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Sox9 Directs Hypertrophic Maturation and Blocks Osteoblast Differentiation of Growth Plate Chondrocytes

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SUMMARY

The transcription factor Sox9 is necessary for early chondrogenesis, but its subsequent roles in the cartilage growth plate, a highly specialized structure that drives skeletal growth and endochondral ossification, remain unclear. Using a doxycycline-inducible *Cre* transgene and *Sox9* conditional null alleles in the mouse, we show that *Sox9* is required to maintain chondrocyte columnar proliferation and generate cell hypertrophy, two key features of functional growth plates. Sox9 keeps *Runx2* expression and β-catenin signaling in check and thereby inhibits not only progression from proliferation to prehypertrophy, but also subsequent acquisition of an osteoblastic phenotype. Sox9 protein outlives *Sox9* RNA in upper hypertrophic chondrocytes, where it contributes with Mef2c to directly activate the major marker of these cells, *Col10a1*. These findings thus reveal that Sox9 remains a central determinant of the lineage fate and multistep differentiation program of growth plate chondrocytes and thereby illuminate our understanding of key molecular mechanisms underlying skeletogenesis.

INTRODUCTION

The skeleton is critically important in vertebrates. It forms the body framework, assists in locomotion, and fulfills key physiological functions. It is subject to prevalent inherited and acquired diseases in humans, many of which remain poorly treatable (Woolf and Pfleger, 2003; Rimoin et al., 2007). To help find efficient cures, it is thus essential that we reach a fuller understanding of the basis of the skeleton complex structure and regulation, and in particular the mechanisms underlying its development.

Building the vertebrate skeleton requires the generation of two main tissues, cartilage and bone, at the right time in over 200 locations in the embryo, and subsequently ensuring their proper growth and maturation (Provot and Schipani, 2005). These hard connective tissues greatly differ in composition, function, and regulation, but develop through closely related, mutually interacting processes. Chondrocytes (cartilage-forming cells) and osteoblasts (bone-making cells) derive from osteochondroprogenitors, bipotent cells that arise from multipotent mesenchyme. Skull vault bones and other flat bones form intramembranously through direct differentiation of osteochondroprogenitors into osteoblasts. Other bones form endochondrally, that is, through a cartilage intermediate. Osteochondroprogenitors first condense into precartilaginous masses. Inner cells differentiate into early chondrocytes and perichondrium cells remain uncommitted. Early chondrocytes then undergo further steps of differentiation in a staggered manner, establishing structures referred to as growth plates in reference to their prime contribution to skeletal elongation. Chondrocytes flatten, stack in longitudinal columns, and proliferate actively until reaching the prehypertrophic stage. This stage is a major step in skeletogenesis because the cells switch to a mature phenotypic program and induce osteogenesis in the perichondrium. Following subsequent hypertrophy, another key step in skeleton elongation, chondrocytes terminally mature, and most if not all undergo apoptosis. Osteoblast precursors, endothelial cells, osteoclasts, and hematopoietic cells migrate from the perichondrium into the cartilage remnant to remodel the tissue and lay down bone and marrow (Maes et al., 2010). Each chondrocyte and osteoblast developmental stage is characterized by expression of specific genes (Lefebvre and Smits, 2005). Typical markers include *Col2a1* (collagen 2) and *Acan* (aggrecan) for early chondrocytes; *Fgfr3* (fibroblast growth factor receptor 3) for columnar cells; *Ppr* (parathyroid hormone-related protein receptor), *Ihh* (Indian hedgehog), and *Col10a1* (collagen 10) for prehypertrophic cells; and *Col10a1* only for hypertrophic cells. Terminal chondrocytes express *Mmp13* (matrix metalloproteinase 13) and *Bsp* (bone sialoprotein), and mineralize the extracellular matrix, as do mature osteoblasts, whereas early osteoblasts express *Osx* (Osterix) and *Col1a1* (collagen 1).

Like other developmental processes, skeletogenesis is spatially and temporally governed by intricate networks of regulatory molecules, among which lineage-specific transcription factors have key fate-determining roles (Karsenty et al., 2009). The Sry-related transcription factor Sox9 is one of them

(Akiyama, 2008). Research on its functions started when *SOX9* heterozygous mutations were found to cause campomelic dysplasia, a severe form of dwarfism affecting all cartilage and endochondral structures (Foster et al., 1994; Wagner et al., 1994). *Sox9* expression is turned on in mesenchymal precursors, maintained in developing chondrocytes until prehypertrophy, but turned off in other lineages. Sox9 is absolutely necessary for chondrocyte specification and early differentiation (Bi et al., 1999; Akiyama et al., 2002). It directly activates all major cartilage-specific extracellular matrix genes expressed by early chondrocytes, and is helped in this function by two distant relatives, Sox5 and Sox6 (Lefebvre and Smits, 2005). The three Sox proteins are needed and sufficient for early chondrogenesis, and thus referred to as the chondrogenic trio (Ikeda et al., 2004). Subsequent differentiation of chondrocytes is directed from the prehypertrophic stage by the Runt domain transcription factors Runx2 and Runx3, and by MADS box transcription factors, mainly Mef2c (Takeda et al., 2001; Yoshida and Komori, 2005; Arnold et al., 2007). Runx2 is also necessary for osteoblast specification and differentiation (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997), along with the zinc finger transcription factor Osx (Nakashima et al., 2002).

Strong expression of *Sox9* in growth plate chondrocytes until prehypertrophy and marked shortening of campomelic dysplasia growth plates strongly suggest that Sox9 has important roles in growth plates. These roles, however, remain unclear. *Sox9* was first proposed to inhibit chondrocyte proliferation and hypertrophy (Akiyama et al., 2002, 2004), but was more recently proposed to be necessary for chondrocyte survival and hypertrophy, and to delay terminal maturation (Hattori et al., 2010; Ikegami et al., 2011). Some of the data in these previous studies were difficult to interpret because the mouse transgenes that were used to inactivate or overexpress *Sox9* were active from the precursor or early chondrocyte stage, causing defects in cartilage primordia that precluded definitive identification of growth plate-specific roles for *Sox9*. To solve this problem and clarify the roles of *Sox9* in the growth plate, we used in this study mice harboring *Sox9* conditional null alleles and a *Cre* transgene inducible in differentiated growth plate chondrocytes. We show that Sox9 continues to fulfill essential roles at several stages of differentiation of these cells to ensure cartilage-mediated skeletal growth and coordinate this process with endochondral ossification.

RESULTS

Generation of a *Cre* Transgene Inducible in Differentiated Chondrocytes

We previously showed that an *Acan* (aggrecan) upstream enhancer was sufficient to activate the *Col2a1* promoter in differentiated chondrocytes in transgenic mice (Han and Lefebvre, 2008). Here, we cloned these regulatory elements into a bigenic template (Utomo et al., 1999) to generate a mouse line expressing an *Acan* enhancer-driven, tetracycline-inducible Cre (*ATC*) transgene (see Figure S1A available online). We characterized transgene activity using a dual $R26^{TG}$ Cre reporter (Muzumdar et al., 2007). This reporter expresses Tomato ubiquitously before Cre recombination and GFP following recombination. $R26^{TG}ATC$ fetuses at embryonic day 17.5 (E17.5) showed Cre activity in few cells in the end of growth plates, nuclei pulposi and bone in absence of tetracycline (Figures S1B–S1D). When their mothers drank water containing the tetracycline compound doxycycline (Dox) from E15.5, they showed Cre-mediated recombination within 2 days in all differentiated chondrocytes (except in epiphyseal lateral sides) and nucleus pulposus cells, and in some myoblasts and bone cells, but none in perichondrium cells and other cell types (Figures S1B–S1E). We concluded that *ATC* should be an excellent model to study gene functions in growth plate chondrocytes independently of functions in precursors and most other cell types.

Sox9 Is Necessary to Maintain Functional Growth Plates

To determine whether Sox9 has specific roles in the growth plate, we bred females carrying *Sox9* conditional null alleles ($Sox9^{fl/fl}$; Kist et al., 2002) with $Sox9^{fl/+}ATC$ males, treated them with Dox from E15.5, and analyzed fetuses daily afterward. *Sox9* RNA remained abundant in nonhypertrophic chondrocytes of $Sox9^{fl/fl}$ and $Sox9^{fl/+}$ control fetuses through E18.5, but it was lost in most growth plate cells of $Sox9^{fl/fl}ATC$ mutants by E17.5 (Figure 1A). Control epiphyses and growth plates kept the same length over time while primary ossification centers elongated, reflecting balanced turnover of cartilage, and $Sox9^{fl/+}ATC$ growth plates remained virtually normal (Figures 1A and 1B). In contrast, columnar zones shortened in $Sox9^{fl/fl}ATC$ growth plates, chondrocytes lost the ability to enlarge, and endochondral bones stopped elongating by E17.5, resulting in severe dwarfism (Figures 1A and 1C). These data thus revealed that *Sox9* is essential to maintain functional growth plates.

Sox9 Is Needed to Maintain Growth Plate Chondrocyte Proliferation and Viability, Delay Prehypertrophy, and Allow Hypertrophy

Shortening of *Sox9* mutant columnar zones could result from chondrocyte slow proliferation, untimely death, or precocious maturation. In bromodeoxyuridine (BrdU) incorporation assays performed after 2 days of fetus treatment with Dox, growth plate chondrocytes from $Sox9^{fl/fl}ATC$ fetuses showed normal proliferation rates in epiphyses, but columnar cells growth arrested about three times as fast as control cells (Figure 2A). This result was confirmed by immunostaining for Ki-67 (Figure S2A). In TUNEL assay, $Sox9^{fl/fl}$ growth plates never showed a significant rate of chondrocyte death until the cells reached the ossification front (Figure 2B). In contrast, $Sox9^{fl/fl}ATC$ growth plates showed 0.1% of dying cells in the columnar zone and 4.5% in the prehypertrophic zone after 2 days on Dox, and 2.8% and 24.8% in the same respective zones by the next day. Immunoreactivity for cleaved caspase 3 indicated that mutant cell death occurred through apoptosis (Figure S2B). Thus, *Sox9* deletion led to premature growth arrest of columnar cells, followed by apoptosis. Consistent with precocious prehypertrophy, $Sox9^{fl/fl}ATC$ chondrocytes turned on *Ppr* and *Ihh* concomitantly with growth arrest (Figure 2C). Surprisingly, however, they failed to activate the hypertrophic markers *Col10a1*, *Bmp6*, and *Has2*, but ultimately expressed the terminal chondrocyte and osteoblast marker *Mmp13* (Figure 2D and Figure S2C). They expressed the chondrocyte proliferation inhibitor *Fgfr3* at a normal level, and chondrocyte maturation inhibitors *Pthrp* (parathyroid hormone related peptide) and *Ptc*

