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Sox5 and *Sox6* are needed to develop and maintain source, columnar, and hypertrophic chondrocytes in the cartilage growth plate

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Sox5 and *Sox6* encode *Sry*-related transcription factors that redundantly promote early chondroblast differentiation. Using mouse embryos with three or four null alleles of *Sox5* and *Sox6,* we show that they are also essential and redundant in major steps of growth plate chondrocyte differentiation. *Sox5* and *Sox6* promote the development of a highly proliferating pool of chondroblasts between the epiphyses and metaphyses of future long bones. This pool is the likely cellular source of growth plates. *Sox5* and *Sox6* permit formation of growth plate columnar zones by keeping chondroblasts proliferating and by delaying chondrocyte prehypertrophy. They allow induction of chondrocyte hypertrophy and permit formation of prehypertrophic and hypertrophic zones by delaying chondrocyte terminal differentiation induced by ossification fronts. They act, at least in part, by down-regulating Ihh signaling, *Fgfr3,* and *Runx2* and by up-regulating *Bmp6*. In conclusion, *Sox5* and *Sox6* are needed for the establishment of multilayered growth plates, and thereby for proper and timely development of endochondral bones.

Introduction

Cartilage has essential roles in endochondral ossification, a multistep process whereby most bones develop in vertebrates. This process starts in the embryo with the formation of cartilage primordia that already prefigure definitive bones. These primordia then develop growth plates that enlarge skeletal elements and are progressively replaced by bone from fetal stages until adulthood.

Cartilage primordia arise upon condensation of prechondrogenic mesenchymal cells. These cells differentiate sequentially into prechondrocytes and chondroblasts. Prechondrocytes activate *Col2a1* (collagen type 2), *Agc1* (aggrecan), and other cartilage-specific extracellular matrix genes. Chondroblasts up-regulate these genes and activate others like *Mat1* (matrilin-1). They produce large amounts of extracellular matrix and actively proliferate. Several types of signaling molecules and transcription factors control skeleton patterning and cell differentiation at these early stages (DeLise et al., 2000; Hall and Miyake, 2000; de Crombrugghe et al., 2001; Karsenty and Wagner, 2002). The latter include three Sry-related HMG box-containing proteins, L-Sox5, Sox6, and Sox9. L-Sox5 (a long product of *Sox5*) and Sox6 are highly identical to each other but related to Sox9 only in the HMG box DNA-binding domain (Lefebvre, 2002). *Sox5*, *Sox6*, and *Sox9* are coexpressed in all cartilage primordia of the mouse embryo and may cooperate to directly activate *Col2a1* (Ng et al., 1997; Zhao et al., 1997; Lefebvre et al., 1998). *Sox9* is required for prechondrogenic cell condensation, prechondrocyte and chondroblast differentiation, and activation of *Sox5*, *Sox6*, and cartilage matrix genes (Bi et al., 1999; Akiyama et al., 2002). *Sox5* and *Sox6* have essential, redundant roles in early chondroblasts (Smits et al., 2001). Although $Sox5^{-/-}$ and $Sox6^{-/-}$ mice are born with minor cartilage defects, $Sox5^{-/-}/Sox6^{-/-}$ embryos develop a severe, generalized chondrodysplasia due to considerable delay and impairment of chondroblast proliferation and expression of cartilage matrix genes. They die around embryonic day 16.5 (E16.5) with rudimentary and matrix-deficient cartilage primordia.

The fate of each chondroblast is determined by its location in cartilage primordia (Karsenty and Wagner, 2002; Kronenberg, 2003). Although epiphyseal chondroblasts are

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Abbreviations used in this paper: BMP, bone morphogenetic protein; E, embryonic day; NA, null allele.

