HES factors regulate specific aspects of chondrogenesis and chondrocyte hypertrophy during cartilage development

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Abstract

RBPjk-dependent Notch signaling regulates multiple processes during cartilage development including chondrogenesis, chondrocyte hypertrophy, and cartilage matrix catabolism. Select members of the HES/HEY family of transcription factors are recognized Notch signaling targets that mediate specific aspects of Notch function during development. However, whether particular HES/HEY factors play any role(s) in the processes of cartilage development is unknown. Here, for the first time, we have developed unique in vivo genetic models and in vitro approaches demonstrating that the RBPjk-dependent Notch targets, HES1 and HES5, suppress chondrogenesis and promote the onset of chondrocyte hypertrophy. HES1 and HES5 may have some overlapping function in these processes, although only HES5 directly regulates Sox9 transcription to coordinate cartilage development. HEY1 and HEYL play no discernable role in regulating chondrogenesis or chondrocyte hypertrophy, while none of the HES/HEY factors appear to mediate Notch regulation of cartilage matrix catabolism. This work identified important candidates that may function as downstream mediators of Notch signaling in both normal skeletal development and Notch-related skeletal disorders.

Introduction

The limb skeleton is largely comprised of endochondral bones, which initially form as cartilage templates and are ultimately replaced by bone. Cartilage formation of the limb skeleton begins with the migration of mesenchymal progenitor cells (MPCs) from the lateral plate mesoderm into the developing limb field. MPCs undergo rapid proliferation expanding the limb-bud, followed by the formation of mesenchymal condensations that will give rise to individual cartilage elements via chondrogenesis. This process, which generates mature chondrocytes or cartilage cells from MPCs via differentiation, is primarily driven by the expression and activity of the transcription factor SOX9. SOX9 induces and maintains the expression of numerous cartilage-related genes including collagen type II (Col2a1) and aggrecan (Acan) as well as drives growth of cartilage elements (Akiyama et al., 2002; Horton, 2003). As cartilage rudiments continue to develop, chondrocytes near the center of the elements undergo phenotypic and molecular changes known as pre-hypertrophy and
hypertrophy, which are regulated and marked by the sequential activation of genes including *indian hedgehog* (*Ihh*), *runt related transcription factor 2* (*Runx2*), *collagen type X* (*Col10a1*), *matrix metalloproteinase 13* (*Mmp13*), and the concomitant down-regulation of *Sox9*. The cartilage matrix is ultimately removed via the activity of terminally hypertrophic chondrocytes, which secrete MMP13 to catabolize or degrade the cartilage matrix creating a scaffold for newly formed osteoblasts to lay down bone matrix (Zuscik et al., 2008).

The Notch signaling pathway is a known regulator of chondrogenesis, chondrocyte hypertrophy, cartilage matrix catabolism, and osteoblastogenesis (Dong et al., 2010; Zanotti and Canalis, 2010; Kohn et al., 2012; Liu et al., 2015). Activation of the Notch pathway requires receptor-ligand interactions that initiate a cascade of cleavage events leading to the release of the Notch intracellular domain (NICD) and translocation to the nucleus where it forms a ternary transcriptional complex with Recombination Signal Binding Protein for Immunoglobulin Kappa J Region (*Rbpj*) and Mastermind-like (MAML) to activate downstream target genes (Bray, 2006). Recently, several groups have utilized various Notch pathway component loss-of-function (LOF) and gain-of-function (GOF) genetic approaches to study the roles of Notch signaling during cartilage and bone development. For example, utilization of the *Prx1Cre* transgene to remove *Rbpj* floxed alleles (Notch LOF) within MPCs demonstrated an acceleration in chondrogenic and osteoblastic differentiation within the limb skeleton, while over-expression of NICD (Notch GOF) within MPCs potently inhibited chondrogenesis and osteogenesis while maintaining and expanding MPCs (Dong et al., 2010). Genetic removal of various Notch signaling components (*Presenilin1 and 2, Notch1* and 2, and *Rbpjk*) within MPCs using the *Prx1Cre* or within cartilage progenitor cells using a *Col2Cre* transgene delayed the onset and progression of chondrocyte hypertrophy and cartilage matrix catabolism (Hilton et al., 2008; Kohn et al., 2012), while activation of NICD in committed chondrocytes both *in vivo* and *in vitro* promoted chondrocyte hypertrophy and cartilage matrix catabolism (Mead and Yutzey, 2009; Kohn et al., 2012). Recently, we also demonstrated that several of the Notch-mediated effects on cartilage development occur in an RBPJK-dependent manner (Dong et al., 2010) and are likely the consequence of an indirect transcriptional regulation of *Sox9* (Kohn, 2015). While the importance of Notch signaling in cartilage development has been well documented, the precise molecular mechanism(s) by which Notch regulates these distinct processes remain unclear or unknown.

Hairy and Enhancer of Split (HES) and Hairy and Enhancer of Split Related (HEY) proteins are bHLH transcription factors, of which several are molecular targets of RBPJK-dependent Notch signaling and mediate aspects of Notch function within cells. The most well
documented RBPjκ-dependent Notch targets are Hes1, Hes3, Hes5, Hes7, Hey1, and HeyL. HES/HEY transcription factors are largely classified as transcriptional repressors that bind unique N-box (CACNAG) and E-box (CANNAG) DNA sequences in the promoters of target genes (Kageyama et al., 2007). To determine whether specific HES/HEY factors may function in cartilage development and mediate some aspects of Notch signaling in this process, we 1) analyzed Hes/Hey gene expression using an in vitro model of chondrogenesis and chondrocyte hypertrophy followed by further gene and protein expression analyses using in vivo models, 2) developed and analyzed individual and combined Hes or Hey gene LOF and GOF mouse models for defects in cartilage development, and 3) tested the ability of specific HES factors to transcriptionally regulate Sox9 during chondrogenic differentiation in vitro and in vivo.