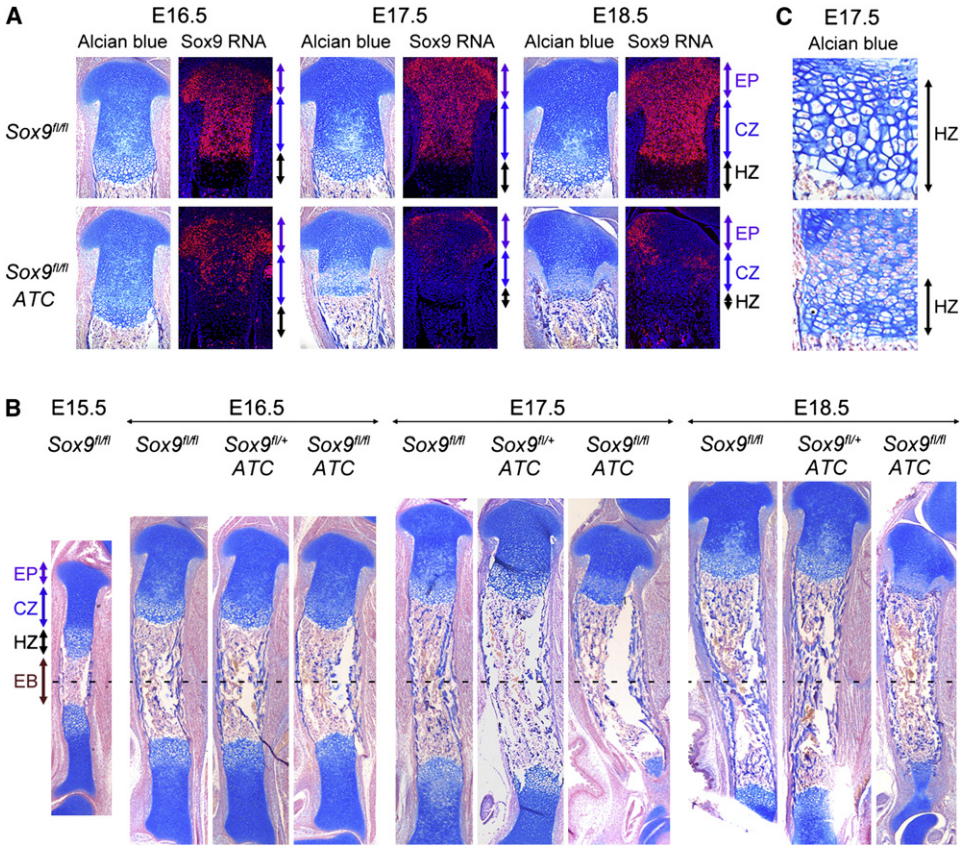

Figure 1. Sox9 Is Required to Maintain Functional Growth Plates

(A) Histological aspect and *Sox9* expression of proximal tibia growth plates from *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* fetuses treated with Dox from E15.5 and collected at E16.5, E17.5, and E18.5. Sections were stained with Alcian blue (which binds aggrecan) and nuclear fast red, or hybridized with a *Sox9* RNA probe (red) and DNA-binding DAPI dye (blue). CZ, columnar zone; EP, epiphyses; HZ, hypertrophic zone.

(B) Histological aspect of the entire tibia of *Sox9*$^{fl/fl}$, *Sox9*$^{fl/+}$*ATC*, and *Sox9*$^{fl/fl}$*ATC* littermates treated as described in (A). The dotted line designates the middle of the bone. EB, endochondral bone.

(C) High-magnification images of hypertrophic zones at E17.5.

See also Figure S1.

(Patched) at a high level (Figure 2E). They strongly expressed *Runx2* and *Mef2c*, which encode transcription factors required for chondrocyte maturation, and *Hdac4*, encoding a histone deacetylase inhibitor of these factors (Vega et al., 2004), indicating that *Sox9* may delay prehypertrophy by downregulating *Runx2* and *Mef2c*, but must control hypertrophy differently. Overall, these data thus showed that *Sox9* is required to maintain growth plate chondrocyte proliferation, delay prehypertrophy, and allow hypertrophy before terminal maturation and apoptosis.

Sox9 Contributes with Mef2c to Directly Activate *Col10a1*

Based on the facts that *Col10a1* is expressed exclusively in prehypertrophic and hypertrophic chondrocytes and that Sox9 is required for hypertrophy, we investigated whether Sox9 directly controls *Col10a1*. As a prerequisite to this function, Sox9 protein must be present in *Col10a1*-expressing chondrocytes. Using RNA in situ hybridization, we confirmed (Lefebvre et al., 1998) that *Sox9* and *Col10a1* RNAs overlap each other in prehypertrophic cells, and immunostaining revealed that the Sox9 protein outlives its RNA and remains nuclear in upper hypertrophic chondrocytes (Figures 3A and 3B). Concomitant loss of *Sox9* RNA and protein in Dox-treated *Sox9*$^{fl/fl}$*ATC* growth plates ascertained antibody specificity. Thus, Sox9 protein is present in cells activating *Col10a1*.

To test whether Sox9 could transactivate *Col10a1*, we searched for putative Sox9 binding sites in the *Col10a1* promoter and hypertrophic enhancer (Zheng et al., 2009). We found only one sequence that was evolutionarily conserved and matched a Sox9 binding site: a pair of sites resembling the Sox consensus $C^A/_TTTG^A/_T{}^A/_T$, oriented head to head, and separated by 3–5 nucleotides (Sock et al., 2003). This sequence was located at −186/−169 (Figures 3C and 3D), near a Mef2c binding site (Arnold et al., 2007). Electrophoretic mobility shift assays showed the ability of each protein to bind its site (Figure 3E), but no evidence of cooperative binding (Figure 3F). Chromatin immunoprecipitation assays revealed efficient binding of both proteins to the *Col10a1* promoter in primary growth plate chondrocytes forced to upregulate *Col10a1* by treatment with okadaic acid, a potent inhibitor of Pp2a phosphatase (Kozhemyakina et al., 2009) (Figures 3G and 3H).

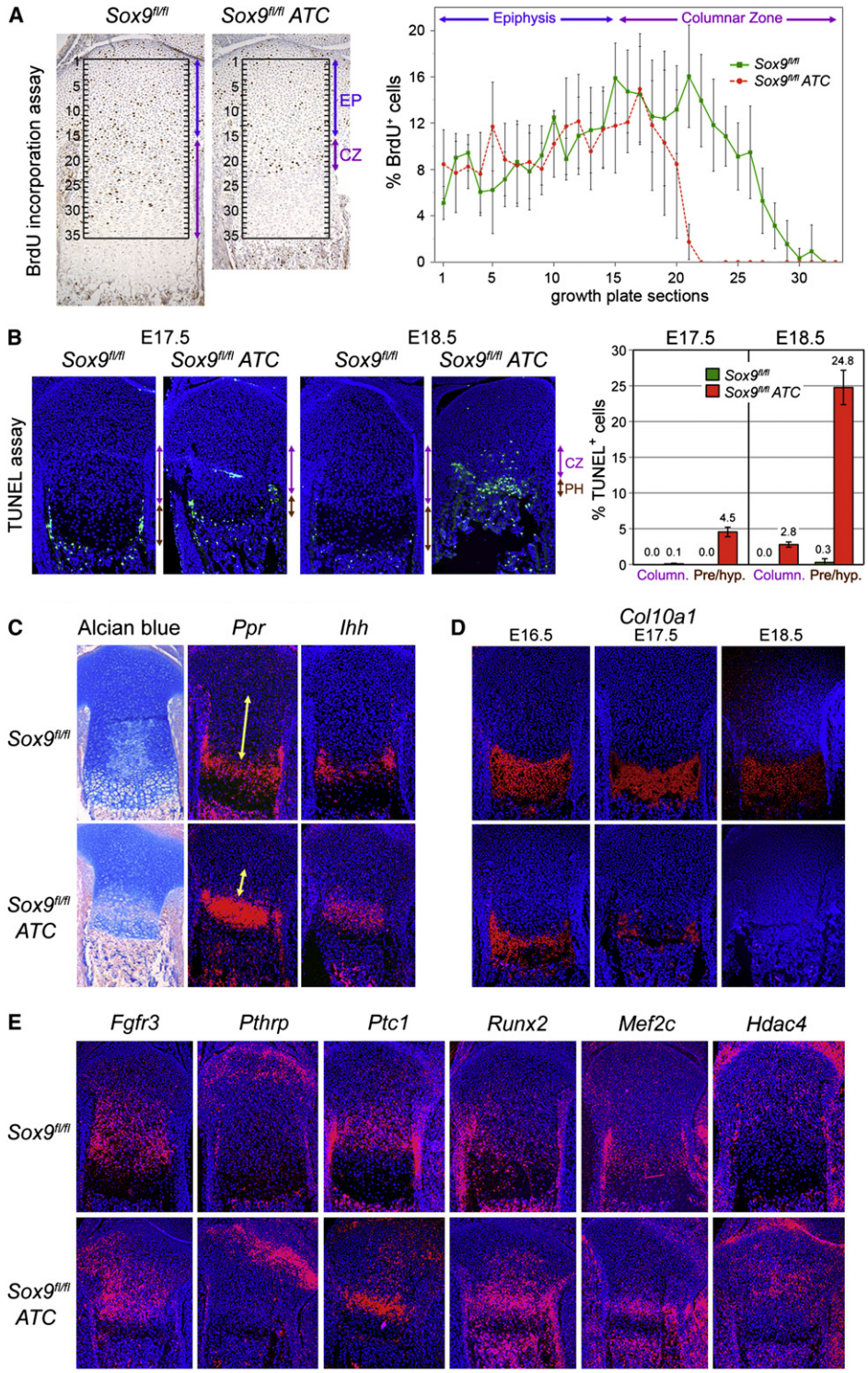

Figure 2. *Sox9* Is Required for Chondrocyte Proliferation, Survival, and Hypertrophy but Delays Prehypertrophy

(A) BrdU incorporation assay in tibia proximal growth plates of *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5 and harvested at E17.5. Left, representative images of BrdU antibody staining. The box shows 35 segments in the epiphysis (EP) and columnar zone (CZ), in which BrdU-positive cells (dark brown nuclei) were counted. Right, graph showing the percentage of positive cells in control and mutant growth plates in each segment. Data are presented as the average with SD of measurements made in three nonadjacent sections from each of three embryos per genotype.

(B) TUNEL assay in embryos similar to those shown in (A), but collected at E17.5 and E18.5. Left, representative images of data. Dying cells are seen as green dots; cell nuclei are seen as blue dots. Right, quantification of the percentage of TUNEL-positive cells in the columnar (CZ) and prehypertrophic/hypertrophic (PH) zones. Data are shown as the averages with SD obtained for three nonadjacent sections in each of three embryos per genotype.

To test whether Sox9 binding to the *Col10a1* promoter resulted in transactivation, we constructed *Col10a1/βgeo/EGFP* reporters (Figure S3A). As expected, the activity of the *Col10a1* promoter was upregulated in primary chondrocytes when the reporters contained the *Col10a1* hypertrophic enhancer and the cells were treated with okadaic acid (Figure 4A). Forced expression either human SOX9 or mouse Mef2c enhanced transactivation, and forced expression of both proteins resulted in additive or slightly stronger effects. The proteins acted similarly in nonchondrocytic 293T cells, even though the enhancer was virtually inactive in these cells (Figure S3B). In agreement with the protein binding locations, promoter mutagenesis experiments revealed that SOX9 and Mef2c largely mediated their activity through the *Col10a1* −210/−94 sequence (Figure S3C). The mutation of either protein site reduced reporter activity in chondrocytes in absence and presence of exogenous protein, and the mutation of both sites abolished reporter activity (Figure 4B). Taken together, these results support the concept that Sox9 and Mef2c transactivate *Col10a1*, and act additively.

