrocyte proliferation, differentiation and perichondrial bone formation (Chung et al., 2001). Tibial sections from each of the three cartilage-specific Notch mutants exhibited a reduction in the number of *Ihh* expressing prehypertrophic and hypertrophic chondrocytes by E18.5. Interestingly, IHH responsiveness throughout the columnar chondrocytes of the cartilage-specific Notch mutants was dramatically reduced as gauged by *Ptc1* (*Ptch1* – Mouse Genome Informatics) expression, a direct transcriptional target of IHH signaling (Fig. 6E-L). Furthermore, the perichondrial cells surrounding the Notch mutant cartilage showed a significant increase in *Ptc1* expression (Fig. 6E-L). In contrast to the columnar zone reduction in *Ihh* responsiveness, periarticular chondrocytes of the cartilage-specific Notch mutants exhibited a robust enhancement in *Pthrp* (*Pthlh* – Mouse Genome Informatics) expression, with the cartilage-specific RBPjk-deficient Notch GOF mutants (Fig. 6M-P) displaying the

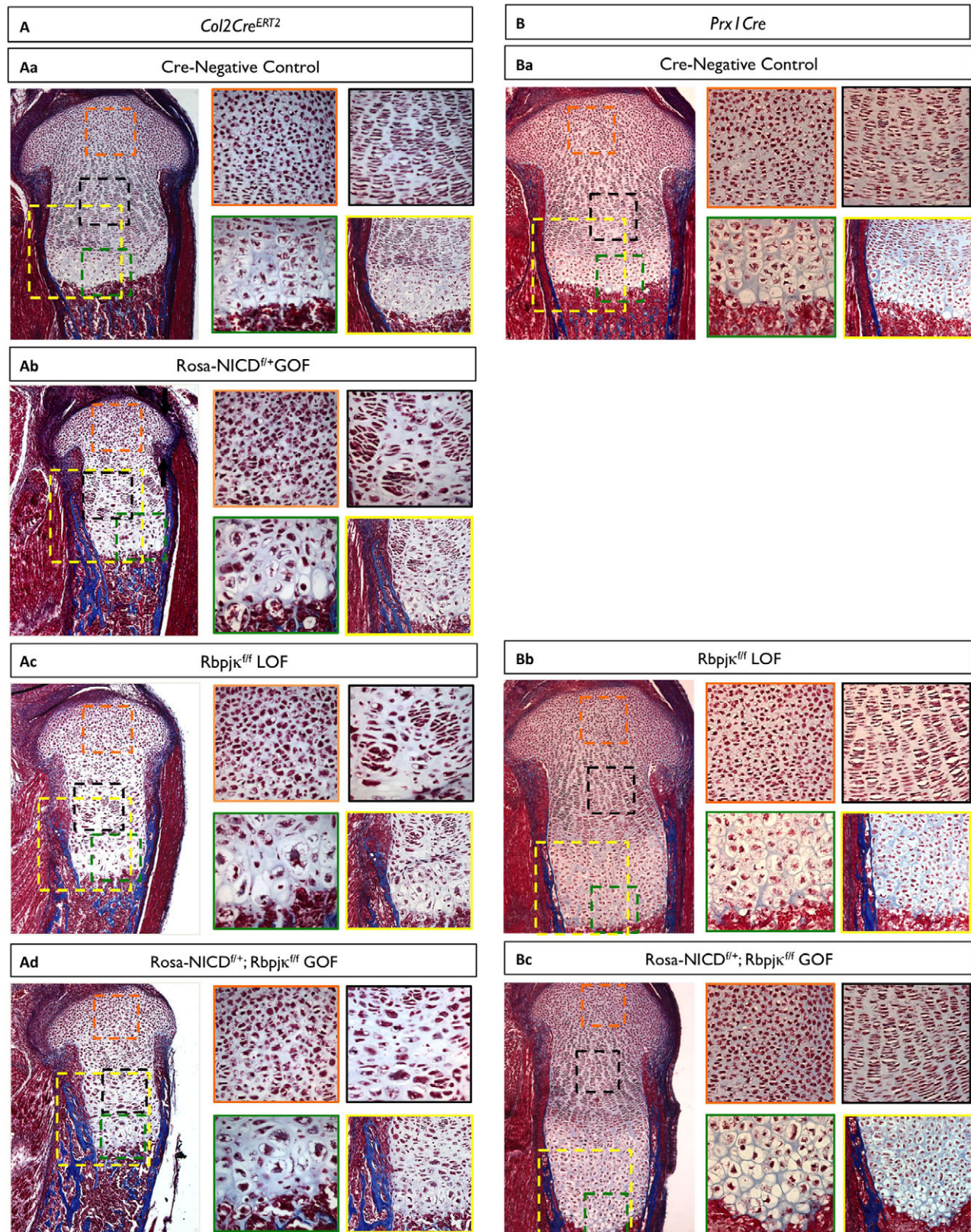


Fig. 4. Cellular architecture of endochondral bones from cartilage-specific (*Col2Cre^{ERT2}*) and MPC-targeted (*Prx1Cre*) Notch mutant mice. (A) Masson's trichrome staining of control (a), *Col2Cre^{ERT2};Rosa-NICD^{fl/+}* (*Rosa-NICD^{fl/+}* GOF) (b), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (c) and *Col2Cre^{ERT2};Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (d) embryonic tibia sections at E18.5. (B) Masson's trichrome staining of wild-type (a), *Prx1Cre;Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (b) and *Prx1Cre;Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (c) embryonic tibia sections at E18.5. Orange box: 40× magnification of resting zone; black box: 40× magnification of columnar zone; green box: 40× magnification of hypertrophic zone; yellow box: 20× magnification of perichondrium.