Results

**Hes and Hey genes are expressed during chondrogenesis and chondrocyte hypertrophy.**

Previously, we analyzed the expression and function of Hes1, Hey1, and Heyl during in vitro chondrogenesis using limb-bud micromass cultures and shRNA knock-down experiments. These data suggested that Hes1 was important in suppressing MPC differentiation and chondrogenesis, while Hey1 and Heyl were dispensable during chondrogenic differentiation (Dong et al., 2010). Here, we have further analyzed the expression of several RBPjκ-dependent Notch target genes of the Hes and Hey families throughout both the processes of chondrogenesis and chondrocyte hypertrophy using a different in vitro system. ATDC5 cells were cultured in the presence of insulin-transferrin-selenium (ITS) supplements in order to induce chondrogenic differentiation and maturation. RNA was isolated for gene expression analysis at 7, 14, 21, and 28 days following chondrogenic induction. To monitor the progression of chondrogenesis and chondrocyte hypertrophy, we first examined the expression of the early chondrogenic markers, Sry box 9 (Sox9), Collagen type II (Col2a1), and Aggrecan (Acan) via quantitative PCR (qPCR). All early chondrogenic genes peaked in expression at day 21 and decreased by day 28, correlating with the ramping up of chondrogenic differentiation and the transition to hypertrophy (Fig. 1A). The hypertrophic chondrocyte markers, Col10a1 and Mmp13, were most highly expressed at day 28 indicating that the majority of cells had reached hypertrophy (Fig. 1A). We next analyzed the expression of Hes1, Hes3, Hes5, Hes7, and Hey1. Hes1 reached peak expression at day 21 and then decreased at day 28 (Fig. 1A). Hes5 was highly expressed at day 7 followed by a reduction in expression at days 14 and 21, and then was
elevated again at day 28 (Fig. 1A). When comparing the expression of *Hes5* to *Sox9*, there appears to be an inverse relationship between the two genes such that if one is highly expressed the other is decreased in expression. *Hey1* expression was highest at day 28 and lower at earlier stages of chondrogenesis, suggesting a potential role in chondrocyte hypertrophy (Fig. 1A). Other *Hes* genes were also analyzed but largely could not be detected during chondrogenesis and chondrocyte hypertrophy of ATDC5 cells in culture.

To determine whether HES factors demonstrated similar expression profiles during chondrogenesis *in vivo*, we isolated RNA and protein from wild-type (WT) embryonic day 10.5 and 11.5 (E10.5 and E11.5) limb-buds. Using qPCR, we observed a similar trend between *Hes1* and *Sox9* as well as between *Hes5* and *Sox9*: that is, increased *Hes1* expression was observed as *Sox9* increases from E10.5-E11.5, while *Hes5* expression decreases as *Sox9* increases from E10.5-E11.5 (Fig. 1B). We then analyzed HES1 and HES5 protein expression in E10.5 and E11.5 limb-buds using Western blot analysis. Similar to the qPCR data, we again observed increased HES1 as SOX9 increases from E10.5-E11.5, while HES5 decreased as SOX9 increased from E10.5-E11.5 (Fig. 1C). Collectively, these data demonstrate that both *Hes1* and *Hes5* are expressed throughout chondrogenesis and chondrocyte hypertrophy, and suggests that HES5 may be a negative regulator of *Sox9*, consistent with the transcriptional repressive role for most HES factors and the suppressive role of RBPjκ-dependent Notch signaling during chondrogenesis (Kageyama et al., 2005; Dong et al., 2010).

**HES1 is dispensable for MPC differentiation, potentially due to compensatory expression of HES5.**

As stated previously, HES1 is an RBPjκ-dependent Notch target gene capable of suppressing *in vitro* chondrogenesis in limb-bud micromass cultures (Dong et al., 2010). To determine whether the specific loss of *Hes1* in MPCs can induce a similar acceleration of chondrogenesis *in vivo*, we generated and analyzed *Prx1Cre;Hes1<sup>f/f</sup>* (Hes1 LOF) embryos. This genetic targeting strategy allows for the specific removal of *Hes1* floxed alleles within MPCs of the developing limbs. Using whole-mount *in situ* hybridization (WISH), we analyzed WT and Hes1 LOF mutant embryos at E12.5; however, we did not observe any change in *Sox9* (Fig. S1Aa-b) or *Col2a1* (Fig. S1Ac-d) expression. RNA was then isolated from whole limb-buds of both WT and Hes1 LOF E12.5 embryos. Using qPCR, we did not observe any change in expression of the chondrogenic markers *Sox9*, *Col2a1*, and *Acan* (Fig. S1B). This data was surprising as we expected to observe an acceleration in chondrogenesis...
as previously observed in Hes1 LOF in vitro models of chondrogenesis and other Notch LOF in vivo models (Dong et al., 2010). To determine whether other Hes or Hey genes may be compensating for the loss of Hes1, we isolated RNA from E11.5 WT and Hes1 LOF whole limb-buds. Hes and Hey genes are prominently expressed in undifferentiated MPCs of the developing limb-bud at E11.5 (Dong et al., 2010). Using qPCR, we observed increased Hes5 gene expression in Hes1 LOF limb-buds compared to WT controls (Fig. S1B). Additionally, we analyzed the expression of Hes3, Hes7, Hey1, and Heyl, but either did not observe any change in expression between Hes1 LOF limb-buds compared to WT controls or the expression levels were too low to be reliably detected. These data suggest the potential for HES5 to compensate for the lack of HES1 during MPC differentiation in our Hes1 LOF embryos. This compensatory effect of HES factors has been well documented in other cell systems, such as neural progenitor cells (Hatakeyama et al., 2004).

Removal of multiple HES factors in MPCs accelerates differentiation and chondrogenesis.