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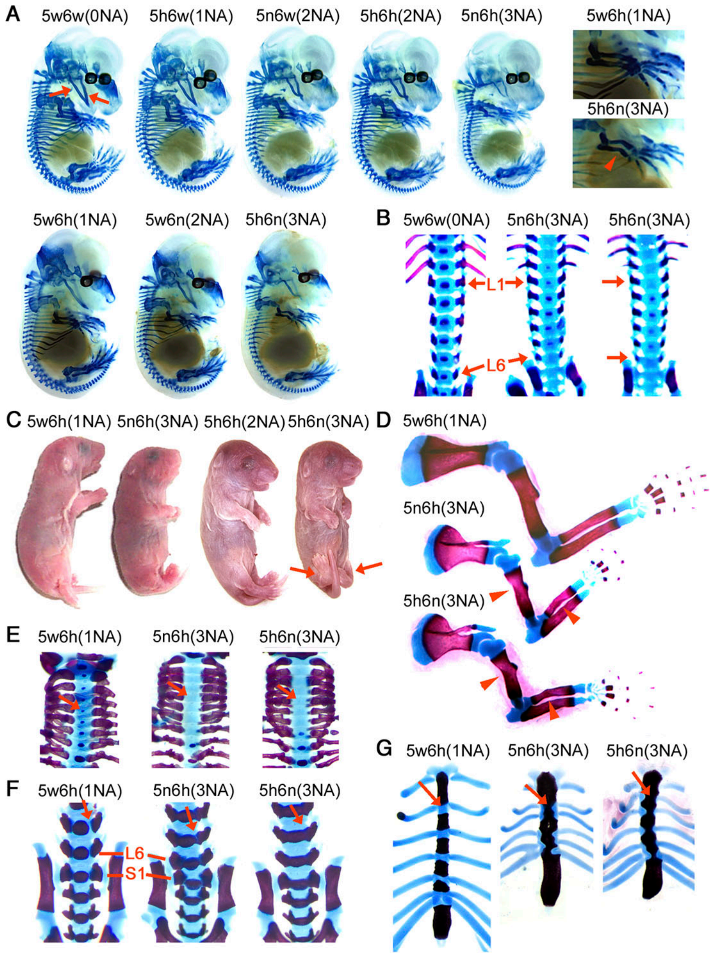


Figure 1. **Chondrodysplasia features of 3NA mice.** Genotypes are designated as follows: 5, *Sox5*; 6, *Sox6*; w, wild type; h, heterozygous null; n, homozygous null. (A) Alcian blue staining of E14.5 embryos. Top and bottom embryos are from different litters. Arrows, Meckel's cartilages; arrowhead, bent radius and ulna. (B) Skeletal preparations of vertebrae from E17.5 littermates. Alcian blue stains nonmineralized cartilage and alizarin red stains mineralized cartilage and bone. (Arrows) L1 and L6, first and sixth lumbar vertebrae, respectively. (C) Pictures of newborns. The first two and the last two mice are from separate litters. Arrows, bent hindlimbs. (D–G) Skeletal preparations of newborn littermates. (D) Forelimbs. Arrowheads, bent humeri and radii. (E) Cervical vertebrae. Arrows, vertebral bodies. (F) Lumbar and sacral vertebrae. L6, sixth lumbar vertebra; S1, first sacral vertebra. Arrows, vertebral bodies and arches separated by nonmineralized cartilage in controls and mineralized cartilage or bone in mutants. (G) Sternum. Arrows, intersternebrae made of nonmineralized cartilage in the control and mineralized bone or cartilage in the mutants.

maintained at an early differentiation stage throughout gestation, diaphyseal and metaphyseal chondroblasts mature to form growth plates and induce the formation of primary ossification centers. Upon undergoing prehypertrophy, they stop proliferating and sequentially activate *Ppr* (receptor for parathyroid hormone and parathyroid hormone–related peptide), *Ihh* (Indian hedgehog), and *Col10a1* (collagen type 10). They also induce the flanking perichondrium to form a bone collar. Next, they undergo hypertrophy, stop expressing *Col2a1*, *Agc1*, and other early markers, progressively down-regulate *Ppr* and *Ihh*, up-regulate expression of *Col10a1*, and induce cartilage matrix mineralization. As they terminally differentiate, they stop expressing *Col10a1*, activate *Opn* (osteopontin) and *Mmp13* (matrix metalloproteinase-13), and undergo apoptosis. Bone collar chondro/osteoclasts, osteoblasts, and blood vessels then invade and replace the cartilage by bone. Various signaling molecules control these processes. They include Ihh, PTHrP (parathyroid hormone–related peptide), and PPR (Karaplis et al., 1994; Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999), FGFR3 (fibroblast growth factor receptor-3) and its ligand FGF18 (Colvin et al., 1996; Deng et al., 1996; Liu et al., 2002; Ohbayashi et al., 2002), bone morphogenetic proteins (BMPs; Zou et al., 1997), and Wnt proteins (Hartmann and Tabin, 2000; Yang et al., 2003). Few transcription factors have yet been demonstrated to control these processes. *Cbfa1/Runx2* is expressed in late chondroblasts and in prehypertrophic and hypertrophic chondrocytes (Inada et al., 1999; Kim et al., 1999). It induces prehypertrophy as shown by a block of chondrocyte differentiation before prehypertrophy in several skeletal elements of *Runx2*$^{-/-}$ mouse embryos and by maturation of transgenic chondroblasts forced to express *Runx2* (Takeda et al., 2001). However, because *Runx2*$^{-/-}$ chondrocytes properly mature in some skeletal elements, additional factors must contribute to induce chondrocyte maturation. *Sox5*, *Sox6*, and *Sox9* are turned off upon chondrocyte prehypertrophy (Lefebvre et al., 1998). *Sox9*$^{+/-}$ embryos precocious mineralize cartilage primordia and develop enlarged hypertrophic zones (Bi et al., 2001). Thus, *Sox9* might delay chondrocyte hypertrophy. When *Sox5*$^{-/-}$/*Sox6*$^{-/-}$ fetuses die, their cartilage primordia contain few prehypertrophic chondrocytes but no hypertrophic cells. They are being invaded by thick bone collars but feature no cartilage growth plates and no ossification centers (Smits et al., 2001). Therefore, these fetuses do not allow for a full assessment of the roles of *Sox5* and *Sox6* in the growth plate.