To test this concept in vivo, we generated *Sox9/Mef2c* compound mutants. Single and double heterozygotes obtained using *Prx1Cre*, a transgene active in limb bud mesenchyme, including chondrocyte precursors (Logan et al., 2002), displayed no major histological defects at E18.5 (Figure 4C). *Col10a1* RNA level was slightly but not significantly decreased in single heterozygotes, but was significantly reduced by 2.4-fold in double heterozygotes. Further, and consistent with the reported roles of *Mef2c* (Arnold et al., 2007), $Mef2c^{fl/fl}ATC$ fetuses on Dox since E15.5 showed greatly expanded growth plates by E17.5 and severely delayed *Col10a1* activation (Figure 4D). $Sox9^{fl/+}Mef2c^{fl/fl}ATC$ littermates had similar histological defects, but virtually failed to express *Col10a1*. Thus, Sox9 and Mef2c also act together to activate *Col10a1* in vivo. Interestingly, $Mef2c^{fl/fl}ATC$ mutants initiated *Ppr* and *Runx2* expression in a timely manner and delayed *Ppr* deactivation and *Ihh* activation, whereas $Sox9^{fl/+}Mef2c^{fl/fl}ATC$ littermates exhibited stronger and earlier activation of *Ppr*, and a partial rescue of the *Ihh* activation delay. Thus, Sox9 inhibits chondrocyte prehypertrophy from the *Ppr* expression stage, Mef2c activates it from the *Ihh* expression stage, and both proteins are then required for chondrocyte hypertrophy and *Col10a1* expression.

Sox9 Prevents Osteoblastic Differentiation of Prehypertrophic Chondrocytes

Besides *Col10a1* transactivation, we considered the possibility that Sox9 could use additional, indirect mechanisms to account for its absolute requirement for chondrocyte hypertrophy. Since *Runx2* is necessary for osteoblast differentiation and chondrocyte maturation, its inability to cause hypertrophy of *Sox9*-deficient chondrocytes, despite being strongly expressed, made us wonder whether $Sox9^{fl/fl}ATC$ prehypertrophic chondrocytes were switching lineage. After 2 days on Dox, control and mutant prehypertrophic cells, as identified by expression of their exclusive marker, *Ihh*, still contained RNA for *Col2a1*, but none for *Sox9*, *Sox5*, and *Sox6* (Figure 5A and data not shown). Since the Sox trio is required for *Col2a1* transcription, the presence of *Col2a1* RNA in these cells was unlikely due to recent production, but more likely due to earlier production of this slowly decaying RNA (Murakami et al., 2000). Importantly, this result attested to the chondrocyte origin of the cells. Strikingly, mutant prehypertrophic cells upregulated not only *Runx2*, but also *Osx*, which is required for osteoblast differentiation. *Osx* RNA is normally present at an extremely low level in prehypertrophic chondrocytes compared to osteoblasts, but *Sox9*-deficient chondrocytes contained as much *Osx* RNA as periosteum and endochondral bone osteoblasts. Further, they contained low levels of *Col1a1* RNA, which is abundant in osteoblasts but absent in chondrocytes, and *Bsp* RNA (bone sialoprotein), a marker of osteoblasts and terminal chondrocytes. $Sox9^{fl/fl}ATC$ prehypertrophic chondrocytes were thus transitioning into early osteoblasts within 2 days on Dox. One day later, these mutant cells still contained *Col2a1* RNA, but no longer contained *Ihh* RNA, and they were expressing *Col1a1* and *Bsp* RNA as strongly as wild-type osteoblasts (Figure 5B). Furthermore, they were strongly expressing genes involved in matrix mineralization, such as *Alpl* (alkaline phosphatase) and *Ank* (ankylosis), and were surrounded by mineralized matrix (Figure 5C). They had thus completed differentiation into osteoblasts and reached the mature stage in this lineage. Confirming conversion into a single lineage, $Sox9^{fl/fl}ATC$ growth plate cells treated for 3 days with Dox were negative for markers of other mesenchyme-derived cell types, including *Gdf5* (joint precursors), *MyoD* (myoblasts), *Pparg* (adipocytes), and *Scx* (tenocytes) (Figure S4). We concluded that *Sox9* is necessary to maintain the lineage fate of prehypertrophic cells and prevent acquisition of an osteoblastic phenotype.

Sox9 Secures the Fate of Growth Plate Chondrocytes by Reducing β-Catenin Activity

Sox9 and β-catenin oppose each other in osteochondroprogenitors to specify the chondrocyte and osteoblast lineages, respectively (Day et al., 2005; Hill et al., 2005). We thus considered the possibility that Sox9 blocks expression of an osteoblastic phenotype by growth plate chondrocytes by opposing β-catenin signaling. We observed that $Sox9^{fl/fl}ATC$ prehypertrophic chondrocytes on Dox for 2 days were avidly expressing *Tcf1* (T cell-specific transcription factor 1) and *Ccnd1* (cyclin D1), two targets of β-catenin signaling very active in osteoblasts (Figure 6). Inactivation of β-catenin along with *Sox9* in $Sox9^{fl/fl}Ctnnb^{fl/fl}ATC$ fetuses resulted in repression of these targets and partial downregulation of *Osx*. Importantly, it prevented *Col1a1* and *Bsp* activation, thus osteoblastic differentiation, but did not prevent precocious activation of *Ihh* and upregulation of *Runx2*, and did not rescue cell hypertrophy and *Col10a1* expression (Figure 6 and Figure S5A). Thus, Sox9 blocks expression of an osteoblast phenotype by chondrocytes at least in part by suppressing β-catenin signaling, but does not use this mechanism to keep *Runx2* expression low and permit hypertrophy. In

(C) *Ppr* and *Ihh* RNA in situ hybridization of sections similar to those in (A). The double arrow shows the length of the columnar zone.
(D) *Col10a1* RNA in situ hybridization of sections from $Sox9^{fl/fl}$ and $Sox9^{fl/fl}ATC$ littermates treated with Dox from E15.5 and collected at E16.5, E17.5, and E18.5.
(E) RNA in situ hybridization of sections similar to those in (A). Probes are indicated.
See also Figure S2.

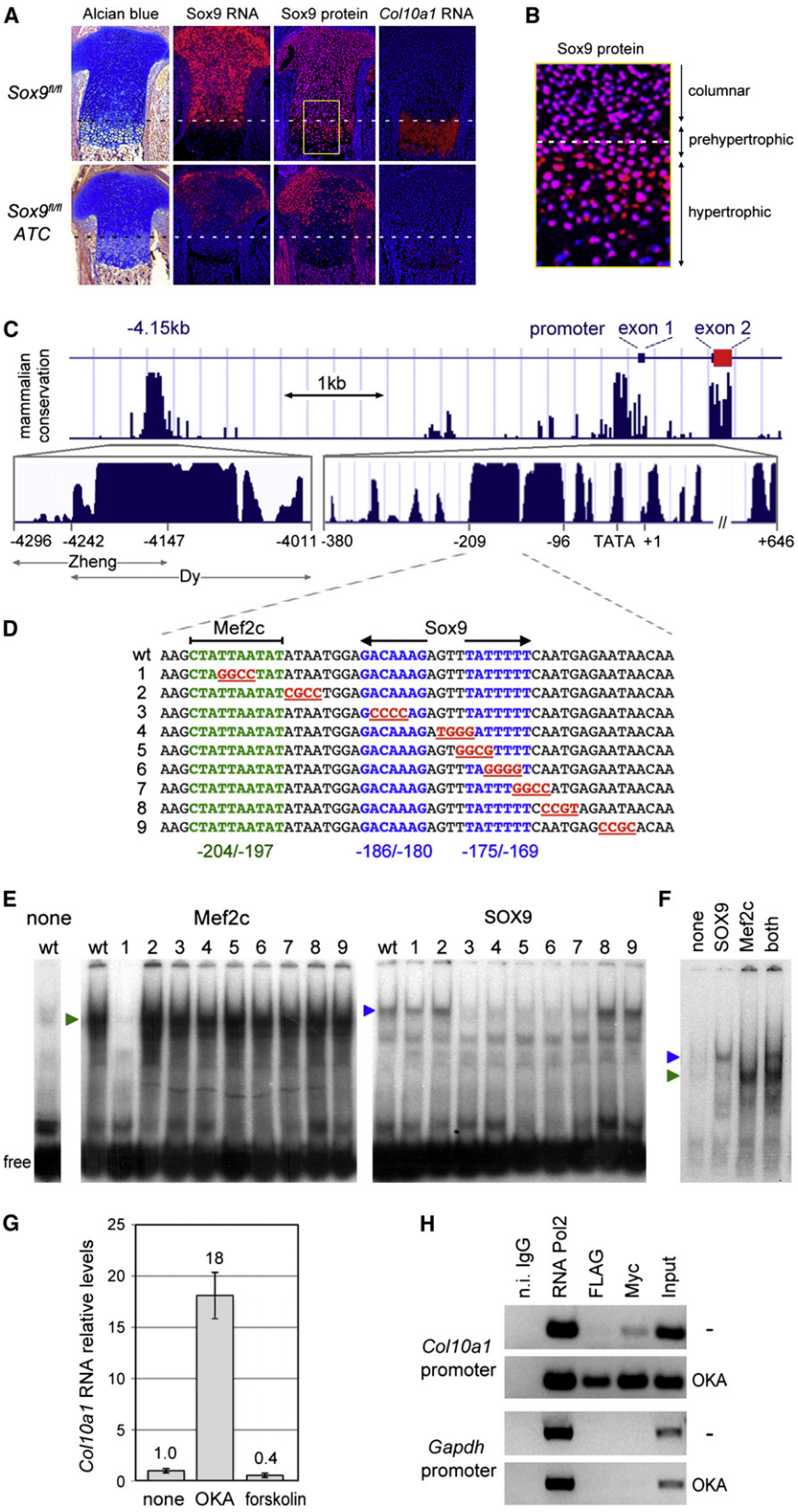

Figure 3. Sox9 Protein Outlives Its RNA in Upper Hypertrophic Chondrocytes and Binds Next to Mef2c on the *Col10a1* Promoter

(A) Sox9 RNA in situ hybridization and protein immunostaining in tibia sections from E17.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. The dotted line designates the middle of the prehypertrophic zone.

(B) High-magnification image of the growth plate region boxed in yellow in (A).

(C) Top, mammalian conservation plot for the mouse *Col10a1* locus from −5.0 to +1.5 kb relative to the transcription start site (+1). Peaks denoting high conservation are seen at −4.15 kb, and in the promoter, exon 1, and exon 2. Bottom left, enlargement of the −4.15 kb region. Double arrows designate the enhancers described by Zheng et al. (2009) and used in this study in *Col10a1* reporters (Dy). Bottom right, enlargement of the −380/+646 bp region used in this study in *Col10a1* reporters.

(D) Alignment of the −207/−154 bp *Col10a1* upper-strand wild-type sequence (wt) with sequences (1–9) harboring four consecutive nucleotide changes. Mef2c and Sox9 binding sites and mutant nucleotides are highlighted. Arrows show Sox-like sites oriented head to head. Double-stranded oligonucleotides corresponding to these sequences were used in EMSA.

(E) EMSA with extracts from Cos1 cells forced to express no exogenous protein (none), SOX9, or Mef2c. Probes are indicated on top of the lanes. Arrowheads indicate SOX9/DNA (blue) and Mef2c/DNA (green) complexes. Unbound probes (free) are also shown.

(F) EMSA performed as in (E) with the wild-type probe and combinations of empty, SOX9, and Mef2c extracts. Gels were run longer than in (E) to separate SOX9/DNA from Mef2c/DNA complexes.

(G) Real-time RT-PCR quantification of *Col10a1* RNA present in primary chondrocytes treated with no drug, okadaic acid (OKA), or forskolin for 24 h. Each bar corresponds to the average with SD of measurements made in triplicate cultures in a representative experiment. *Col10a1* RNA levels were normalized to *18S* rRNA levels.