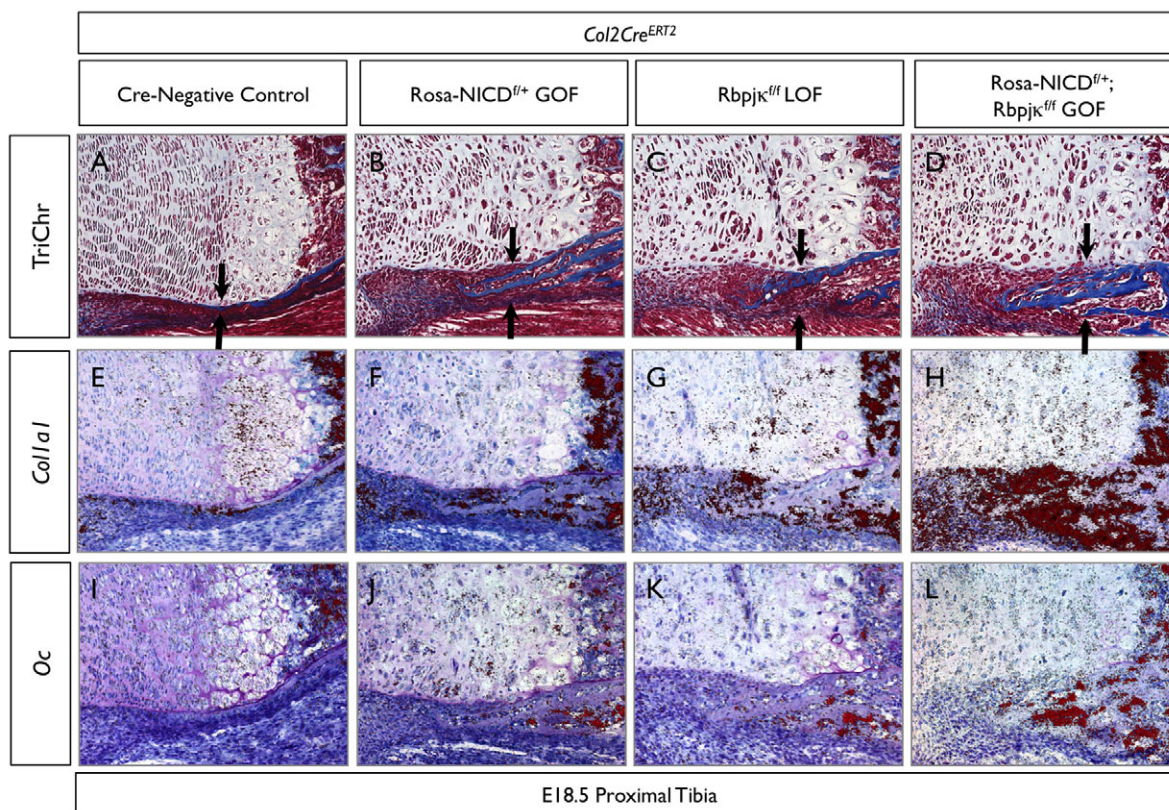


Fig. 5. Cartilage-specific RBPjk-independent Notch signaling enhances perichondrial bone formation. (A–D) Masson's trichrome staining of control (A), *Col2Cre^{ERT2};Rosa-NICD^{fl/+}* (*Rosa-NICD^{fl/+}* GOF) (B), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (C) and *Col2Cre^{ERT2};Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (D) embryonic tibia sections at E18.5. (E–L) In situ hybridization for *Col1a1* (E–H) and *Oc* (I–L). Space between black arrows: thickness of the perichondrial bone (stained blue).

largest increase in periarticular *Pthrp* expression. As the mutant mice generated using the *Prx1Cre* transgene did not share similar patterns of expression for *Ihh*, *Ptc1* and *Pthrp*, these data suggest that changes in IHH signaling may require the uncoupling of Notch signals between the cartilage and overlying perichondrium (data not shown).