Due to the compensatory increase in Hes5 expression following the conditional removal of Hes1 (Fig. S1B), we analyzed embryos in which both Hes factors were deleted in MPCs. We generated Prx1Cre;Hes1fl/fl;Hes5-/- (Hes1,5 LOF) and WT embryos to analyze cartilage development of the limb skeleton. We first confirmed genetic removal of Hes1 and Hes5 by analyzing their expression via qPCR on RNA isolated from E10.5 Hes1,5 LOF and WT limb-buds (Fig. 2A). Using SOX9 immunohistochemistry (IHC) on E10.5 Hes1,5 LOF and WT control limb-bud sections, we observed a subsequent increase in the expression of SOX9 in Hes1,5 LOF limb-buds as compared to WT (Fig. 2Ba-b). These data demonstrate an early acceleration in MPC differentiation and chondrogenesis. We next analyzed E11.5 Hes1,5 LOF and WT embryos. Similar to E10.5 limb-bud sections, we performed SOX9 IHC and observed increased SOX9 in the Hes1,5 LOF limb-buds as compared to WT (Fig. 2Bc-d). We also performed qPCR on RNA isolated from E11.5 Hes1,5 LOF and control limb-buds. At E11.5, we observed a significant increase in both Sox9 and Col2a1 expression in Hes1,5 LOF limb-buds (Fig. 2A), further suggesting an acceleration in MPC differentiation and chondrogenesis in the absence of Hes1 and Hes5. Lastly, we analyzed expression of COL2A1 and ACAN protein and RNA at E12.5 in WT and Hes1,5 LOF limb-buds. Alcian Blue/Hematoxylin/Orange G (ABH/OG) staining, which stains cartilage matrix blue, showed that Hes1,5 LOF mutant limbs displayed enhanced or accelerated cartilage formation in the radius and ulna, with more clearly defined joint formation in regions of the developing
humerus and ulna when compared to WT sections (Fig. 2Ca-b). Protein analysis using IHC demonstrated altered COL2A1 expression associated with advanced secondary chondrogenesis and joint formation (Fig. 2Cc-f) and an increase in ACAN expression (Fig. 2Cg-j) in E12.5 Hes1,5 LOF forelimbs as compared to WT controls. Further analysis at this time point revealed a change in cellular morphology. As chondrocytes mature prior to hypertrophy, they begin to flatten (Zuscik et al., 2008). In E12.5 Hes1,5 LOF limb buds, we observed more flattened cells compared to WT controls (Fig. 2Ci-j). This indicates that the cells in the Hes1,5 LOF limb-bud are beginning to form the columnar zone of proliferating chondrocytes, which occurs just prior to hypertrophy. This is not observed as clearly in the WT control sections. Lastly, we analyzed RNA from E12.5 wild-type and Hes1,5 LOF mutant limb-buds using qPCR. Gene expression analysis showed that the loss of Hes1 and Hes5 resulted in a significant increase in Col2a1 and Acan expression (Fig. 2A). We did not observe any change in Sox9 at this time point, which is likely due to the fact that as chondrocytes begin to mature, Sox9 levels decrease (Fig. 2A). Collectively, these data demonstrate that Hes1 and Hes5 are necessary for appropriate MPC differentiation and chondrogenesis.

**HES1 over-expression in MPCs delays chondrogenesis and induces MPC proliferation.**

To determine whether Hes1 is sufficient to suppress chondrogenesis, we analyzed PrxlCre;Rosa-Hes1ff [Hes1 gain-of-function (Rosa-Hes1)] embryos across multiple time points. These experiments were conducted using a mouse line in which Hes1 was targeted to the Rosa26 locus containing a transcriptional stop sequence flanked by loxP sites upstream of the Hes1 cassette. When crossed with the PrxlCre mouse line, Hes1 is continuously overexpressed in MPCs of the developing limb-buds (Kobayashi and Kageyama, 2010). We first analyzed Hes1 and chondrogenic gene expression from E12.5 Rosa-Hes1 and WT limb-buds using qPCR. We observed a significant over-expression of Hes1 and a significant decrease in both Col2a1 and Acan expression in Rosa-Hes1 limb-buds as compared to WT (Fig. 3A). Interestingly, Sox9, Sox5, and Sox6 gene expression was largely unchanged in Rosa-Hes1 limb-buds as compared to WT (Fig. 3A). Histological analyses of E12.5 Rosa-Hes1 and WT limb-bud sections using ABH/OG staining demonstrated a reduction in proteoglycan content in Rosa-Hes1 developing hindlimbs as compared to WT sections (Fig. 3Ba-b). Similar to the decrease in ABH/OG staining, IHC analyses showed decreased expression of COL2A1 (Fig. 3Be-d) and ACAN (Fig. 3Be-f) in the Rosa-Hes1 hindlimbs compared to WT sections.
To determine whether overexpression of Hes1 also affects MPC proliferation, we used BrdU IHC to analyze Rosa-Hes1 and WT forelimbs. Similar to Notch gain-of-function in MPCs (Dong et al., 2010), we observed an increase in BrdU+ MPCs in the Rosa-Hes1 forelimbs compared to WT (Fig. 3Ca-b). The red box outlines the area in which cells (both BrdU+ and BrdU-) were counted. This region of the limb bud is just beyond the highly proliferative apical zone, where MPCs normally begin the differentiation process. Statistical analysis verified a significant increase in the percentage of BrdU+ cells in the Rosa-Hes1 forelimbs compared to WT controls (Fig. 3Cc). We also isolated protein from E11.5 Rosa-Hes1 and WT limb-buds to perform Western blot analysis of the proliferative marker, CyclinD1 (CYCD1). Rosa-Hes1 limb-buds demonstrated increased CYCD1 protein as compared to WT controls further validating the observed increase in proliferation (Fig. 3D).

Collectively, these data demonstrate that Hes1 is sufficient to delay chondrogenesis by suppressing Col2a1 and Acan gene expression, and is also capable of expanding the MPC population during early limb development.

**HES, but not HEY, factors regulate early chondrocyte hypertrophy potentially via suppression of SOX9.**

We have shown previously that RBPjκ-dependent Notch signaling is an important regulator of the onset and progression of chondrocyte hypertrophy (Hilton et al., 2008; Kohn et al., 2012; Kohn, 2015). To determine whether HES1 regulates the onset and progression of chondrocyte hypertrophy, we first analyzed Hes1 LOF and WT embryos at E14.5. Hes1 LOF forelimbs were analyzed using histological staining and in situ hybridization for markers of chondrocyte maturation. ABH/OG staining of E14.5 Hes1 LOF mutant forelimbs exhibit a mild and largely inconsistent decrease in the length of the hypertrophic zone (Fig. S1Ca-b), which is also observed via reduced domains of Col10a1 (Fig. S1Cc-d) and Mmp13 (Fig. S1Ce-f) expression as compared to WT controls. Collectively, these data suggest that Hes1 may play a limited role in regulating the onset of chondrocyte hypertrophy at E14.5 or that compensation by other HES factors, such as HES5, may be blunting the effect of Hes1 LOF alone.

To determine whether Hes5 compensatory expression may also be affecting chondrocyte hypertrophy in Hes1 LOF mutants, we generated and analyzed E13.5 Hes1,5 LOF mutant and WT embryos via histological approaches. Hematoxylin and Eosin (H&E) staining of humerus sections demonstrate that Hes1,5 LOF mutants have a smaller hypertrophic zone compared to WT controls (Fig. 4Aa-b). In situ hybridization for the
hypertrophic chondrocyte marker, *Col10a1*, indicated a clear lack of expression in E13.5 Hes1,5 LOF mutant humerus sections compared to WT (Fig. 4Ac-d). To understand the potential mechanism underlying this delay in the onset of hypertrophy, we used IHC to analyze the protein expression of SOX9. Analysis of E13.5 Hes1,5 LOF mutant and WT humerus sections demonstrated that Hes1,5 LOF mutants exhibited more continuous expression of SOX9 compared to WT controls within cells of the central regions of elements poised for hypertrophy (Fig. 4Ac-f). These data suggest that the delay in the onset of chondrocyte hypertrophy may be due to the maintenance of SOX9 expression.