(H) Chromatin immunoprecipitation in primary chondrocytes transfected with FLAG-SOX9 and Myc-Mef2c expression plasmids and treated without (−) or with OKA. Antibodies were nonimmune (n.i.), or directed against RNA polymerase 2, FLAG, or Myc. PCR products for *Col10a1* and *Gapdh* promoters were obtained from input material and precipitated chromatin and resolved by electrophoresis.

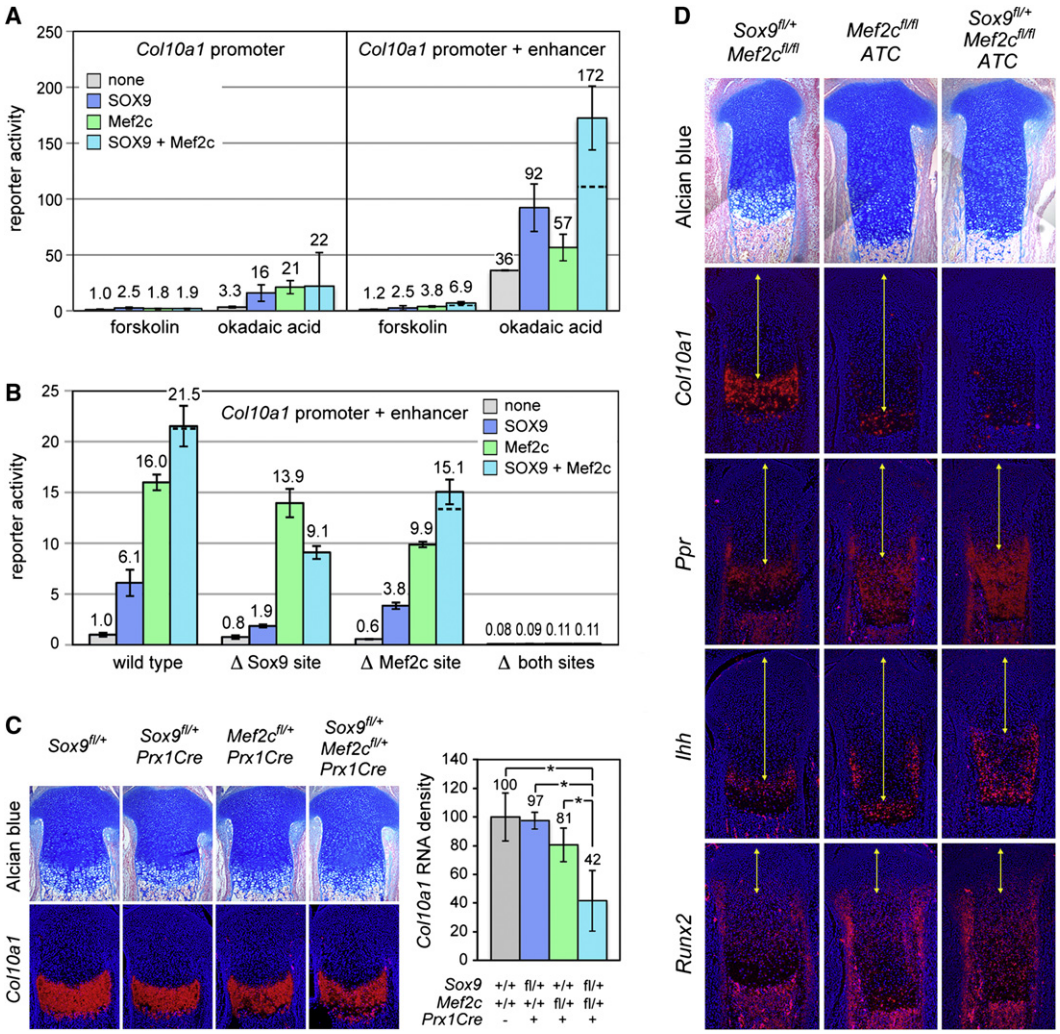

Figure 4. *Sox9* and Mef2c Act Cooperatively to Activate *Col10a1*

(A) Relative activity of *Col10a1* reporters transfected in primary chondrocytes along with SOX9 and Mef2c expression plasmids. Cells were treated with forskolin or okadaic acid. Reporter activities were normalized for transfection efficiency and are presented as average with SD of biological triplicates in one representative experiment. Dotted lines designate expected values from additive effects of SOX9 and Mef2c.

(B) Effect of mutations (Δ) in the Sox9 and Mef2c binding sites on the relative activity of a *Col10a1* reporter transfected in primary chondrocytes treated with okadaic acid. Reporter activities were normalized for transfection efficiency and are presented as average with SD of biological triplicates in one representative experiment. Dotted lines designate expected values from additive effects of SOX9 and Mef2c.

(C) Histology and *Col10a1* expression in tibia proximal growth plates of E18.5 *Sox9/Mef2c* mutants generated using *Prx1Cre*. Left, images of sections stained with Alcian blue and hybridized with a *Col10a1* RNA probe. Right, quantification of the density of *Col10a1* RNA level in hypertrophic zones. Data are presented as the averages with SD obtained for three sections per embryo. *$p < 0.05$ (Student's t test).

(D) Histology and gene expression analysis of tibia proximal growth plates of E18.5 *Sox9/Mef2c* mutants generated using *ATC* and treated with Dox from E15.5. Double arrows designate the distance from the articular surface to the onset of marker gene expression.

See also Figure S3.

a complementary experiment, we asked whether forced upregulation of β-catenin signaling in growth plate chondrocytes would suffice to change the fate of the cells. For this purpose, we generated *ATC* fetuses harboring *Ctnnb*^{flEx3}, an allele that allows constitutive activation of β-catenin (Harada et al., 1999). The growth plates of these fetuses exhibited expanded hypertrophic zones, but looked otherwise normal after 2 days on Dox (Figure S5B). Strong expression of *Tcf1* and *Ccnd1* in columnar and prehypertrophic chondrocytes proved upregulation of β-catenin signaling (Figure S5C). *Ihh* and *Runx2* were upregulated and their expression domains were broadened, as was that of *Col10a1*. *Osx* was slightly expressed, but *Col1a1* remained silent. Not surprisingly, therefore, mutant chondrocytes still contained high levels of *Sox9* RNA and protein (Figure S5D). Thus, upregulation of β-catenin signaling in a *Sox9* wild-type context promotes chondrocyte maturation, as previously shown (Tamamura et al., 2005; Guo et al., 2009), but is insufficient to induce osteoblastic differentiation.

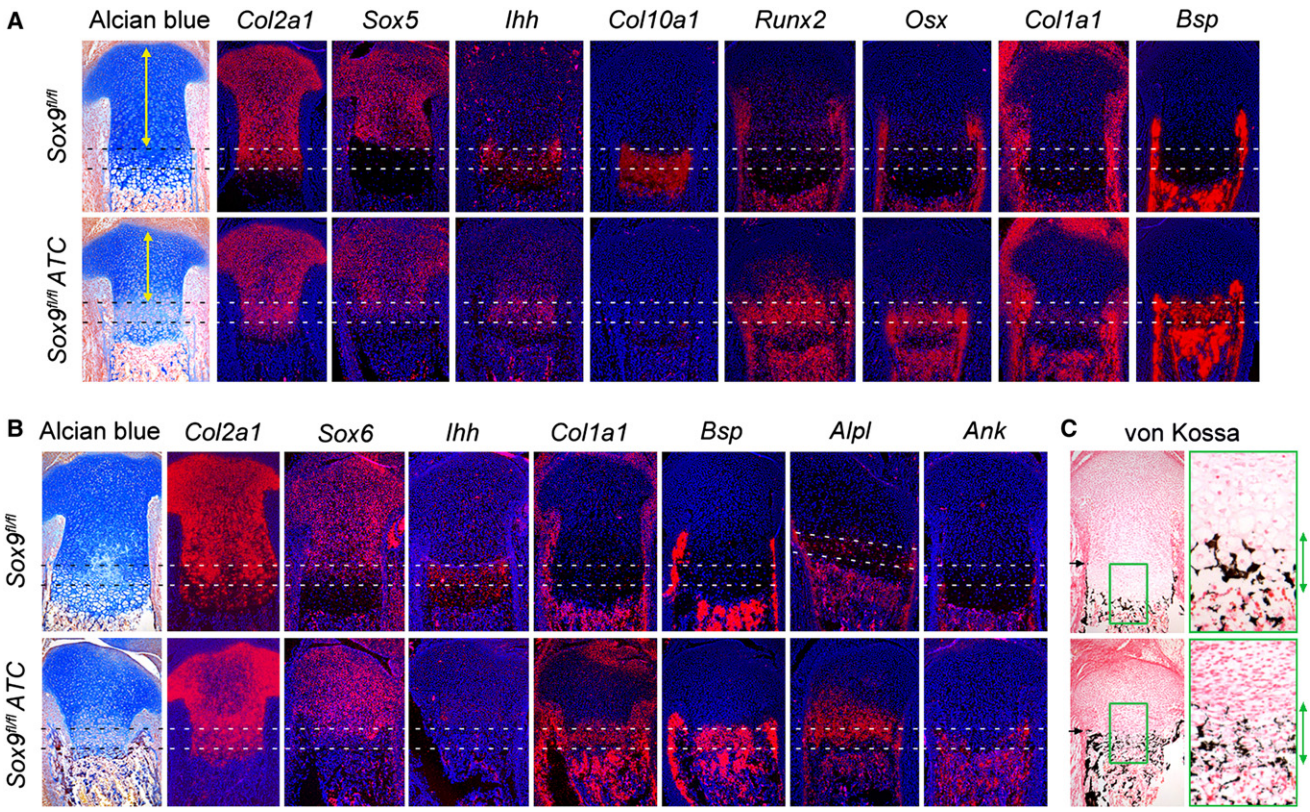

Figure 5. *Sox9* Prevents Osteoblastic Differentiation of Chondrocytes

(A) RNA in situ hybridization of tibia proximal growth plates from E17.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. Double arrows designate the distance from the articular surface to the prehypertrophic zone. Dotted lines delineate the prehypertrophic/osteoblastic zone.

(B) RNA in situ hybridization of tibia proximal growth plates from E18.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5.

(C) Staining with the von Kossa reagent of the same growth plates as in (B). The region boxed in green on the left is shown at high magnification on the right. Black arrows indicate the upper edge of the prehypertrophic/osteoblastic zone. Double green arrows designate mineralized cartilage matrix (dark brown signal).

See also Figure S4.

DISCUSSION

This study clarified and provided novel insights on the roles of Sox9 in the growth plate (Figure 7). It showed that Sox9 maintains columnar proliferation, delays prehypertrophy, and then prevents osteoblastic differentiation of chondrocytes by lowering β-catenin signaling and *Runx2* expression. Further, Sox9 is required for chondrocyte hypertrophy, both indirectly by keeping the lineage fate of chondrocytes and directly by remaining present in upper hypertrophic cells and transactivating *Col10a1* along with Mef2c. It is thus essential to form and maintain functional growth plates.

Most of our findings were made using an *ATC* conditional deletion system, which allows for precise temporal and spatial deletion in differentiated chondrocytes upon induction by doxycycline. The drug was delivered for several days without adverse effects, whereas tamoxifen, which is used for many inducible transgenes, is often abortive in pregnancy. *ATC* was instrumental to uncover growth plate-specific roles of *Sox9* independently of roles in precursor, perichondrium, and joint cells and should be equally useful to study the roles of many other genes in differentiated chondrocytes.