Cartilage-specific RBPjk-independent Notch signaling suppresses chondrocyte proliferation and induces immature chondrocyte apoptosis

Finally, we assessed alterations in chondrocyte proliferation and apoptosis in both the *Col2Cre^{ERT2}* and the *Prx1Cre* mutants to determine whether Notch signals regulate these processes during cartilage development. We performed BrdU labeling experiments to determine how altering Notch signals in committed chondrocytes (Fig. 7A,B) compared with MPCs (Fig. 7C,D) affects chondrocyte proliferation. At E15.5 (Fig. 7A) proximal humerus sections from cartilage-specific *Rosa-NICD^{fl/+}* GOF and RBPjk-deficient mutants both displayed a significant decrease (~40%) in BrdU-positive staining cells compared with controls. This was exacerbated in the cartilage-specific RBPjk-deficient Notch GOF sections, which displayed a decrease in BrdU-positive staining cells of approximately 50%. The trend is also seen in E18.5 sections (Fig. 7B), where again there is a decrease in BrdU-positive cells seen in the cartilage-specific *Rosa-NICD^{fl/+}* GOF and RBPjk-deficient mutants and a significant decrease by approximately 50% was observed in RBPjk-deficient Notch GOF sections. At both E15.5 and E18.5 the RBPjk-deficient Notch GOF embryos show a

more robust decrease in proliferation than the *Rosa-NICD^{fl/+}* GOF and RBPjk-deficient mutants, suggesting that the primary Notch-mediated mechanism governing proliferation in chondrocytes may be RBPjk-independent in nature. Interestingly, when examining the *Prx1Cre* mutants at E14.5 (Fig. 7C) we found that both the RBPjk-deficient and RBPjk-deficient Notch GOF embryos had reductions in proliferation approaching 20%, whereas at E18.5 (Fig. 7D) both mutants had increased proliferation of approximately 20% of the columnar chondrocyte population. Collectively, these data support a role for RBPjk-dependent Notch signaling in the perichondrium that indirectly inhibits chondrocyte proliferation during later development, while cartilage-specific RBPjk-independent Notch signals may suppress chondrocyte proliferation in a cell-autonomous manner throughout cartilage development.

We also performed TUNEL labeling experiments to detect any apoptotic changes in mutant chondrocytes in E18.5 proximal tibia sections. When we assessed the cartilage-specific Notch mutants for TUNEL staining we found that all three mutants had at least a 150-fold increase in the number of TUNEL-positive cells (Fig. 8Ae). Furthermore, apoptotic cells were spread throughout all regions of the growth plate instead of their normal localization in the deepest layers of the hypertrophic zone (Fig. 8Aa–d). Interestingly, analyses of *Prx1Cre*-mediated RBPjk-deficient mutants did not result in any significant changes in chondrocyte apoptosis compared with controls (Fig. 8Aa,b). However, the *Prx1Cre*-mediated RBPjk-deficient Notch GOF mutants had a significant increase (~twofold) in the number of apoptotic cells (Fig. 8Ba–d), with a normal localization pattern in the terminal hypertrophic zone. These data indicate a