To determine whether the loss of *Hes1* and *Hes5* results in a continuous delay in chondrocyte hypertrophy, we analyzed E14.5 Hes1,5 LOF mutant and WT limb skeletons. H&E staining revealed a decrease in the length of the hypertrophic zone in E14.5 Hes1,5 LOF humerus sections as compared to WT (Fig. 4Ba-b). Furthermore, Hes1,5 LOF forelimbs exhibit a decrease in the *Col10a1* (Fig. 4c-d) and *Mmp13* (Fig. 4Be-f) expression domains at E14.5. To determine if this continued delay in hypertrophy is due to altered SOX9 expression, we used IHC analysis. Similar to the Hes1,5 LOF data at E13.5, we observed maintenance of SOX9 expression deeper into the hypertrophic zone of E14.5 mutant forelimbs (Fig. 4Bg-j). To determine if this decrease size of the hypertrophic zone was statistically significant, we measured the length of the hypertrophic zones and normalized to total lengths of the cartilage elements for WT and Hes1,5 LOF mutants. Quantitative analysis showed a significant decrease in the length of the hypertrophic zone in the Hes1,5 LOF mutant forelimbs compared to WT (Fig. 4C). To ensure this delay in hypertrophy was not due to changes in proliferation, we utilized BrdU IHC analysis, and did not observe any change in the percentage BrdU+ cells in Hes1,5 LOF humerus sections as compared to WT (Fig. S2A-B). Combined, these data demonstrate that HES1 and HES5 control the pace of chondrocyte hypertrophy, potentially via SOX9 regulation.

We next analyzed terminal chondrocyte hypertrophy and cartilage matrix catabolism using E18.5 Hes1,5 LOF and WT humerus sections. Interestingly, no delay in terminal chondrocyte hypertrophy or cartilage matrix turnover was observed between Hes1,5 LOF and WT cartilage elements, as indicated by similar zones of hypertrophy in ABH/OG stained sections (Fig. S2Ca-b) and a lack of any change in MMP13 expression (Fig. S2Cc-d). This was consistent with data demonstrating that genetic removal of *Hes1* alone within MPCs caused no obvious defects in terminal chondrocyte hypertrophy and cartilage matrix turnover, as indicated by similar zones of hypertrophy in E18.5 Hes1 LOF and WT H&E stained sections (Fig. S2Da-b) and similar expression domains of *Ihh* (Fig. S2Dc-d), *Col10a1* (Fig. S2De-f, and *Ihh* expression in Hes1,5 LOF mutants (Fig. S2Df-g).
Surprisingly, neither Hes1 LOF or Hes1,5 LOF mutants appeared to exhibit an expanded hypertrophic zone at E18.5, which was observed previously in several Notch LOF mutant mice and is indicative of a continuous delay in terminal chondrocyte hypertrophy and cartilage matrix catabolism (Hilton et al., 2008; Mead and Yutzey, 2009; Kohn et al., 2012; Kohn, 2015). Based on these data combined with the observation that HEY factor expression increases in maturing and hypertrophic chondrocytes (Fig. 1A), we obtained and analyzed E14.5 and E18.5 Hey1−/−; HeyL−/− double mutant (Hey1,HeyL LOF) and control embryos for defects in the onset and progression of chondrocyte hypertrophy and cartilage matrix catabolism. H&E staining (Fig. S3Aa-b, Ba-b), and in situ hybridization for Ihh (Fig. S3Ac-d, Bc-d), Col10a1 (Fig. S3Ae-f, Be-f), and Mmp13 (Fig. S3Ag-h, Bg-h) on tibia sections at E14.5 and E18.5 demonstrate no obvious changes in the hypertrophic zones between Hey1,HeyL LOF and control cartilage elements.

Collectively, these data demonstrate that HES factors (particularly HES5) primarily control the onset of chondrocyte hypertrophy during cartilage maturation, potentially via SOX9 regulation, while neither the HES or HEY factors appear to control terminal chondrocyte hypertrophy or cartilage matrix catabolism.

**HES1 over-expression in MPCs delays chondrocyte hypertrophy and inhibits skeletal growth.**

To determine whether Hes1 over-expression in MPCs affects chondrocyte proliferation and hypertrophy during cartilage development and maturation, we first examined Rosa-Hes1 and WT E14.5 skeletal preparations via alcian blue staining. Rosa-Hes1 cartilage rudiments were shorter, and the limbs as a whole, were smaller than WT controls (Fig. 5Aa-d). In the most severely affected Rosa-Hes1 forelimbs (Fig. 5Ab) and hindlimbs (Fig. 5Ad), we observed a hypoplastic or missing radius and/or fibula (black arrows). Distal cartilage rudiments appeared to be more severely affected as compared to proximal elements, and hindlimbs were more affected than forelimbs (Fig. 5Aa-d). Analysis of alcian blue stained hindlimbs shows the formation of a defined hypertrophic zone in WT cartilage rudiments (Fig. 5Ac) (red asterisks), although these were largely absent in severely affected Rosa-Hes1 mutants at this stage (Fig. 5Ad). ABH/OG staining of E14.5 humerus sections (the least affected proximal element) (Fig. 5Ba-b) demonstrated that Rosa-Hes1 mutants exhibited only a mild delay in chondrocyte hypertrophy as compared to WT controls, with only minor changes in Col10a1 (Fig. 5Bc-d) and Mmp13 (Fig. 5Be-f) expression within the hypertrophic zone.
To assess whether changes in chondrocyte proliferation could contribute to the Rosa-Hes1 skeletal phenotype, we performed BrdU staining on E14.5 Rosa-Hes1 and WT humerus sections (Fig. 5Ca-b). Consistent with the reduced size observed in most elements of Rosa-Hes1 mutants, we observed a decrease in the percentage of BrdU positive chondrocytes (Fig. 5Cc). We next analyzed overall growth changes of skeletal elements at E18.5 using Rosa-Hes1 and WT forelimb (FL) and hindlimb (HL) skeletal preparations (Fig. 5Da,Db,Dd,De,Dg,Dh,Dj,Dk). Analyses indicated that the total lengths of these bones are significantly shorter in Rosa-Hes1 mutants as compared to WT controls, with the most prominent effects occurring on distal elements (Fig. 5Dc,Df,Di,Dl). Interestingly, Rosa-Hes1 mutant mice survive to adulthood and present with various skeletal anomalies including alterations to skeletal patterning, bone ridge or tuberosity development, and digit number. These phenotypes are the likely result of the early and broad effects of the Prx1Cre transgene controlling Hes1 over-expression in skeletal progenitors. The precise cellular and molecular mechanisms underlying each of these peripheral phenotypes will be explored and described elsewhere. These data presented here suggest that Hes1 over-expression in MPCs can delay chondrocyte hypertrophy and reduce chondrocyte proliferation, although these effects may be secondary to the delay in chondrogenesis described earlier.