To determine such roles, we mostly analyzed here embryos with three null alleles (3NA) of *Sox5* and *Sox6* (*Sox5*$^{+/-}$/*Sox6*$^{-/-}$ and *Sox5*$^{-/-}$/*Sox6*$^{+/-}$). These embryos live until birth and show severe growth plate chondrocyte defects. We show that *Sox5* and *Sox6* are needed to form and maintain a pool of highly proliferating chondroblasts between epiphyses and metaphyses, to form columnar chon-

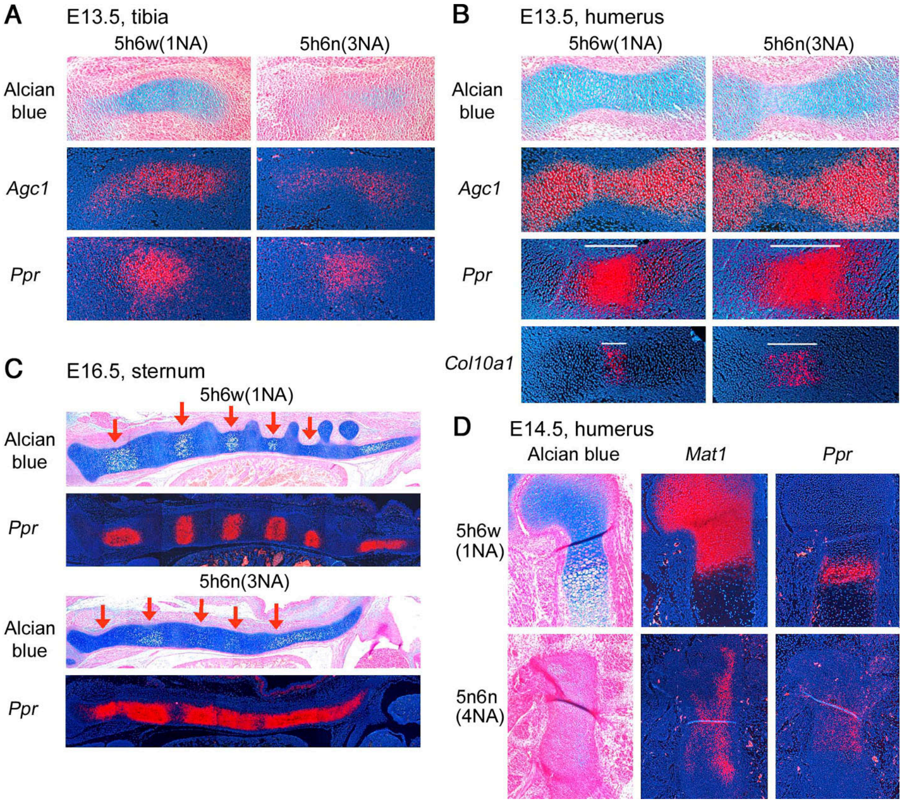


Figure 2. **Premature prehypertrophy of 3NA and 4NA chondroblasts.** Longitudinal sections through the tibias (A), humeri (B and D), or sternums (C) of E13.5 to E16.5 control and mutant embryos were stained with Alcian blue or hybridized in situ with RNA probes, as indicated. White bars, prehypertrophic chondrocyte domains. Sternum prehypertrophic chondrocytes are recognized by a white cytoplasm. Arrows, sternebrae. For E14.5 humeri, the control proximal half and the entire mutant element are shown.

droblasts, delay chondrocyte prehypertrophy but promote hypertrophy, and to delay terminal differentiation of chondrocytes on contact with ossification fronts. *Sox5* and *Sox6* act in part by controlling the domain of action and expression of major regulatory factors.