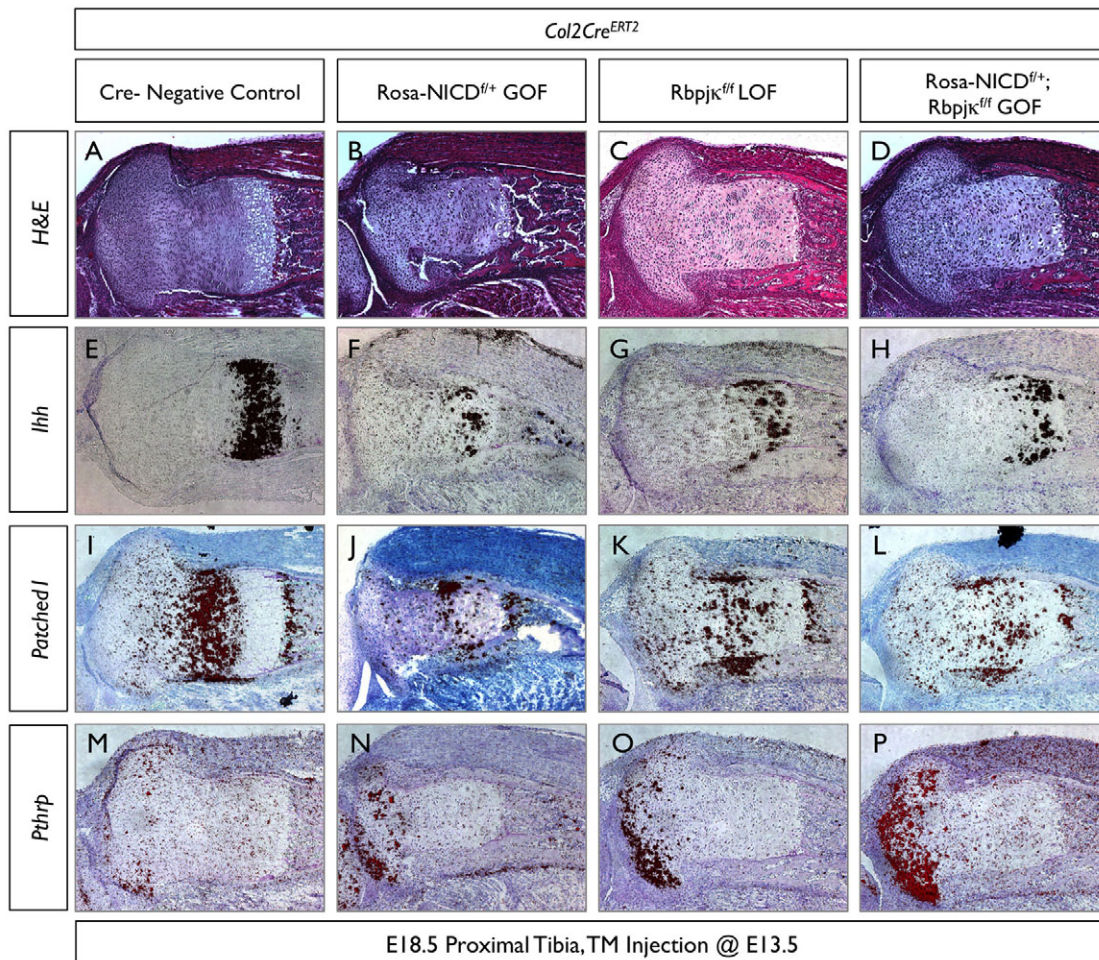


Fig. 6. Cartilage-specific RBPjk-independent Notch signaling alters IHH signaling and responsiveness. (A-D) H&E staining of control (A), *Col2Cre^{ERT2};Rosa-NICD^{fl/+}* (*Rosa-NICD^{fl/+}* GOF) (B), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (C) and *Col2Cre^{ERT2};Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (D) embryonic tibia sections at E18.5. (E-P) In situ hybridization for *Ihh* (E-H), *Ptc1* (I-L) and *Pthrp* (M-P).

potential role for the RBPjk-independent pathway in regulating hypertrophic cell death. Collectively, these data demonstrate that uncoupling Notch signals between the chondrogenic and osteogenic cell populations by utilizing the temporal and cartilage-specific *Col2Cre^{ERT2}* transgene leads to severe chondrocyte cell death throughout the developing cartilage.

DISCUSSION

In this study, we provided the first conclusive genetic data demonstrating that cartilage-specific RBPjk-dependent Notch signaling promotes the onset and progression of chondrocyte maturation using both in vivo and in vitro GOF and LOF approaches. These data also uncovered the previously unknown and crucial role that cartilage-specific Notch signaling plays in coordinating perichondrial osteoblast differentiation and bone formation. Furthermore, the RBPjk-independent Notch signaling pathway is implicated as a potential coupling mechanism in the communication between chondrocytes and perichondrial osteoblasts, and in the regulation of chondrocyte proliferation and apoptosis; a mechanism that likely involves IHH signaling.

The exact role for Notch in regulating chondrogenesis and chondrocyte maturation in vivo has been controversial, as multiple conditional genetic mouse models have produced some apparently

conflicting results. For example, when Notch signaling is activated in early cartilage progenitor cells of the chick limb using a *DIII* replication-competent retrovirus, the maturation and hypertrophic differentiation program of chondrocytes was inhibited (Crowe et al., 1999). Genetic studies using Notch GOF approaches in the mouse later demonstrated a similar effect on inhibiting chondrocyte and osteoblast maturation when Notch was constitutively activated in COP cells (Dong et al., 2010; Mead and Yutzey, 2009). Furthermore, Dong et al. (Dong et al., 2010) showed that Notch activation in MPCs inhibits all skeletal cell differentiation in an RBPjk-dependent manner, whereas inactivation of RBPjk-dependent Notch signaling in MPCs accelerated MPC differentiation. Mead and Yutzey (Mead and Yutzey, 2009) also performed LOF experiments in which *Rbpjk* floxed alleles were removed from COPs using the standard *Col2Cre* transgene, which demonstrated an increase in committed chondrocyte proliferation and an early acceleration in chondrocyte hypertrophy, followed by delayed progression to terminal maturation. These data were in contrast to a previous study by Hilton et al. (Hilton et al., 2008), which demonstrated that MPC-specific loss of ‘upstream’ Notch signaling components (*Psen1* and *Psen2* or *Notch1* and *Notch2*), and therefore both RBPjk-dependent and -independent Notch signaling, led to decreased chondrocyte proliferation and a delay in both the onset and progression of chondrocyte maturation.