**HES5 directly regulates Sox9 expression.**

Since chondrogenic differentiation from MPCs and early chondrocyte hypertrophy are coordinated by the expression and activity of SOX9, we next examined whether HES factors could transcriptionally regulate Sox9 expression. Earlier we demonstrated that Hes1 over-expression in MPCs in vivo is sufficient to repress Col2a1 and Acan expression without affecting Sox9 expression (Fig. 3A). To determine whether HES5 is capable of regulating Sox9 expression, we first transfected ATDC5 chondrogenic cells with either Flag (control) or Hes5 over-expression constructs. After seven days in chondrogenic differentiation media, RNA was isolated for qPCR analysis from each group. Hes5 over-expression resulted in a notable reduction of Sox9, Sox5, and Sox6, as well as chondrogenic genes such as Col2a1 and Acan, although to a lesser degree at this time-point (Fig. 6A). These data suggest HES5 is sufficient to down-regulate or delay chondrogenesis in vitro and may directly regulate Sox9 expression.

Previous studies have demonstrated RBPj-dependent Notch regulation of Sox9, however, the exact mechanism remains unknown or controversial (Mead and Yutzey, 2009; Dong et al., 2010; Kohn et al., 2012; Chen et al., 2013; Kohn, 2015). Recent data has
suggested that the Notch-mediated transcriptional regulation of Sox9 occurs indirectly via secondary effectors (Kohn, 2015). Therefore, we first used a bioinformatics approach to search the Sox9 promoter for HES binding sites, N-box/E-box sequences (Fig. S4A). We identified two N-box/E-box sequences within the first kilobase of the Sox9 promoter that were 100% conserved between the mouse and human genomes (Fig. S4C). To determine whether HES5 directly binds this region of the Sox9 promoter in MPCs and chondrogenic cells in vivo, we utilized chromatin immunoprecipitation (ChIP) assays on DNA isolated from WT E10.5 and E11.5 limb-buds. As previously indicated, Hes5 and Sox9 expression demonstrated an inverse relationship in E10.5 and E11.5 limb-buds (Fig. 1Ba-b). Primers were designed to amplify the region of the Sox9 promoter containing the N-box/E-box (Fig. S4B [red arrows]) and a negative control region approximately 19kb upstream of N-box/E-box sequences (Fig. S4B [green arrows]). ChIP analysis at E11.5 using a HES5 antibody revealed amplification of DNA when using primers flanking the N-box/E-box sequence (Primer 2; P2)(Lane 8), and no amplification when using primers targeting an upstream region of the Sox9 promoter (Primer 1; P1)(Lane 7) (Fig. 6Ba). Interestingly, no amplification of either primer set was observed when ChIP analyses were performed with a HES1 antibody (Lanes 5 and 6) (Fig. 6Ba), indicating specificity of binding to the N-box/E-box site for HES5. We also observed positive control amplification from the sheared genomic DNA (Lanes 1 and 2) when using P1 and P2 primers, while IgG pull downs showed no amplification (Lanes 3 and 4) (Fig. 6Ba). By performing qPCR on DNA pulled down during ChIP assays from E10.5 and E11.5 limb-buds, we were able to determine that the occupancy of HES5 on the Sox9 promoter region was greater at E10.5 than E11.5 (Fig. 6Bb). These data suggest that RBPj-dependent Notch regulation of Sox9 works in part through direct HES5 transcriptional activity.

Finally, we utilized a 1.0kb Sox9 promoter driven luciferase construct that includes the N-box/E-box sequence to demonstrate the direct transcriptional regulation of Sox9 by HES5. When this construct was co-transfected with Flag, NICD1, and Hes5 expression vectors, we observed a significant and similar level of luciferase activity suppression between NICD1 and Hes5 transfected groups when compared to the Flag transfected control (Fig. 6Ca). However, when we co-transfected a 1.0kb Sox9 promoter driven luciferase construct containing a mutated N-box sequence with Flag or Hes5 expression vectors, we observed no change in luciferase activity (Fig. 6Cb). Collectively, these data demonstrate that the RBPsj-dependent Notch target, HES5, directly binds the Sox9 promoter via the N-box sequence and is capable of down-regulating Sox9 expression in MPCs and chondrogenic cells.
Discussion

HES/HEY factors are well known RBPjk-dependent Notch target genes, which are capable of mediating several aspects of Notch function in various settings (Cau et al., 2000; Hirata et al., 2001; Zine et al., 2001; Kageyama et al., 2007). Prior in vitro studies have implicated HES1 as a potential suppressor of chondrogenesis, as well as a potential transcriptional regulator of the Col2a1 and Acan promoters (Grogan et al., 2008; Dong et al., 2010); however, the in vivo evidence for HES regulation of cartilage development was lacking. Our results demonstrate that MPC-specific deletion of Hes1 alone is not sufficient to affect cartilage development, and that the additional removal of Hes5 is required to alter both chondrogenesis and the onset of chondrocyte hypertrophy, potentially due to the compensatory expression of Hes5 in vivo. Interestingly, mutant mice with deletion of Hes1 in more committed chondro-osteo progenitors combined with conventional deletion of Hes5 (Col2Cre;Hes1^{f/f};Hes5^{-/-}) failed to show any defects in cartilage development (Karlsson et al., 2010). Importantly, this study only analyzed embryos at E16.5 and later time-points during endochondral bone development, thereby potentially missing the earlier defects we have described here. Alternatively, Col2Cre;Hes1^{f/f};Hes5^{-/-} mutant mice may not have developed defects such as those observed in our study at E10.5-E15.5, since the Col2Cre transgene targets a more committed chondro-osteoprogenitor population. Prx1Cre;Hes1^{f/f};Hes3^{-/-};Hes5^{-/-} mutant mice have also previously been generated and shown to have increased postnatal bone mass consistent with other Notch LOF mutant mice (Hilton et al., 2008; Tu et al., 2012), although only limited late stage embryonic skeletal analyses were performed showing no obvious phenotype (Zanotti et al., 2011). Similarly, Hey1^{+/-}; HeyL^{-/-} mutant mice were shown to have increased bone mass as compared to controls at late postnatal and adult time-points (Tu et al., 2012). Therefore, while these studies have implicated HES/HEY factors in the regulation of postnatal bone development and homeostasis, they missed the important roles of specific HES factors during cartilage development of the limb skeleton.