In contrast to inhibition of chondrocyte proliferation, which was inferred from the analysis of fetuses overexpressing SOX9 from the *Col2a1* locus (Akiyama et al., 2004), we found that Sox9 sustains columnar cell proliferation. Akiyama and colleagues compared cell proliferation in control and mutant fetuses using an average value for entire cartilage elements, without considering differences in cell developmental stages that existed between controls and mutants. We measured cell proliferation in discrete areas succeeding each other from the top to the end of the growth plate to account for cell differentiation changes, and we did this in established growth plates promptly after *Sox9* inactivation. This precise assay pinpointed to premature growth arrest of columnar cells, coincident with prehypertrophy, as reported in *Sox5/6* mutants (Smits et al., 2004). Since the Sox trio transactivates cartilage matrix genes, it may influence chondrocyte destiny via regulatory events taking place in this matrix. In addition, it may interact with Ihh or Pthrp signaling, which control the transition from proliferation to prehypertrophy (Karsenty et al., 2009). Supporting this idea, *Sox9* is a target of Pthrp signaling (Huang et al., 2001) and pathway components are normal or upregulated in *Sox9* and *Sox5/6* mutants.

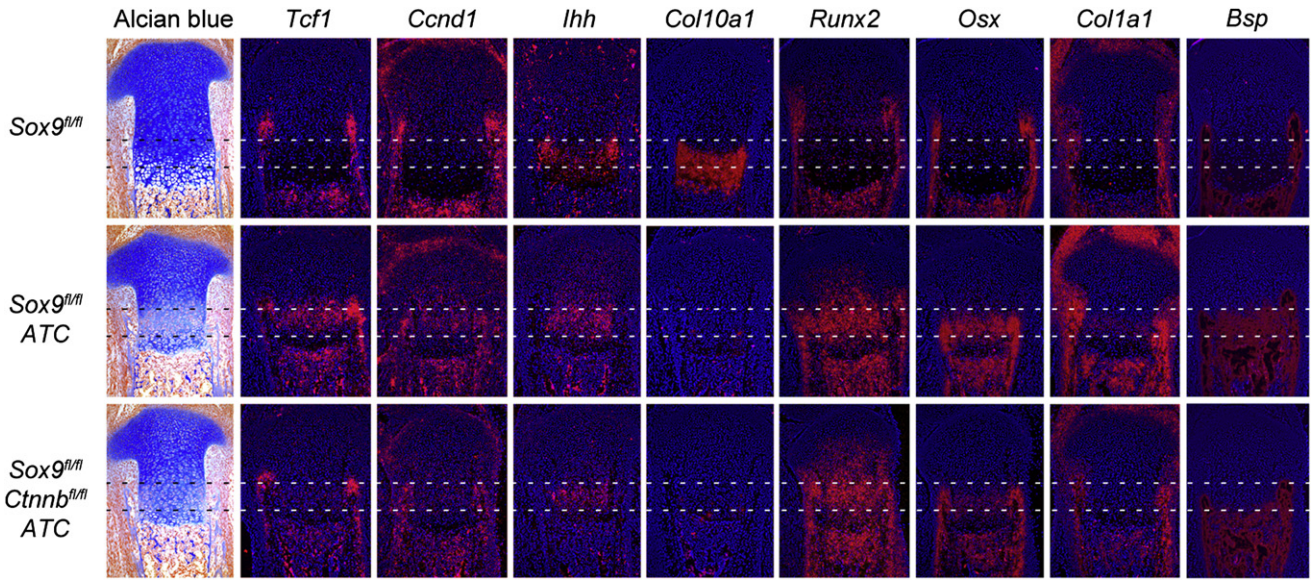

Figure 6. Sox9 Inhibits β-Catenin Signaling in the Growth Plate

Histology analysis and RNA in situ hybridization of tibia proximal growth plates from E17.5 *Sox9*$^{fl/fl}$, *Sox9*$^{fl/fl}$*ATC*, and *Sox9*$^{fl/fl}$*Ctnnb*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. Dotted lines delineate the prehypertrophic/osteoblastic zones.

See also Figure S5.

We observed extensive apoptosis in *Sox9*-deficient growth plates, but only 3 days after *Sox9* inactivation when the cells had converted into mature osteoblasts. This cell fate change and time lapse argue against a direct role for *Sox9* in chondrocyte survival. This conclusion contrasts with a recent report by Ikegami et al. (2011) that Sox9 sustains chondrocyte survival through Pik3ca-Akt pathways. These authors showed that control chondrocytes were positive for pAkt and Sox9 until prehypertrophy. The cells then underwent hypertrophy and terminal maturation before apoptosis. Similarly, mutant chondrocytes lost pAkt upon losing Sox9, and as in our study, went on to undergo prehypertrophy and mineralize their matrix before dying. It is thus unclear that Sox9 and pAkt directly control chondrocyte viability.

Concurring with Ikegami et al. (2011), we found that *Sox9* is required for chondrocyte hypertrophy, including *Col10a1* expression. The presence of Sox9 protein in prehypertrophic and upper hypertrophic chondrocyte nuclei, Sox9 binding to the *Col10a1* promoter in hypertrophic cells, and upregulation of *Col10a1* reporters by Sox9 through this site point to a direct role for Sox9 in this process. The late onset of *Col10a1* expression in chondrocytes implies that early and hypertrophic chondrocytes use distinct mechanisms to specify Sox9 activity. Candidate partners of Sox9 in prehypertrophy and hypertrophy include Runx2/3 and Mef2c. Several Runx2 and Mef2c binding sites in the *Col10a1* enhancer, promoter, and intervening region are needed for reporter activity, and whereas Mef2c is sufficient to activate a full-length *Col10a1* reporter, Runx2 is not (Zheng et al., 2003; Arnold et al., 2007; Li et al., 2011). Runx2 was insufficient in our experiments as well, even in combination with Sox9 and Mef2c (data not shown). In contrast, we found that Sox9 and Mef2c act cooperatively to activate *Col10a1* and *Col10a1* reporters. They bind to adjacent sites in the promoter, and the simultaneous mutation of their sites inactivated reporters. The mutation of either site, however, only partially repressed reporter activation by either or both proteins. This suggests that a functional transactivation complex can be assembled on the *Col10a1* promoter as long as one of the two proteins is present and allowed to bind DNA. This possibility would explain that *Col10a1* might still be transcribed in lower hypertrophic chondrocytes, where Sox9 protein is no longer detected. However, since *Sox9* inactivation abolishes *Col10a1* expression, despite upregulation of *Mef2c*, we proposed and provided evidence that *Sox9* contributes to chondrocyte hypertrophy through both direct and indirect mechanisms.

A major function of Sox9 revealed in this study is to maintain the lineage decision of chondrocytes during prehypertrophy to prevent osteoblastic differentiation and allow hypertrophy. Osteoblastic differentiation of chondrocytes during wild-type endochondral ossification has been debated for decades. Using inducible *CreER* transgenes and an *R26*^{lacZ} reporter, Maes and colleagues (2010) recently showed that chondrocytes accumulate at chondro-osseous junctions while precursor cells emerge from the perichondrium to contribute to the trabecular osteoblast population. The time frame of the experiments was unfortunately too short to definitively conclude on the ultimate fate of chondrocytes, i.e., death or contribution to the trabecular osteoblast population. Because it remains uncertain that chondrocytes can turn into osteoblasts in vivo, we used gold standard markers for cell lineages and differentiation stages to unmistakably prove that *Sox9* mutant chondrocytes acquired a fully differentiated osteoblastic phenotype. This included demonstration that cells containing residual amounts of *Col2a1* RNA, thus of chondrocyte origin, also contained a specific signature of osteoblasts (i.e., both *Col1a1* RNA and high levels of *Osx* RNA). No markers for other mesenchyme-derived lineages were

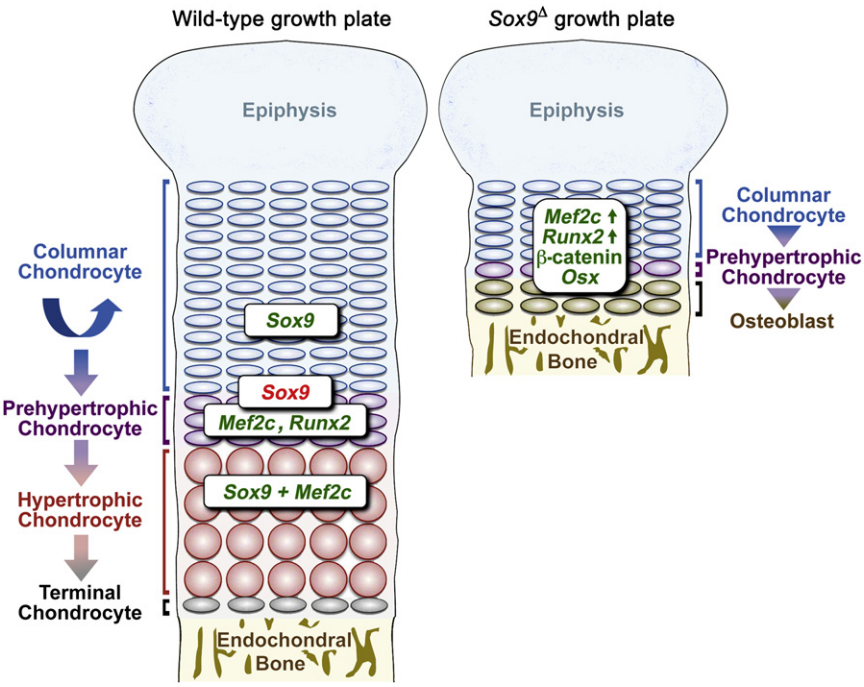

Figure 7. Proposed Roles of Sox9 in the Growth Plate

In a wild-type growth plate (left), *Sox9* is expressed in the columnar, prehypertrophic, and upper hypertrophic zones. It promotes columnar chondrocyte proliferation (green) and prevents prehypertrophy (red) by keeping expression of the major chondrocyte maturation regulators *Runx2* and *Mef2c* in check. Sox9 then contributes, along with Mef2c, to activate hypertrophy (green). *Sox9* deletion in growth plate chondrocytes (right) swiftly leads to columnar cell growth arrest, prehypertrophy, and osteoblastic differentiation through upregulation of *Runx2*, *Osx*, and *Mef2c* expression and increased β-catenin signaling.

expressed in these cells. Although osteoblastic differentiation was thus irrefutable, it occurred only in prehypertrophic cells, indicating that permissive conditions were met only at that stage. These conditions undoubtedly included *Ihh* expression, as Ihh induces osteoblastogenesis of adjacent perichondrial cells (St-Jacques et al., 1999). A key difference that normally allows prehypertrophic chondrocytes to maintain a distinct lineage fate from perichondrium neighbors is their expression of *Sox9*. Wild-type terminal chondrocytes resemble osteoblasts in that they express *Mmp13* and induce matrix mineralization, but they do not express RNA for *Col1a1* or the osteoblast differentiation factor *Osx*. They may thus contribute to bone formation as mature osteoblast-like cells rather than genuine osteoblasts, while perichondrium-derived cells undergo full osteoblastogenesis.

Searching for mechanisms whereby Sox9 prevents osteoblastic conversion of chondrocytes, we focused on β-catenin, because the two factors specify the fate of osteochondroprogenitors in opposite ways. Whereas *Sox9* inactivation in neural crest led to bone formation in prospective cartilage sites (Mori-Akiyama et al., 2003), β-catenin inactivation in skull vault precursors and endochondral bone perichondrium led to ectopic chondrogenesis (Day et al., 2005; Hill et al., 2005). Moreover, endogenous and constitutively activated β-catenin were shown to silence *Sox9* in undifferentiated synovial joint and limb bud mesenchyme, respectively (Hill et al., 2006; Später et al., 2006). In addition to β-catenin blocking *Sox9* expression, the two proteins were shown to oppose each other's action by mutually inducing their proteasomal degradation (Akiyama et al., 2004; Topol et al., 2009). Our study shows that endogenous Sox9 inhibits β-catenin signaling in the growth plate and thereby blocks osteoblastic conversion of prehypertrophic cells. This result is consistent with Sox9 inducing β-catenin degradation.