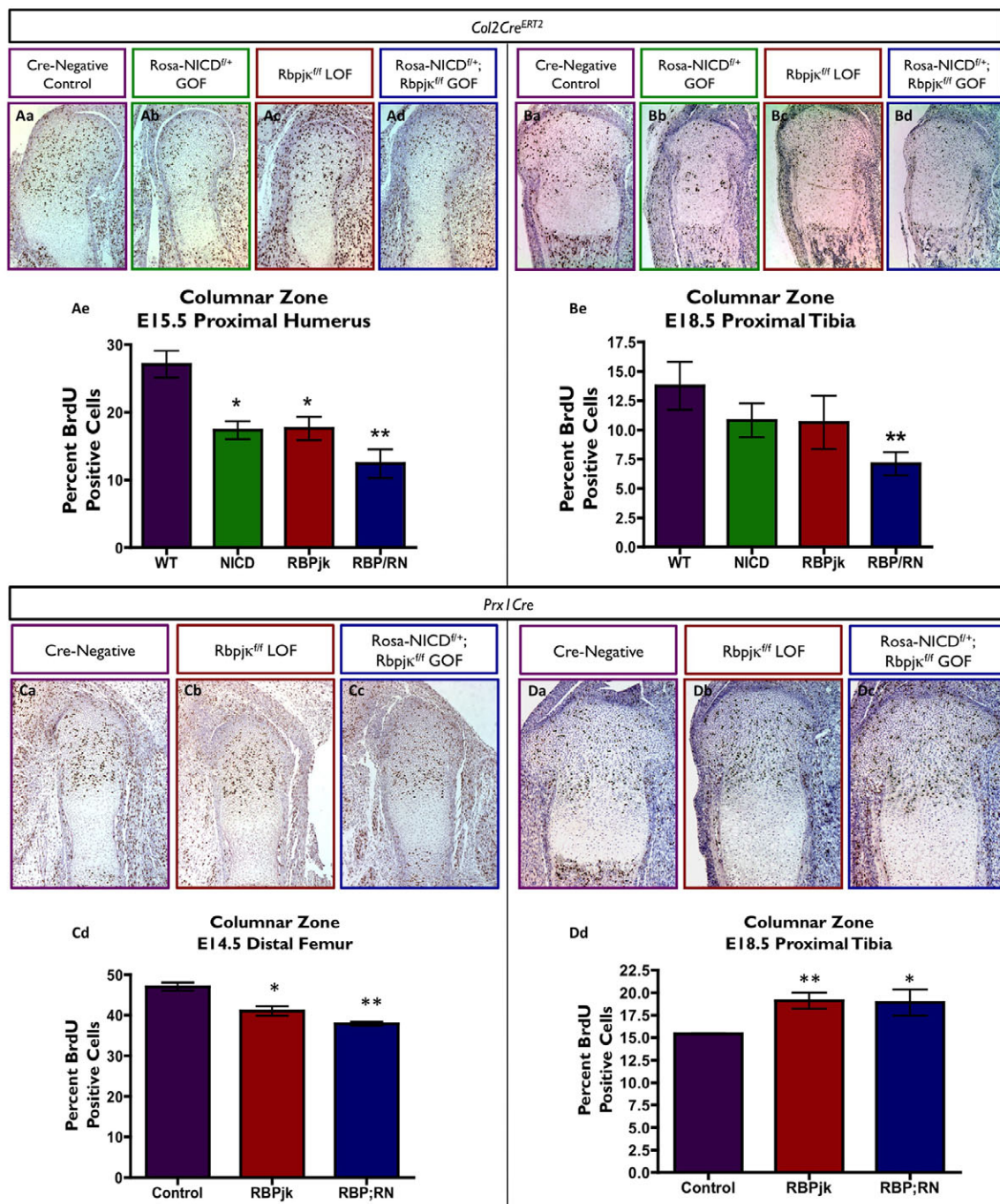


Fig. 7. Cartilage-specific RBPjk-independent Notch signaling decreases chondrocyte proliferation. (A) BrdU staining of control (a), *Col2Cre^{ERT2};Rosa-NICD^{fl/+}* (*Rosa-NICD^{fl/+}* GOF) (b), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (c), and *Col2Cre^{ERT2};Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (d) embryonic humerus sections at E15.5. (B) BrdU staining of control (a), *Rosa-NICD^{fl/+}* GOF (b), *Rbpjk^{fl/fl}* LOF (c), and *Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF (d) embryonic tibia sections at E18.5. (C) BrdU staining of control (a), *Prx1Cre;Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (b) and *Prx1Cre;Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF) (c) embryonic femur sections at E14.5. (D) BrdU staining of control (a), *Rbpjk^{fl/fl}* LOF (b) and *Rosa-NICD^{fl/+};Rbpjk^{fl/fl}* GOF (c) embryonic tibia sections at E18.5. (Ae,Be,Cd,Dd) Quantification of percent BrdU-positively stained cells out of total number of cells in the humeral, femoral or tibial cartilages. *, $P < 0.05$; **, $P < 0.01$.

These apparently conflicting reports suggest that RBPjk-dependent and -independent Notch signals may regulate different aspects of chondrocyte proliferation and maturation or that the timing at which Notch signals are removed or activated in the skeletal lineages (progenitor versus committed cell) differentially affects cartilage development.

Our data demonstrate that RBPjk-dependent Notch signaling in committed chondrocytes promotes both the onset and progression of chondrocyte maturation, although Notch regulation of terminal maturation also appears to occur via the osteogenic lineage. We compared *Prx1Cre*-mediated RBPjk-deficient and RBPjk-deficient Notch GOF mutant sections to cartilage-specific (*Col2Cre^{ERT2}*)