Results

Chondrodysplasia of 3NA embryos

By E14.5, all cartilage primordia of 3NA embryos were hypoplastic and weakly stained with Alcian blue (Fig. 1 A). Some were also bent. In comparison, only the Meckel's, costal, and a few other cartilages were hypoplastic in 2NA embryos (Fig. 1 A), and all 4NA cartilage primordia were rudimentary and hardly stained with Alcian blue (Smits et al., 2001). 1NA and wild-type embryos were indistinguishable from each other at this and later stages, and therefore were used as controls throughout this work. The onset of mineralization was delayed in all 3NA endochondral skeletal elements, such as in vertebral bodies (Fig. 1 B). The two types of 3NA mice died at birth from respiratory distress and featured virtually identical skeletal defects (Fig. 1, C–G). They had a foreshortened snout, micrognathia, short trunk, and short, bent limbs (Fig. 1 C). Forelimb (Fig. 1 D) and other long bones were shorter than normal, but not always thinner, and some were bent. Cervical vertebrae start to ossify later than lumbar and sacral vertebrae. In 3NA newborns, the former had not yet started to mineralize (Fig. 1 E), whereas the latter were ectopically mineralized (Fig. 1 F). The sternum was the most affected 3NA element (Fig. 1 G). It was short and thick, and intersternebrae were ectopically mineralized. Thus, 3NA embryos developed generalized cartilage primordium hypoplasia and skeleton growth and mineralization defects that strongly suggested roles for *Sox5* and *Sox6* in chondrocyte maturation.

Precocious prehypertrophy of chondrocytes in 3 and 4NA cartilage primordia

3NA chondroblasts started to differentiate with a significant delay compared with control cells but ultimately formed close-to-normal cartilage primordia. For instance, 3NA tibia chondroblasts were surrounded by less cartilage extracellular matrix and were expressing *Agc1* at a lower level than control cells at E13.5 (Fig. 2 A). In contrast, 3NA humerus chondroblasts, which develop earlier, were expressing *Agc1* and other markers at close-to-normal levels and had accumulated a fair amount of cartilage matrix (Fig. 2 B). The onset of chondrocyte prehypertrophy was delayed in all 3NA cartilage primordia, as seen for *Ppr* activation in E13.5 tibias. Interestingly, once the first core cells became prehypertrophic, their neighbors became prehypertrophic more rapidly in mutants than in controls, as seen for *Ppr* and *Col10a1* activation in E13.5 humeri. The sternum was the most affected 3NA element (Fig. 2 C). Sternebral but not intersternebral chondroblasts were prehypertrophic in E16.5 control sternums, as seen by cell enlargement and *Ppr* expression. In contrast, both sternebral and intersternebral chondrocytes were already prehypertrophic in 3NA sternums. 4NA humeri were poorly developed by E14.5 (Fig. 2 D). The expression domain of *Mat1*, a marker of both chondroblasts and prehypertrophic chondrocytes, was wide in control humeri and overlapped only partially with that of *Ppr*. In contrast, few cells were expressing *Mat1* in 4NA humeri and most of them were also expressing *Ppr*. Thus, these data