Interestingly, we showed that increasing β-catenin signaling through constitutive activation of the protein was insufficient to change the fate of Sox9 wild-type growth plate chondrocytes, and in contrast to findings in undifferentiated mesenchyme, these differentiated chondrocytes were protected from β-catenin signaling-mediated *Sox9* gene repression and protein degradation. One possible explanation can be provided based on the facts that Sox9 induces degradation of endogenous β-catenin by recruiting glycogen synthase kinase 3 (Topol et al., 2009) and that the constitutively active form of β-catenin expressed in our mutants is insensitive to this enzyme. Thus, Sox9 protein may have escaped degradation because it would normally be codegraded with β-catenin. This explanation, however, does not explain how β-catenin signaling leads to Sox9 repression unless, as previously proposed (Kumar and Lassar, 2009), Sox9 maintains its own expression in chondrocytes through positive feedback. Upregulation of *Runx2* is certainly another factor that led *Sox9*-deficient chondrocytes toward osteoblastogenesis, since *Runx2* is required for the latter process. However, *Runx2* upregulation occurred to the same extent in *Sox9*/*Ctnnb*-deficient and *Sox9*-deficient chondrocytes, proving that it is not sufficient on its own to change the fate of *Sox9*-deficient chondrocytes. Supporting this conclusion, forced expression of a *Runx2* transgene in chondrocytes was shown to lead to chondrocyte ectopic maturation, but not to osteoblastic differentiation (Takeda et al., 2001). Our constitutively activated β-catenin mutants showed slight upregulation of *Runx2* expression, but increased chondrocyte maturation rather than osteoblastic differentiation, proving that the combination of both events was insufficient to change the fate *Sox9* wild-type chondrocytes. It is possible, however, that it was sufficient in the absence of *Sox9*, as Sox9 may be critical to shift the activities of Runx2 and β-catenin from osteoblast differentiation to chondrocyte maturation.

In conclusion, this study has demonstrated that Sox9 has more roles in promoting chondrogenesis than previously realized. Like other fate-determining transcription factors in their respective lineages (Tapscott, 2005; Karsenty et al., 2009), Sox9 is involved at many steps of the chondrocyte differentiation

pathway. It is necessary both to specify and maintain the lineage choice of the cells and to activate stage-specific markers. *Sox9* inactivation in the growth plate resulted in dwarfism due to shortening of columnar and hypertrophic zones and in advanced ossification due to premature prehypertrophy and matrix mineralization. These typical features of campomelic dysplasia imply that the disease is likely due to growth plate defects in addition to cartilage primordia defects, as previously proposed (Bi et al., 2001). They also suggest that changes in *Sox9* gene expression or protein activity in growth plates may contribute to many other types of defective growth diseases. Further, while conversion of chondrocytes into osteoblastic cells may not to be a normal process, this study has revealed that differentiated chondrocytes maintain a high degree of lineage plasticity, and thus suggests that chondrocyte ectopic hypertrophy and osteophyte formation, typical features of osteoarthritis, could result from changes in Sox9 activity. This study thus illuminates our understanding of the essential roles of Sox9 in chondrogenesis and provides new insights on mechanisms that may underlie both inherited and acquired skeletal diseases.

EXPERIMENTAL PROCEDURES

Mice

Mice were used according to federal guidelines and as approved by the Cleveland Clinic Institutional Animal Care and Use Committee. *ATC* mice were generated as described (Figure S1). Other alleles and transgenes are described in Results. Data were reproduced with two or more pairs of control and mutant littermates. Dox (D9891, Sigma) was administered at 2 mg/ml in drinking water with 5% sucrose.

Assays on Tissue Sections

Sections of paraformaldehyde-fixed paraffin-embedded tissues were analyzed histologically following staining with nuclear fast red and Alcian blue or the von Kossa reagent using standard protocols. RNA in situ hybridization was performed using ^{35}S-labeled RNA probes (Smits et al., 2004; Nakashima et al., 2002; Arnold et al., 2007; Dy et al., 2010; Table S1). Sox9 immunostaining was performed after antigen retrieval in sodium citrate using rabbit polyclonal anti-Sox9 antibody (1/200; AB5535, Chemicon) and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (1/500; Invitrogen). Mounting was performed with DAPI-containing Vectashield medium (Vector Laboratories). TUNEL staining was performed using an alkaline phosphatase In Situ Cell Death Detection Kit (Roche). BrdU incorporation was measured as described (Smits et al., 2004). Data were visualized with a Leica DM2500 microscope, captured with a Qimaging Micropublisher 5.0 RTV digital camera, and processed with Adobe Photoshop CS4 software. RNA in situ signal density was quantified using Image-Pro Plus 6.1 software (Media Cybernetics).

Col10a1 Sequence Analysis and Reporters

Col10a1 sequences and conservation plots were downloaded from the University of California in Santa Cruz genome browser (http://genome.ucsc.edu/). *Col10a1* reporters were constructed as described (Figure S3A).

Transfection and Real-Time RT-PCR

Primary chondrocytes were prepared from newborn mouse rib cages as described (Han and Lefebvre, 2008) and cultured in Dulbecco's modified Eagle's medium/F12 medium with 5% fetal calf serum and 0.5× ITS (GIBCO). For reporter assay, 10^5 cells were plated per 24-well dish in the presence of 4 U/ml hyaluronidase (Sigma). Transfection mixtures contained 1.2 μl FuGENE 6 (Roche), 250 ng *Col10a1* reporter, 40 ng pGL3 control plasmid (Promega), 100 ng expression plasmid encoding no protein, FLAG-SOX9 (30 ng), or Myc-Mef2c (70 ng). Cells were treated with 50 nM okadaic acid or 25 μM forskolin (Kozhemyakina et al., 2009) after 24 hr and assayed for luciferase and β-galactosidase activities (Applied Biosystems) 24 hr later. Reporter activities were normalized for transfection efficiency and are presented as average with SD of biological triplicates in one representative experiment. Total RNA was prepared using TRIzol (Invitrogen) and assayed by real-time RT-PCR as described (Wang et al., 2007).

Electrophoretic Mobility Shift Assay and Chromatin Immunoprecipitation

EMSA, Cos1 protein extracts, and oligonucleotide probes were prepared as described (Dy et al., 2010). Chromatin immunoprecipitation was performed as described (Bhattaram et al., 2010) using newborn rat growth plate primary chondrocytes transiently transfected with FLAG-SOX9 and Myc-Mef2c expression plasmids. Antibodies were mouse anti-FLAG M2 (Sigma), Myc-Tag (Cell Signaling), nonimmune IgG (Sigma), and anti-RNA polymerase II (Upstate). PCR primers were as described (Table S2; Han and Lefebvre, 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and five figures and can be found with this article online at doi:10.1016/j.devcel.2011.12.024.

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Supplemental Information

Sox9 Directs Hypertrophic Maturation and Blocks Osteoblast Differentiation of Growth Plate Chondrocytes

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SUPPLEMENTAL INVENTORY

Table S1. Generation of *Alpl* and *Ank* RNA probes

Table S2. Primers used to amplify the rat *Col10a1* promoter sequence encompassing the Sox9 and Mef2c binding sites in chromatin immunoprecipitation

Figure S1. Generation and characterization of *ATC* transgenic mice. This supplemental figure shows the composition of the ATC transgene and its specific activity in mouse embryos before and upon treatment with doxycycline.

Figure S2. *Sox9* promotes growth plate chondrocyte proliferation, survival, and hypertrophy. This supplemental figure complements the main figure 2 by showing additional assays for cell proliferation and death and additional markers for chondrocyte hypertrophy.

Figure S3. Transactivation of *Col10a1* reporters by Sox9 and Mef2c. This supplemental figure complements the main figure 4 by showing the structure of *Col10a1* reporters, the activity of these reporters in non-chondrocytic cells, and the delineation of the promoter region responsive to SOX9 and Mef2c.

Figure S4. Expression of various lineage markers in *Sox9* mutant growth plates. This supplemental figure complements the main figure 5 by showing that deletion of Sox9 in the

growth plate does not result in activation of markers for presumptive joint cells, myoblasts, adipocytes, and tenocytes.

Figure S5. Histology analysis of growth plates in β-catenin mutants. This supplemental figure complements the main figure 6 by comparing growth plates lacking β-catenin and expressing a constitutively active form of β-catenin.

SUPPLEMENTAL INFORMATION

Table S1. Generation of *Alpl* and *Ank* RNA probes

Gene name	cDNA region	Accession number
Alpl	1030 - 1995	NM_007431.2
Ank	1303 - 2169	NM_020332

Primers, cDNA regions, and accession numbers are indicated. PCR products were obtained from mouse embryo total cDNA, cloned in pCR-TOPO (Invitrogen), and sequence-verified.

Table S2. Primers used to amplify the rat *Col10a1* promoter sequence encompassing the Sox9 and Mef2c binding sites in chromatin immunoprecipitation

Forward primer	CCATCATGAACCAACATTGGAGTCAGAAC
Reverse primer	AGTCCTATTGGGCAGGCGTACAAA