Here we report the first in vivo genetic evidence demonstrating that the RBPj-dependent Notch target genes, Hes1 and Hes5, act as regulators of chondrogenesis and chondrocyte hypertrophy during cartilage development. We have demonstrated that HES1 is dispensable for normal MPC differentiation and chondrogenesis, likely due to compensatory expression of Hes5. However, HES1 is sufficient to delay chondrogenesis by acting downstream of the SOX trio (SOX9, SOX5, SOX6) in addition to inducing MPC
proliferation. Therefore, removal of both Hes1 and Hes5 in MPCs accelerates chondrogenesis and delays the onset of chondrocyte hypertrophy. Consistent with recent Notch LOF studies, the accelerated chondrogenesis and delay in the onset of chondrocyte maturation is likely due to increased SOX9 expression (Kohn, 2015). We identified HES5 as a direct transcriptional modifier of Sox9 gene expression, which in turn has direct influences on Sox5 and Sox6 gene regulation to coordinate chondrogenesis. However, HES1 likely mediates control of chondrogenesis and cartilage development via direct downstream regulation of other chondrogenic genes such as Col2a1 and Acan (Grogan et al., 2008). Our work also demonstrated that neither the removal of individual or multiple Hes or Hey genes alters terminal chondrocyte hypertrophy or cartilage matrix catabolism during normal development, thereby suggesting that the delayed terminal hypertrophy and cartilage matrix metabolism observed in other Notch LOF mutants (Hilton et al., 2008; Mead and Yutzey, 2009; Kohn et al., 2012; Kohn, 2015) are the result of RBPjk-dependent and HES/HEY-independent signaling mechanisms. Interestingly, genetic removal of Hes1 within postnatal cartilages following joint injury in a murine model of osteoarthritis (OA) is capable of reducing Mmp13 expression as well as other cartilage catabolizing enzymes, while prolonged over-expression of Hes1 was also shown to induce some of these same catabolic genes in vitro (Sugita et al., 2015). Therefore, it is possible that HES, and potentially HEY, factor regulation of catabolic gene expression may only be evident in pathological or injury/inflammation settings and that during development Notch signaling regulates terminal chondrocyte hypertrophy and cartilage matrix catabolism via alternative mechanisms. Alternatively, numerous HES/HEY factors may contribute to the regulation of this aspect of Notch function in cartilage, and therefore would require the elimination of nearly all Hes/Hey genes simultaneously to uncover their requisite role in regulating cartilage catabolism during normal development.

While the importance of Notch signaling in skeletal development, injury, and disease has recently come to light, we are just beginning to learn about the underlying Notch-mediated molecular mechanisms that control these distinct events. Identifying these molecular players and their function may not only provide us with additional important molecules to consider when examining skeletal disorders, but may also generate additional drug targets for the treatment of these conditions or ailments. For example, Hajdu Cheney Syndrome (HCS) is a rare, heritable multi-organ connective tissue disorder that presents with significant skeletal features such as skull deformities, short stature, joint laxity, and a severe reduction in bone mass or osteoporosis and is caused by heterozygous mutations in the NOTCH2 receptor (Majewski et al., 2011; Simpson et al., 2011). While it has been recently
discovered that these mutations lead to Notch GOF within connective tissue cells (Majewski et al., 2011; Simpson et al., 2011), the precise downstream effectors that drive the pathology are unknown. Adams-Oliver Syndrome (AOS) is another rare heritable disorder characterized by skin and limb defects including hypoplastic or shortened digits, absence of bones in hands or the feet, as well as, partial or complete absence of the lower legs (tibia/fibula/digits). AOS is an autosomal dominant disorder caused by mutations in \textit{RBPjk} and/or \textit{NOTCH1} genes resulting in Notch LOF, although no additional molecular mechanisms underlying this disease is known (Hassed et al., 2012; Stittrich et al., 2014). Notch signaling defects, either GOF or LOF, have also been implicated in osteoarthritis (Mahjoub et al., 2012; Hosaka et al., 2013; Mirando et al., 2013; Sassi et al., 2014; Liu et al., 2015), rheumatoid arthritis (Nakazawa et al., 2001; Park et al., 2015), osteoporosis (Engin et al., 2008; Hilton et al., 2008; Majewski et al., 2011; Simpson et al., 2011), and a predisposition to pathologic fractures (Kung et al., 2010). Studies like the one presented here furthers our understanding of the molecular players and events in which Notch signaling may control normal skeletal development, as well as, contribute to the pathology of certain skeletal diseases and injury processes.

\textbf{Materials and Methods}

\textit{Mouse Strains}

The \textit{Prx1Cre} mouse line was previously described (Logan et al., 2002). The \textit{Hes1}\textsuperscript{floxed}, \textit{Hes1}\textsuperscript{floxed}/\textit{Hes1}\textsuperscript{floxed}, and \textit{Rosa-Hes}\textsuperscript{floxed} strains were a generous gift from Dr. Ryoichiro Kageyama (Institute for Virus Research, Kyoto University) and were described previously (Cau et al., 2000; Hirata et al., 2001; Imayoshi et al., 2008; Tateya et al., 2011) (Kobayashi et al., 2009). \textit{Hey1} \textsuperscript{-/-}; \textit{HeyL} \textsuperscript{-/-} mutant and control embryos were provided by Dr. Manfred Gessler (Biozentrum Universitat Wurzburg) and have been previously described (Fischer et al., 2007). All animal work has been approved by both the Duke University and University of Rochester Institutional Animal Care and Use Committees (IACUC).

\textit{Real-time RT-PCR}

Isolation of RNA from limb-bud tissues and ATDC5 cultures were performed as previously described (Kohn, 2015). Real-time RT-PCR was used to analyze relative gene expression using the Bio-Rad CFX Connect Real-Time System. Gene expression was normalized to \textit{\beta-actin} before being normalized to control samples. Mouse specific primers for \textit{Sox9}, \textit{Sox5},
Sox6, Col2a1, Acan, Hes1, Hes3, Hes5, Hes7, and Hey1 were designed as described previously (Dong et al., 2010). Primer sequences are available upon request. Gene expression analyses from limb-buds are from representative experiments of at least three biological replicates from pooled genotypes with statistical analyses performed on technical replicates of an individual experiment.