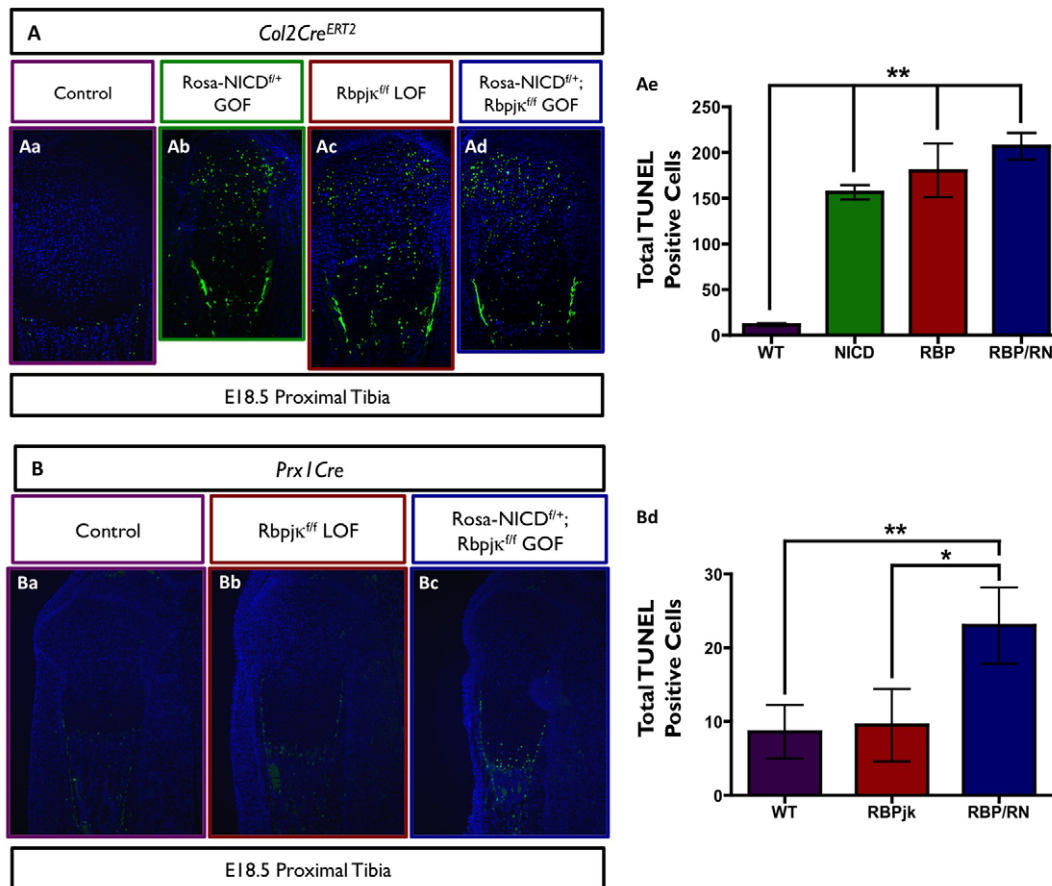


Fig. 8. Cartilage-specific RBPjk-independent Notch signaling increases chondrocyte apoptosis. (A) TUNEL staining of control (a), *Col2Cre^{ERT2};Rosa-NICD^{fl/fl}* (*Rosa-NICD^{fl/fl}* GOF) (b), *Col2Cre^{ERT2};Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (c) and *Col2Cre^{ERT2};Rosa-NICD^{fl/fl};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/fl};Rbpjk^{fl/fl}* GOF) (d) embryonic tibia sections at E18.5. (B) TUNEL staining of control (a), *Prx1Cre;Rbpjk^{fl/fl}* (*Rbpjk^{fl/fl}* LOF) (b) and *Prx1Cre;Rosa-NICD^{fl/fl};Rbpjk^{fl/fl}* (*Rosa-NICD^{fl/fl};Rbpjk^{fl/fl}* GOF) (c) embryonic tibia sections at E18.5. (Ae,Bd) Quantification of the total number of TUNEL-positive cells in the proximal tibia. *, $P < 0.05$; **, $P < 0.01$.

RBPjk-deficient and RBPjk-deficient Notch GOF mutant sections at E14.5, E15.5, and E18.5 for similarities and differences in the onset and progression of chondrocyte maturation. Each mouse model showed similar delays in the onset of chondrocyte maturation at E14.5/E15.5 (Fig. 1A, Fig. 3B, data not shown). Interestingly, the cartilage-specific RBPjk-deficient and RBPjk-deficient Notch GOF mutant sections did not show a phenotype consistent with delayed progression to terminal maturation (a dramatically elongated hypertrophic and terminal hypertrophic cartilage region) at E18.5 (Fig. 1B, Fig. 3C, supplementary material Fig. S2). We therefore speculate that terminal chondrocyte maturation and turnover of the cartilage matrix is regulated at some level via the perichondrial osteoblasts. Previous work has indeed demonstrated that the perichondrium exerts significant negative influence on chondrocyte proliferation and is a positive regulator of chondrocyte terminal maturation (Colnot et al., 2004; Long and Linsenmayer, 1998). Consistent with this, we observed a significant increase in perichondrial and osteoblastic *Mmp13* expression surrounding the terminal hypertrophic cartilage in both our cartilage-specific RBPjk-deficient and RBPjk-deficient Notch GOF mutant sections (Fig. 3Co,p), which may promote turnover of cartilage matrix in these Notch mutant mice. Interestingly, both our cartilage-specific Notch GOF in vivo and in vitro models showed significant acceleration and progression of chondrocyte maturation, an effect opposite to that observed in Notch GOF mutant mice