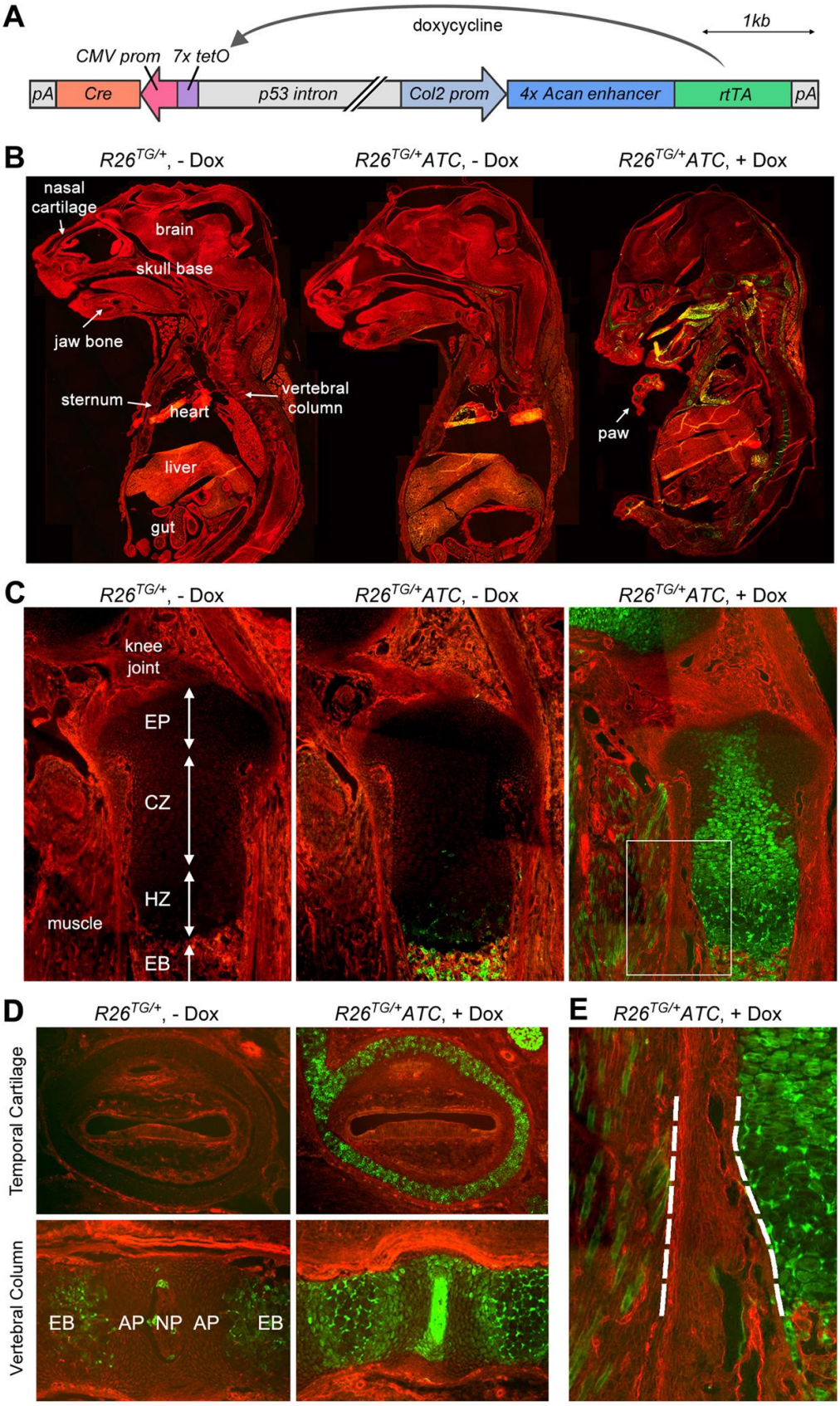

A
doxycycline
1kb
CMV prom
7x tetO
pA
Cre
p53 intron
Col2 prom
4x Acan enhancer
rtTA
pA
B
R26TG/+, - Dox
R26TG/+ATC, - Dox
R26TG/+ATC, + Dox
nasal cartilage
brain
skull base
jaw bone
sternum
heart
vertebral column
liver
gut
paw
C
R26TG/+, - Dox
R26TG/+ATC, - Dox
R26TG/+ATC, + Dox
knee joint
EP
CZ
HZ
muscle
EB
D
R26TG/+, - Dox
R26TG/+ATC, + Dox
Temporal Cartilage
Vertebral Column
EB
AP
NP
AP
EB
E
R26TG/+ATC, + Dox

Figure S1. Generation and characterization of *ATC* transgenic mice

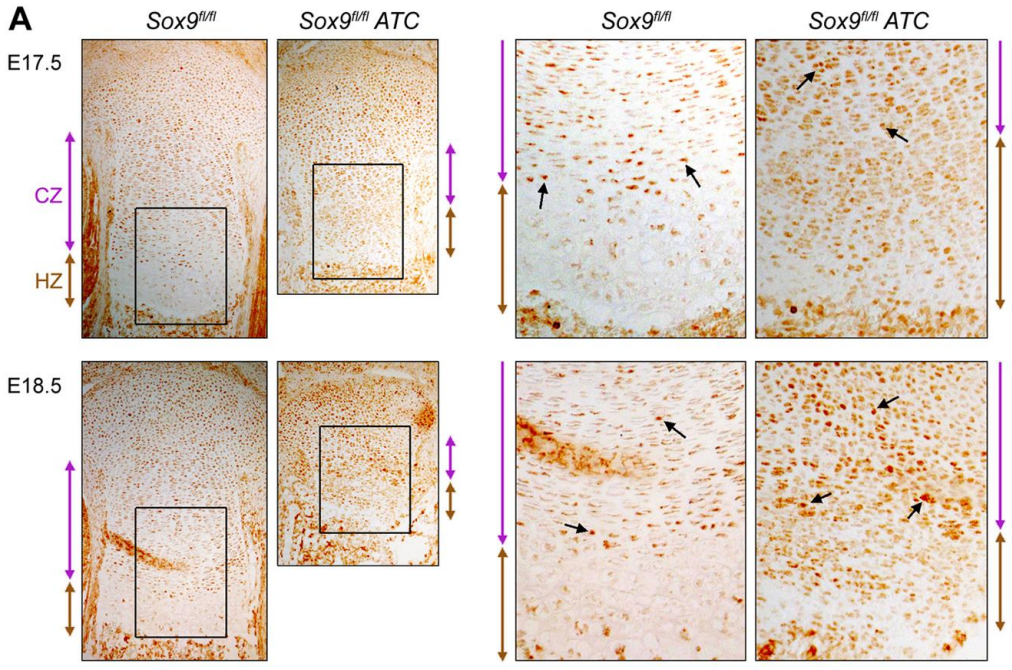
A. Schematic of *ATC*. *ATC* was constructed using a previously described bigenic template (Utomo et al., 1999). One gene was comprised of the -608/+309bp mouse *Col2a1* region (*Col2 prom*), four tandem copies (4x) of a cartilage-specific *Acan* enhancer (Han and Lefebvre, 2008) cloned in an intron, a splice acceptor site (not shown), the reverse tetracycline-controlled transactivator coding sequence (rtTA), and a polyadenylation site (pA). The other gene contained 7 tandem copies of tetracycline-responsive element (tetO), a widely expressed human cytomegalovirus promoter (*CMV prom*), the Cre coding sequence, and a polyadenylation site. A 4kb *p53* intron separated the two genes to avoid mutual interference. Upon treatment with doxycycline (Dox), rtTA is activated and binds tetO to induce *Cre* expression.

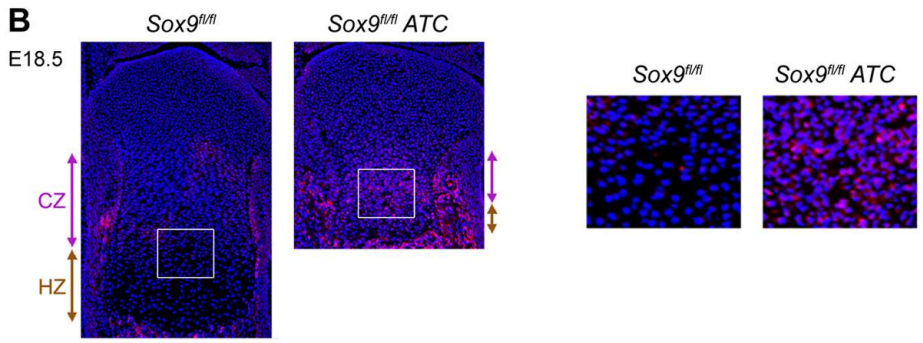
B. Analysis of *ATC* activity in mid-sagittal sections of E17.5 mouse fetuses carrying the $R26^{TG}$ allele. Red and green fluorescence emitted by the products of *Tomato* and *GFP* genes, respectively, was visualized on frozen sections of tissues fixed with 4% paraformaldehyde. + Dox, treatment with doxycycline from E15.5.

C. Proximal tibia growth plate and knee region of the same fetuses as in panel B. EP, epiphysis; CZ, columnar zone; HP, hypertrophic zone; EB, endochondral bone.

D. Sagittal sections through the temporal cartilage and lumbar vertebral column of the same fetuses as in panel B. AP, annulus fibrosus cartilage; NP, nucleus pulposus.

E. High-magnification of the boxed region in panel C. Dashed lines delineate the perichondrium.


A
Sox9fl/fl
Sox9fl/fl ATC
Sox9fl/fl
Sox9fl/fl ATC
E17.5
CZ
HZ
E18.5


B
Sox9fl/fl
Sox9fl/fl ATC
E18.5
CZ
HZ
Sox9fl/fl
Sox9fl/fl ATC

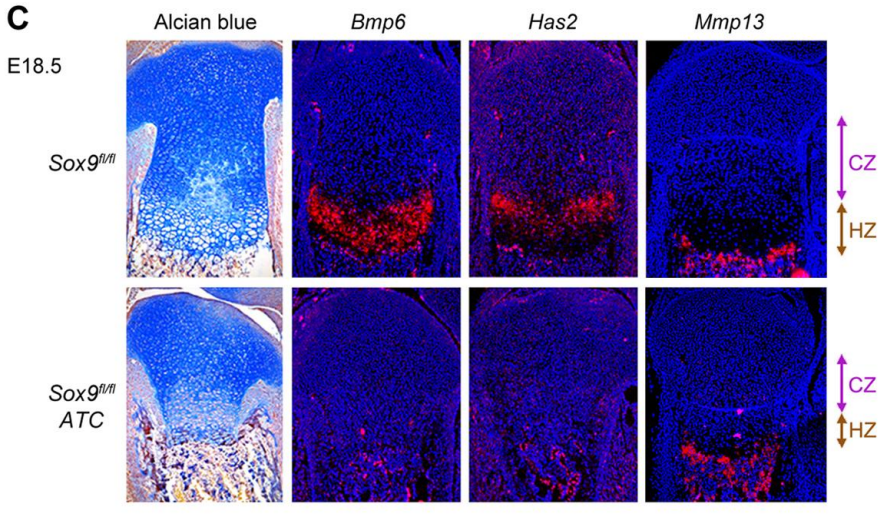

C
Alcian blue
Bmp6
Has2
Mmp13
E18.5
Sox9fl/fl
Sox9fl/fl ATC
CZ
HZ
CZ
HZ

Figure S2. *Sox9* promotes growth plate chondrocyte proliferation, survival, and hypertrophy

A. Immunostaining of Ki-67 in paraffin sections of tibia proximal growth plates from E17.5 and E18.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. After antigen retrieval, Ki-67 was detected using rabbit polyclonal anti-Ki-67 IgG (1/1000; AB9260, Millipore), biotinylated goat anti-rabbit (H+L) IgG (1/1000; Vector Laboratories), and Vectastain Elite ABC kit (PK-6100, Vector Laboratories). Both control and mutant sections show strong signals (brown/red) in many cells of the epiphyses and columnar zones, but weak or no signal in prehypertrophic and hypertrophic zones. The boxed regions on the left are shown at higher magnification on the right. Examples of positive cells are shown with black arrows. CZ and purple arrow, columnar zone. HZ and brown arrow, hypertrophic zones.

B. Immunostaining of cleaved caspase 3 in frozen sections of tibia proximal growth plates from E18.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. The protein was revealed using rabbit polyclonal anti-cleaved caspase 3 IgG (1/200; 9661S, Cell Signaling) and Alexa Fluor 594-conjugated goat anti-rabbit IgG. Positive signal (red) within the growth plates is seen at the level of the mutant prehypertrophic zone only. Cell nuclei are stained with DAPI. Boxed regions in the left pictures are shown at high magnification on the right.

C. Expression of hypertrophic markers in proximal tibia growth plates from E18.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. The left pictures were stained with Alcian blue. The other pictures show adjacent sections hybridized with RNA probes.