Tissue Analysis

Embryos were harvested at E10.0-E18.5 in cold 1X phosphate buffered saline (PBS), fixed in 10% neutral buffered formalin (NBF), and then processed. Embryos >E12.5 were treated overnight with 14% ethylenediaminetetraacetic acid (EDTA). After processing, tissues were paraffin embedded. For embryos E10.0-E11.5, the whole embryo was embedded; limbs from E12.5-E14.5 embryos were dissected off of whole embryo before embedding. Tissue was then sectioned at 4μm and 5μm for E10.0-E12.5 and E13.5-E18.5 limbs, respectively. To analyze cartilage composition and general cellular morphology, standard histological stains using ABH/OG and H&E were performed. To analyze protein expression, IHC was performed using the VectaStain ABC kits and developed with ImmPACT DAB (Vector Labs). The following primary antibodies were used for IHCs: ACAN (Chemicon), COL2A1 (Thermo Scientific), SOX9 (Santa Cruz Biotechnology), and MMP13 (Thermo Scientific). Standard heat induced and sodium citrate antigen retrievals were performed for the previously listed antibodies, while no antigen retrieval was performed for SOX9 IHC.

Relative quantification of some IHC staining intensities were calculated using the IHC image analysis toolbox developed in ImageJ. Briefly, DAB stained pixels were first “trained” by the software to build a statistical positive stain detector. By selecting a region of interest in the image, pixels of the original image were displayed with original color values, while all other pixels were set to 255 as background and thus filtered out during the color detection process. Finally, only DAB stained pixels were quantified and compared between groups. BrdU IHC was performed as previously described (Dong et al., 2010). For in situ hybridization, embryos were prepared, fixed, processed, and sectioned as described previously (Hilton et al., 2005; Hilton et al., 2007; Hilton et al., 2008; Dong et al., 2010). Dig-labeled whole-mount in situ hybridizations were performed as described (Rutkowsky et al., 2014). Skeletal staining was performed using the protocol as previously described (Dong et al., 2010).
**Chromatin Immunoprecipitation assay (ChIP)**

The ChIP assay was performed using the MAGnify Chromatin Immunoprecipitation System (Invitrogen) on E10.5 and E11.5 limb-buds. Limb-buds were homogenized in cold PBS using a 24g syringe and immediately frozen using liquid nitrogen. Sonication was performed using a Covaris S2 sonicator according to manufacturers instructions in order to shear chromatin to the lengths of 100-300 base pairs. The protocol was optimized for the use of 6-10 limb-buds. Antibodies for HES1 and HES5 (Santa Cruz Biotechnology) were used at a concentration of 10μg. Data analysis was performed using qPCR with primers specifically designed to amplify the region of interest within the Sox9 gene promoter.

**ATDC5 Cell Analysis**

ATDC5 cells (RIKEN BRC, Japan) were grown in a 12-well plate with DMEM/F12 1:1 (Invitrogen) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Once cells were 70-80% confluent, they were treated with ITS media: standard DMEM/F12 media supplemented with 1X ITS Premix [insulin (5μg/ml), transferrin (5μg/ml), and selenous acid (5ng/ml)] (BD Biosciences). Treatment with ITS supplement has been previously reported to induce chondrocyte differentiation (Watanabe et al., 2001). Cells were incubated for 4 days with ITS media before transfection with Flag, NICD1, and/or Hes5 over expression plasmids. Transfection was achieved using FuGENE HD (Promega) with 500ng of each construct. Cells were cultured for 7 days changing media every 48 hours. Luciferase assays were also performed in ATDC5 cells using a 1kb Sox9 luciferase construct and a N-box mutant 1kb Sox9 luciferase construct using the same protocol and reagents as described previously (Kohn, 2015). Western and luciferase analyses are representative experiments of at least three biological replicates with statistical analyses performed on technical replicates of an individual experiment. Real-time RT-PCR statistical analyses were performed on the means of three biological replicates.

**Western Blot**

Total proteins were isolated from WT and mutant limb-buds. Limb-buds were dissociated in a standard lysis buffer and protease inhibitor solutions. Approximately, 10μg of protein was separated using NuPAGE Novex 4-12% Bis-Tris pre-cast gels (Invitrogen) and the fractionated protein lysates were transferred onto a nitrocellulose membrane using the iBlot
system (Invitrogen). Antibodies for HES1 (Santa Cruz Biotechnology), HES5 (Abcam), SOX9 (Santa Cruz Biotechnology), CYCD1 (Cell Signaling), and β-ACTIN (Sigma) were used with the appropriate secondary antibody following the manufacturer’s protocol. Quantitation was performed on individual blots and representative blots are shown. Western blot images were first converted to 8-bits and then analyzed using Image J (NIH). Band intensity peak values were calculated and normalized to loading control (β-ACTIN) for comparison.

**Statistical analysis**

Statistical analyses were performed using two-tailed Student’s t-test and one way ANOVA; a $P$ value < 0.05 was considered significant.
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Author contributions
Conception and study design: MJH, TPR, and AK; Study conduct: TPR, AK, DS, AJM, and YR; Analysis and interpretation of the data: TPR, AK, DS, AJM, YR, and MJH; Drafting and/or editing manuscript: TPR, AK, and MJH; Approving final version of the manuscript: TPR, AK, DS, AJM, YR, and MJH.
References


Figure 1. HES/HEY expression during chondrogenesis and chondrocyte hypertrophy. (A) qPCR gene expression analyses for Sox9, Col2a1, Acan, Col10a1, Mmp13, Hes1, Hes5, and Hey 1 on RNA isolated from ATDC5 cells cultured for 7-28 days in ITS supplemented media. (B) qPCR analysis for Sox9, Hes1, and Hes5 on RNA isolated from E10.5 and E11.5 WT limb-buds. The y-axis represents relative gene expression normalized to β-actin then to Day 7 cultures. Bars represent means ± SD. ** denotes P<0.05. Bars under asterisks identify relevant statistical comparisons. (C) Western blot analyses for HES5, HES1, SOX9, and β-ACTIN using protein lysates from E10.5 and E11.5 WT limb-buds. Numbers represent the change in protein abundance as compared to E10.5 samples for the representative blot.
Figure 2. Loss of *Hes1* and *Hes5* in MPCs accelerates chondrogenesis.