when Notch was activated in progenitor cell populations (Dong et al., 2010; Mead and Yutzey, 2009). As the chondrocyte maturation phenotypes observed in E14.5 and E18.5 *Prx1Cre;Rbpjk^{fl/fl}* and *Prx1Cre;Rbpjk^{fl/fl};Rosa-NICD^{fl/fl}* mutants (Fig. 1) phenocopy age-matched *Prx1Cre;Notch1^{fl/fl};Notch2^{fl/fl}* and *Prx1Cre;Psen1^{fl/fl};Psen2^{-/-}* mutants (Hilton et al., 2008), the data strongly suggest that the phenotypes generated by removal of *Rbpjk* floxed alleles led to impaired RBPjk-dependent Notch signaling and not enhanced RBPjk-independent Notch signaling. Further, enhancing Notch signals in the absence of *Rbpjk* floxed alleles did not exacerbate the chondrocyte maturation phenotype, indicating that this process is mediated solely via RBPjk-dependent mechanisms. In fact, Notch GOF effects on accelerating chondrocyte maturation were abolished upon removal of *Rbpjk* floxed alleles.

The Notch pathway can control bone formation and osteoblast differentiation in a cell-autonomous and RBPjk-dependent manner, primarily via regulation of osteoblast differentiation from MPCs and in regulating proliferation and differentiation of committed osteoblasts (Engin et al., 2008; Hilton et al., 2008; Tao et al., 2010; Zanotti et al., 2008). Notch signals are propagated not only in a cell-autonomous manner but also via long-range cell-non-autonomous mediated mechanisms, which have been described in various tissues from *Drosophila* to mammals (Fiuza and Arias, 2007). In mouse epidermal lineages, Notch GOF in a single hair follicle lineage can induce aberrant differentiation of neighboring

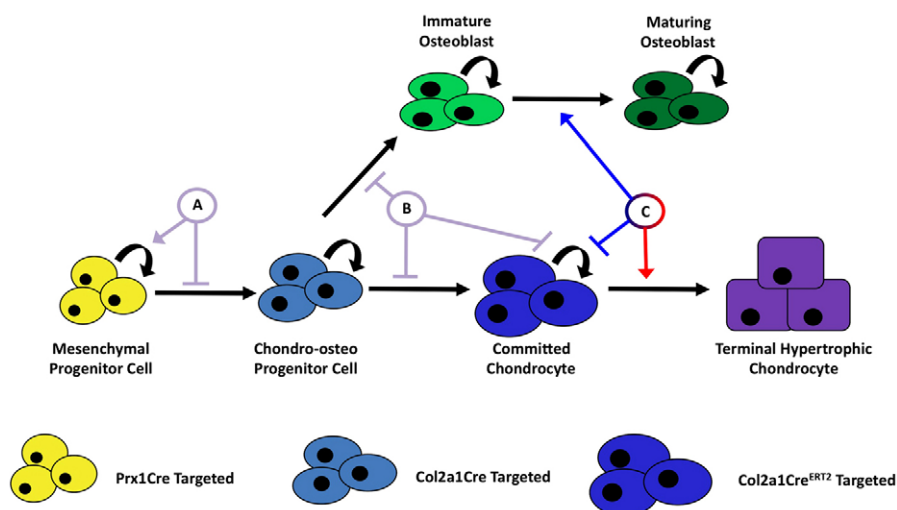


Fig. 9. A unifying model for the Notch regulation of MPC, COP and committed chondrocyte proliferation and differentiation during cartilage development. Cartilage and bone development begins with a common precursor cell, the MPC (yellow). MPCs differentiate into COPs (light blue), which are lineage restricted to adopt either an osteoblastic (light and dark green) or committed chondrocyte (dark blue) cell fate. Arrows indicate induction of proliferation or differentiation; perpendicular lines indicate suppression. (A) RBPjk-dependent regulation of MPC proliferation and differentiation (Dong et al., 2010). (B) Notch regulation of chondrocyte proliferation and COP differentiation (Mead and Yutzey, 2009). (C) Cartilage-specific RBPjk-dependent and -independent Notch signaling effects on chondrocyte maturation, proliferation and perichondrial bone formation (Figs 1-8). RBPjk-dependent Notch functions: red line/arrow. RBPjk-independent Notch functions: blue lines/arrows.

cell types with little cell-cell interaction (Lin et al., 2000), whereas Notch1 activation in post-mitotic keratinizing nail cells stimulates the proliferation of basally located matrix cells via long-range signals (Lin and Kopan, 2003). In each of these examples it is unclear whether the long-range cell-non-autonomous Notch effects are mediated via RBPjk-dependent or -independent signaling mechanisms. Consistent with these types of cell-non-autonomous functions for Notch signaling, we have demonstrated that alterations in cartilage-specific Notch signals can exert long-range cell-non-autonomous effects promoting perichondrial osteoblast differentiation and bone formation. As each of our cartilage-specific Notch mutant mice exhibit enhanced perichondrial bone formation, with the RBPjk-deficient Notch GOF mouse displaying the most robust effect, the long-range cell-non-autonomous effects of Notch on bone formation appear to be coordinated in an RBPjk-independent manner. In this context, we hypothesize that loss of the *Rbpjk* floxed alleles in our cartilage-specific RBPjk-deficient mutant not only prevents NICD from signaling via RBPjk, but frees it from its highest affinity binding partner to associate with and signal through RBPjk-independent signaling mechanisms (Kopan and Ilagan, 2009). Therefore, our data suggest that RBPjk-independent signaling specifically in chondrocytes can induce a paracrine signal(s) that elicits osteoblastic differentiation and a bone formation response from surrounding perichondrial osteoprogenitors. Alternatively, it remains possible that both cartilage-specific RBPjk-dependent and -independent mechanisms regulate these processes in an additive manner, such that RBPjk may suppress certain genes when not engaged with NICD, and that this suppression is important for normal cartilage and bone regulation.