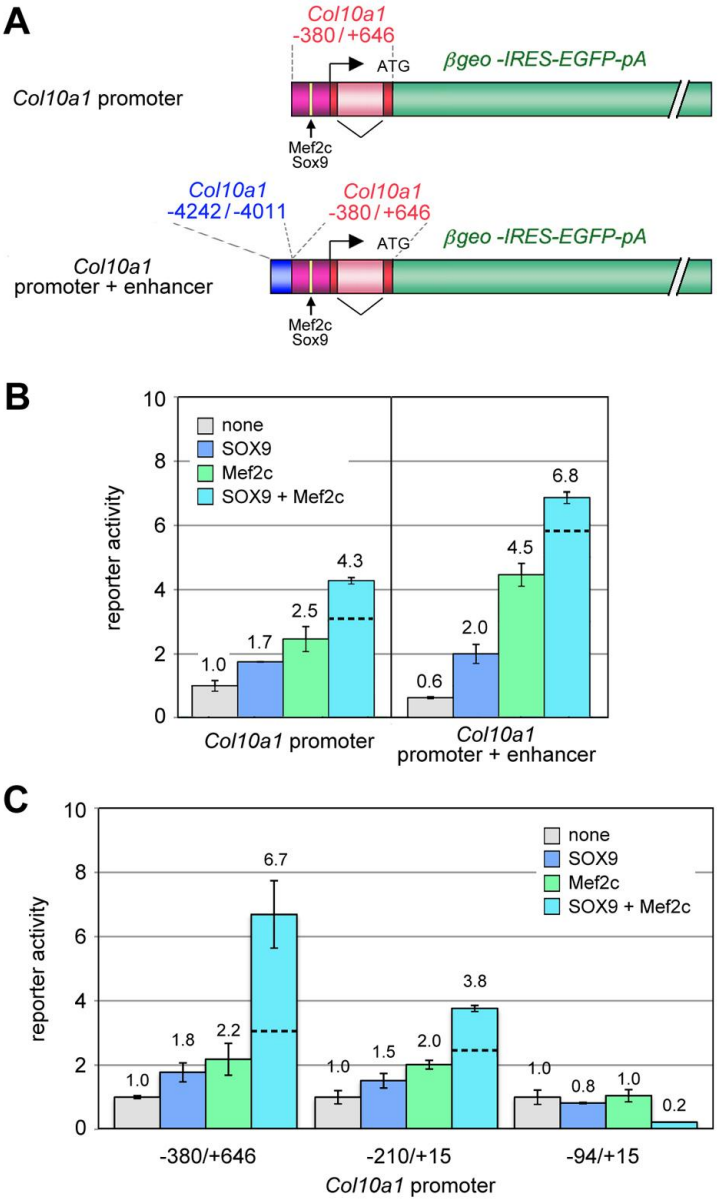


Figure S3. Transactivation of *Col10a1* reporters by Sox9 and Mef2c

A. Schematic of *Col10a1* reporters. Reporters were constructed in the pBluescript KS+ cloning vector (Stratagene). They featured the *βgeo* gene, encoding a fusion protein with a nuclear translocation signal and E. coli β-galactosidase and neomycin resistance activities, and the enhanced green fluorescent protein (EGFP) gene. The two genes were separated by an internal ribosome entry site (IRES) and were followed by a bovine growth hormone polyadenylation site (pA). They were driven by the -380/+646bp mouse *Col10a1* sequence, corresponding to the

conserved promoter region (magenta box), first exon (red), first intron (pink), and 5' part of exon 2 (red) up to the first codon (ATG). Reporters were assembled with or without the -4kb hypertrophic chondrocyte enhancer (-4242/-4011) placed directly upstream of the promoter (blue box). The position of Mef2c and Sox9 binding sites in the promoter is shown (yellow band). Reporters contained either intact or mutant binding sites for these proteins, as described in Fig. 3D. Mutations were introduced using Stratagene Quick Change Mutagenesis kit. Angled arrow, transcription start site; ATG, first codon of the *Col10a1* gene cloned in frame with the *bgeo* coding sequence; V-shaped line, intron.

B. Relative activity of *Col10a1* reporters transfected in 293T cells along with expression plasmids for SOX9 and Mef2c. Reporter activities were normalized for transfection efficiency and are presented as average with standard deviation of biological triplicates in one representative experiment. Dotted lines, expected values from additive effects of SOX9 and Mef2c.

C. Delineation of the *Col10a1* promoter region responsive to SOX9 and Mef2c in transiently transfected 293T cells. Reporters were made as shown in panel A, but with the indicated segments of the promoter region. Reporter activities were normalized for transfection efficiency and are presented as average with standard deviation of biological triplicates in one representative experiment.

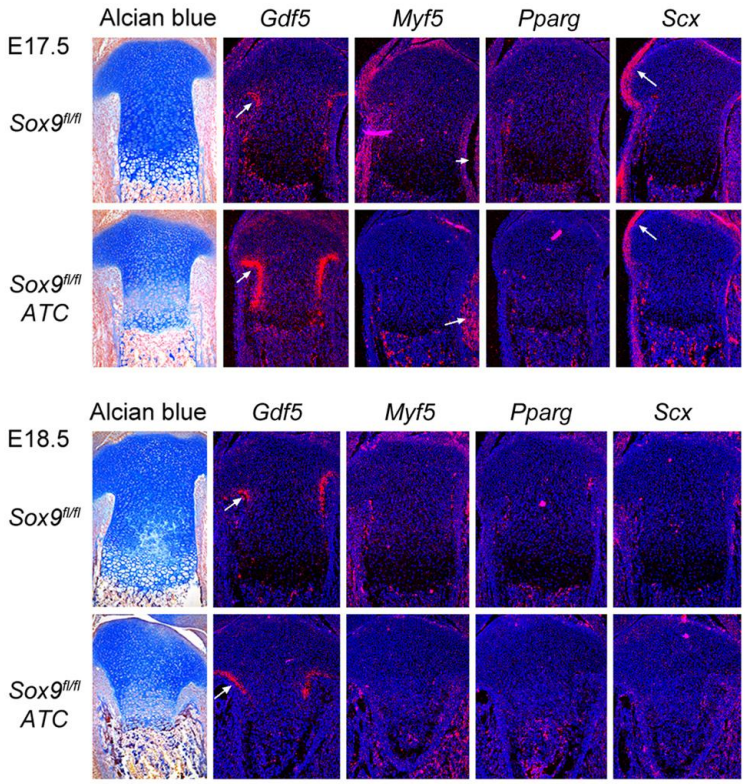


Figure S4. Expression of various lineage markers in *Sox9* mutant growth plates

Sections were made through proximal tibia growth plates from E17.5 and E18.5 *Sox9*$^{fl/fl}$ and *Sox9*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5. Sections were stained with Alcian blue or hybridized with RNA probes for the presumptive synovial joint cell marker *Gdf5*, myogenic marker *Myf5*, adipogenic marker *Pparg*, and tenogenic marker *Scx*. Arrows show typical sites of expression of these genes. None of these genes was expressed in control and mutant growth plates in the prehypertrophic/osteoblastic zone.

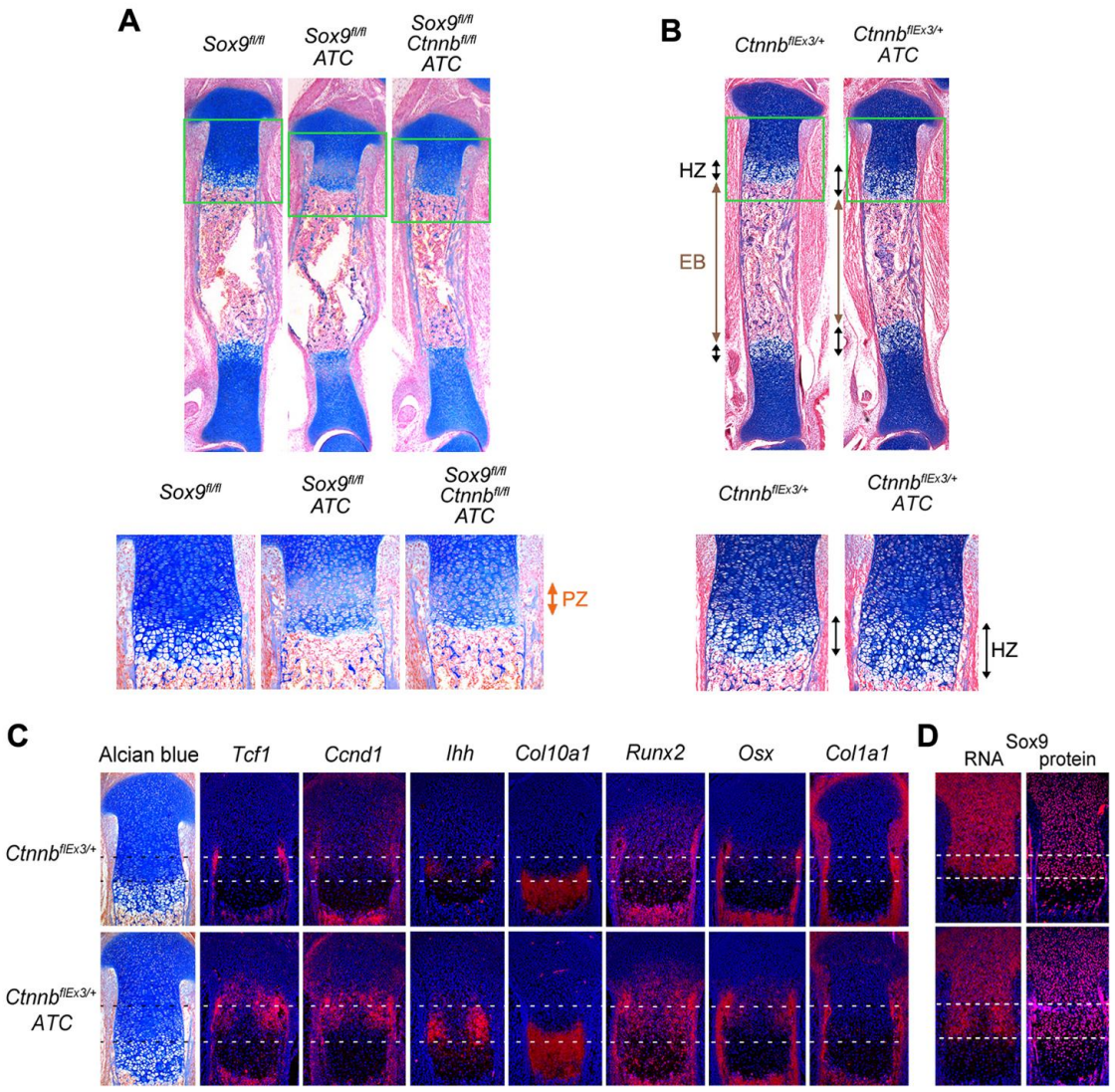


Figure S5. Histology analysis of growth plates in β-catenin mutants

A. Histological analysis of tibias from *Sox9*$^{fl/fl}$, *Sox9*$^{fl/fl}$*ATC*, and *Sox9*$^{fl/fl}$*Ctnnb*$^{fl/fl}$*ATC* littermates treated with Dox from E15.5 and collected at E17.5. Top, the two types of mutants show similar reduction of growth plates. Bottom, high-magnification pictures of the proximal growth plates show a less severe loss of Alcian blue-stained cartilage extracellular matrix in the prehypertrophic zone (PZ) of the *Sox9*$^{fl/fl}$*Ctnnb*$^{fl/fl}$*ATC* mutant than in the *Sox9*$^{fl/fl}$*ATC* mutant. This reduced loss is likely due to lack of osteoblastic differentiation of the cells.

B. Histological analysis of tibias from *Ctnnb*$^{flEx3/+}$ and *Ctnnb*$^{flEx3/+}$*ATC* littermates treated with Dox from E15.5 and collected at E17.5. Top, the hypertrophic zone (HZ) of the mutant is elongated at the expense of endochondral bone (EB) formation. Bottom, high-magnification pictures showing the normal appearance of the mutant proximal growth plate, but for an elongated hypertrophic zone.

C. Histology analysis and RNA in situ hybridization of tibia proximal growth plates from E17.5 *Ctnnb*$^{flEx3/+}$ and *Ctnnb*$^{flEx3/+}$*ATC* littermates treated with Dox from E15.5. Dotted lines delineate the prehypertrophic/osteoblastic zones.

D. Sox9 RNA in situ hybridization and protein immunostaining in similar sections as in panel C.