(A) qPCR gene expression analyses for *Hes1*, *Hes5*, *Sox9*, *Col2a1* and *Acan* from RNA isolated from E10.5, E11.5, and E12.5 WT and *Prx1Cre;Hes1*/*Hes5* (Hes1,5 LOF) forelimbs. The y-axis represents relative gene expression normalized to β-actin then to wild-type control. Bars represent means ± SD. **"** denotes *P*<0.05. (B) IHC analyses of SOX9 expression in E10.5 (Ba-b) and E11.5 (Bc-d) WT and Hes1,5 LOF forelimb sections. (C) Histological and IHC analyses on WT and Hes1,5 LOF forelimb sections at E12.5 using ABH/OG staining (Ca-b), COL2A1 IHC (Cc-f) and ACAN IHC (Cg-j). h, humerus; r, radius; u, ulna. Green box within the joint region (Cc-d) identifies accelerated secondary chondrogenesis and joint formation in Hes1,5 LOF forelimbs compared to WT (Ce-f). Red box within the ulna (Cg-h) annotates region of interest for changes in cellular morphology in Hes1,5 LOF forelimbs compared to WT (Ci-j).
Figure 3: Over-expression of Hes1 increases MPC proliferation and delays chondrogenesis.

(A) qPCR gene expression analyses for Hes1, Sox9, Sox5, Sox6, Col2a1 and Acan from RNA isolated from E12.5 WT and Prx1Cre;Rosa-Hes1<sup>+/−</sup> [Hes1 gain-of-function (Rosa-Hes1)] limb-buds. The y-axis represents gene expression normalized to β-actin then to WT control. Bars represent means ± SD. “*” denotes P<0.05. (B) Histological and IHC analyses on WT and Rosa-Hes1 hindlimb sections at E12.5 using ABH/OG staining (Ba-b), COL2A1 IHC (Bc-d) and ACAN IHC (Be-f). fe, femur; fi, fibula; t, tibia. (C) BrdU IHC analysis on E11.5 sections from WT and Rosa-Hes1 forelimbs (Ca-b). Red box outlines region in which BrdU+ and BrdU- cells were quantified. Statistical analysis shows a significant increase in BrdU+ cells in Rosa-Hes1 limb-buds compared to WT (Cc). The y-axis represents the percentage of BrdU+ cells compared to the total number of cells counted. Bars represent means ± SD. “*” denotes P<0.05. (D) Western blot analysis for CYCD1 and β-ACTIN expression in Rosa-Hes1 limb-buds compared to WT. Numbers represent the change in protein abundance as compared to WT samples for the representative blot.
Figure 4. Loss of *Hes1* and *Hes5* delays chondrocyte hypertrophy due to prolonged SOX9 expression.

(A) H&E staining (Aa-b), *in situ* hybridization for *Col10a1* (Ac-d), and IHC for SOX9 (Ae-f) on E13.5 WT and *Prx1Cre;Hes1*<sup>flo</sup>;*Hes5*<sup>-/-</sup> (Hes1,5 LOF) humerus sections. Black circles outline the hypertrophic zones. (B) H&E staining (Ba-b), *in situ* hybridization for *Col10a1* (Bc-d) and *Mmp13* (Be-f), and IHC for SOX9 (Bg-j) on E14.5 WT and Hes1,5 LOF humerus sections. Red box denotes higher magnification images in Bi-j. (C) Statistical assessment of the hypertrophic zone lengths relative to the total lengths of the cartilage rudiments for WT and Hes1,5 LOF forelimbs. Bars represent means ± SD. “*” denotes *P*<0.05.
Figure 5. Over-expression of Hes1 delays chondrocyte hypertrophy and reduces chondrocyte proliferation and skeletal growth.

A. Alcian blue stained skeletal analysis of E14.5 Prx1Cre;Rosa-Hes1^{f/f} (Rosa-Hes1) and WT forelimbs (Aa-b) and hindlimbs (Ac-d). Black arrows depict the missing radius, in the forelimbs, and missing fibula, in the hindlimbs, of Rosa-Hes1 embryos. The red asterisks depict formation of the hypertrophic zone in the WT hindlimbs. (B) ABH/OG staining (Ba-b) and in situ hybridization for Col10a1 (Bc-d) and Mmp13 (Be-f) on E14.5 WT and Rosa-Hes1 humerus sections. (C) BrdU IHC on E14.5 WT and Rosa-Hes1 humerus sections (Ca-b). Statistical analysis showing the percentage of BrdU-positive cells in E14.5 WT and Rosa-Hes1 humerus sections (Cc). (D) Alcian blue/alizarin red stained skeletal analyses of E18.5 WT and Rosa-Hes1 proximal FL elements (Da-b), distal FL elements (Dd-e), proximal HL elements (Dg-h), and distal HL elements (Dj-k). Statistical analysis of the lengths of WT and Rosa-Hes1 humeri (Dc), ulnae (Df), femurs (Di), and tibiae (Dl). Bars represent means ± SD. “*” denotes P<0.05.
Figure 6. HES5 inhibits Sox9 expression via direct transcriptional regulation and is sufficient to delay chondrogenesis.

(A) qPCR gene expression analyses for Hes5, Sox9, Sox5, Sox6, Col2a1 and Acan from RNA isolated from ATDC5 Day 7 cultures. The y-axis represents relative gene expression normalized to β-actin then to Flag controls. Bars represent means ± SD. *** denotes P<0.05.

(B) ChIP analysis for input genomic DNA (positive control), IgG (negative control) pull down, HES1 pull down, and HES5 pull down (Ba). Lane-1 shows input genomic DNA amplification using primer set 1 (P1). Lane-2 shows input genomic DNA amplification using primer set 2 (P2). Lane-3 shows no amplification using P1 primers with an IgG pull down. Lane-4 shows no amplification using P2 primers with an IgG pull Down. Lane-5 shows no amplification using P1 primers with a HES1 pull down. Lane-6 shows no amplification using P2 primers with a HES1 pull down. Lane-7 shows no amplification using P1 primers with a HES5 pull down. Lane-8 shows amplification using P2 primers with a HES5 pull down. qPCR on DNA from HES5 pull down ChIP assay using E10.5 and E11.5 limb-buds (Bb). The y-axis is relative amplification normalized to the input control then to the E10.5 sample. Bars represent means ± SD. ** denotes P<0.05.

(C) C3H10T1/2 cells or ATDC5 cells co-transfected with (Ca) a Sox9 1kb promoter driven luciferase construct, Renilla plasmid, and over expression plasmids for Flag, NICD1, or Hes5 and (Cb) a Sox9 1kb promoter driven luciferase construct containing a N-box mutation, Renilla plasmid, and over expression plasmids for Flag, NICD1, or Hes5. The y-axis is relative luciferase activity normalized to Renilla then to Flag control. Bars represent means ± SD. *** denotes P<0.05.