The Notch signaling pathway can elicit both positive and negative effects on cell proliferation and survival in a context-dependent manner. Our data demonstrate that cartilage-specific RBPjk-independent Notch signaling inhibits chondrocyte proliferation throughout cartilage development from E14.5 to

E18.5. Interestingly, removal of *Rbpjk* floxed alleles or activation of RBPjk-independent Notch signaling in both chondrocytes and perichondrial osteoblasts inhibited chondrocyte proliferation at E14.5, while enhancing proliferation at E18.5. We speculate that this increase in chondrocyte proliferation is likely to be due to the increase in the number of *Ihh*-expressing cells at this time (Fig. 1B), which may overcome the negative effect of RBPjk-independent Notch signaling on chondrocyte proliferation. It also remains possible that Notch signals in the perichondrium differentially regulate proliferation in the developing cartilage. Although the perichondrium is required for normal chondrocyte organization and growth of the cartilage, it has also been well documented as an inhibitor of chondrocyte proliferation (Colnot et al., 2004; Long and Linsenmayer, 1998). Interestingly, each one of our cartilage-specific Notch mutants, which probably results in aberrant or enhanced RBPjk-independent Notch signaling effects, displays aberrant columnar zone chondrocyte morphology and impaired cell survival. We interpret these effects as being caused by an uncoupling of normal Notch signals between chondrocytes and the perichondrium. Alternatively, since we did observe some mosaic recombination in our cartilage-specific mutant mice (supplementary material Fig. S1), it is also possible that the columnar disorganization, aberrant chondrocyte morphology, and immature chondrocyte apoptosis was caused by disparate Notch signaling among neighboring chondrocytes.

The IHH/PTHrP signaling pathway is a crucial regulator of chondrocyte morphology, proliferation and maturation, and a requisite and potent inducer of perichondrial osteoblast differentiation and bone formation (Chung et al., 2001; Kronenberg, 2003). As IHH is a cartilage-derived paracrine factor that signals to both chondrocytes and osteoblasts coordinating crucial proliferation and differentiation steps during bone development, we analyzed *Ihh* signaling and responsiveness in our cartilage-specific Notch mutants. Each mutant displayed a strong impairment in columnar zone

chondrocyte responsiveness to IHH (decreased *Ptc1* expression) and exhibited aberrant morphology and organization, whereas periarticular chondrocytes demonstrated enhanced *Pthrp* expression and a more normal appearance (Fig. 6). Additionally, perichondrial osteoblasts showed a significant increase in IHH responsiveness that coincided with the advanced osteoblast differentiation and bone formation phenotype. Interestingly, a similar cellular and molecular phenotype has been reported when *Smoothed* (*Smo*) floxed alleles were conditionally removed in a cartilage-specific manner similar to the methodology used for generating our Notch mutant mice (Hilton et al., 2007). When IHH responsiveness was decreased in chondrocytes in this model, the cartilage-specific *Smo* mutants exhibited columnar chondrocytes with abnormal morphology and organization with decreased *Ptc1* expression, increased periarticular *Pthrp* expression due to enhanced IHH diffusion through the columnar zone, and enhanced *Ptc1* expression in perichondrial osteoblasts (Hilton et al., 2007). Collectively, these data suggest an IHH-mediated mechanism by which cartilage-specific RBPjk-independent Notch signaling disrupts normal columnar chondrocyte organization and morphology, inhibits columnar chondrocyte proliferation and induces premature and enhanced perichondrial bone formation.

We have therefore identified a unifying model in which Notch functions within the chondrocyte lineage from MPCs to terminally hypertrophic chondrocytes during cartilage and bone development (summarized above and represented graphically in Fig. 9). Of note, this model demonstrates that Notch regulation of cell proliferation and terminal maturation are not generalizable concepts, as the Notch pathway plays a context dependent function in different cell types and at different time points within a cell lineage. This is highlighted by the fact that activation of the Notch pathway in committed osteoblasts, which leads to enhanced proliferation and inhibition of osteoblast terminal maturation (Tao et al., 2010), results in a general cellular phenotype opposite to that when Notch is activated in committed chondrocytes. These data also provide the first evidence for long-range cell-non-autonomous functions of the Notch pathway in cartilage and bone development, and implicates RBPjk-independent Notch signaling in mediating some of these effects. Finally, we have identified regulation of Hedgehog responsiveness as one of the first potential mechanisms by which RBPjk-independent Notch signaling may function, at least in the context of endochondral bone development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at

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