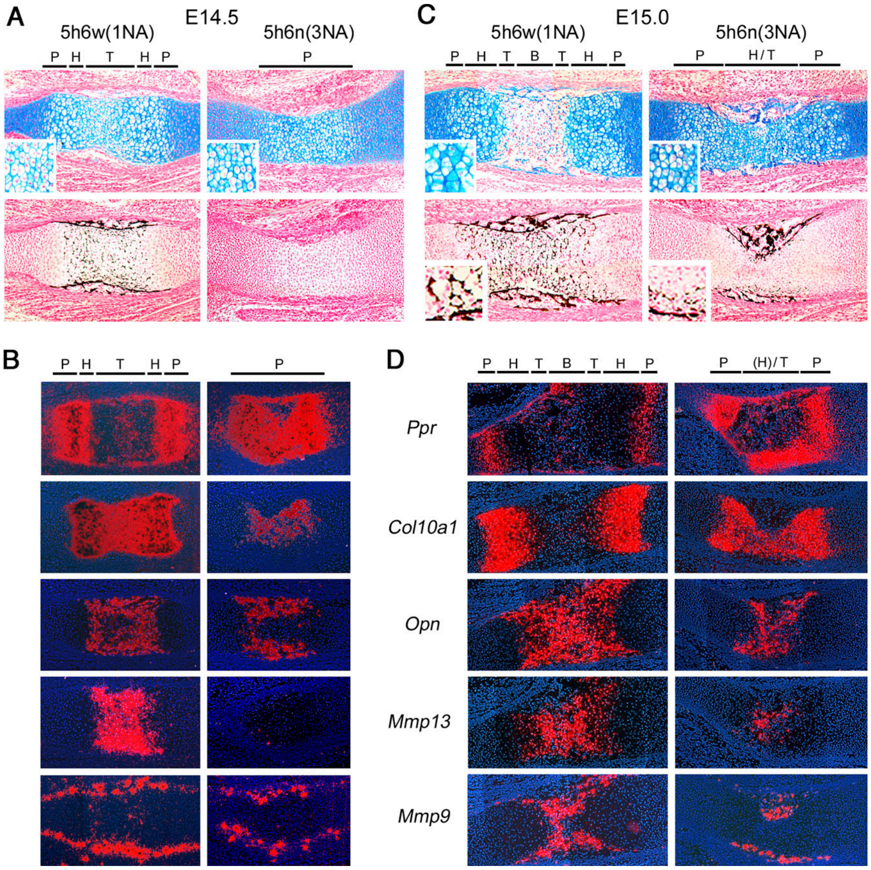


Figure 3. **Delayed hypertrophy and terminal differentiation of 3NA chondrocytes.** (A and C) Longitudinal sections through the shaft of control and 3NA humeri from E14.5 and E15.0 littermates stained with Alcian blue (top) or the von Kossa reagent (bottom). The von Kossa reaction identifies mineral deposits (black signal). (insets) High-magnification pictures showing the size of chondrocytes in the core region at E14.5 and in the flanking region at E15.0 (Alcian blue), and the extent of cartilage mineralization next to the bone collar (von Kossa). (B and D) Sections adjacent to those shown in A and C hybridized in situ with RNA probes, as indicated. The hybridization signal for *Col10a1* is so strong in the E14.5 control hypertrophic zone that it appears black. P, prehypertrophic zone; H, hypertrophic zone; T, terminal zone; B, primary ossification center.

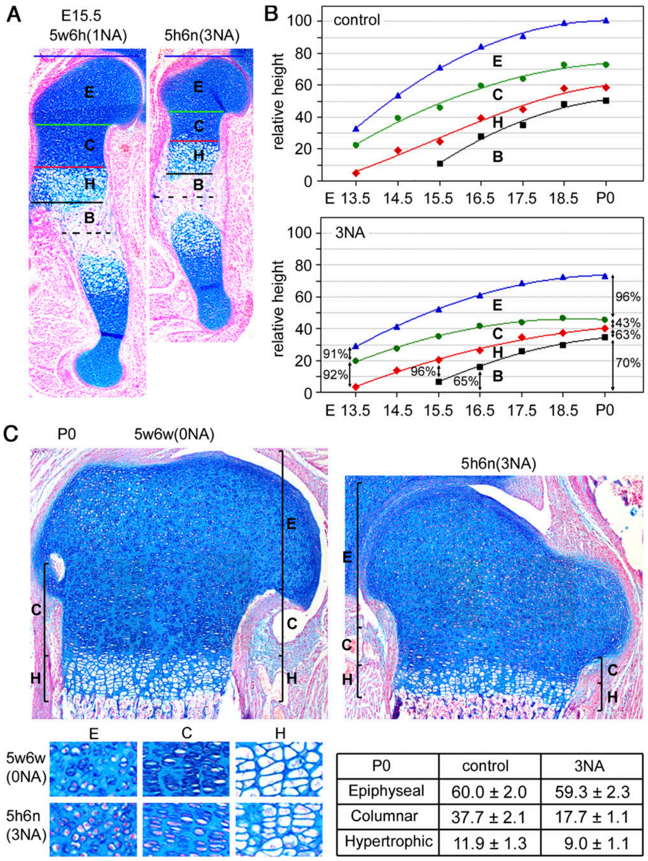
demonstrated that *Sox5* and *Sox6* are needed to prevent chondroblast premature prehypertrophy.

Delayed chondrocyte hypertrophy and terminal differentiation in 3NA cartilage primordia

By E14.5, chondrocytes in the core regions of control humeri still exhibited a moderate size, typical of prehypertrophic cells, but they had already induced matrix mineralization (Fig. 3 A). They had inactivated *Ppr*, were still expressing *Col10a1*, and had activated *Opn* and *Mmp13* (Fig. 3 B). Thus, these chondrocytes had rapidly progressed from prehypertrophy to terminal differentiation. Flanking chondrocytes were fully enlarged, had induced cartilage mineralization, and were expressing *Col10a1* at the highest level. Thus, they were overtly hypertrophic. Both core and flanking cells were surrounded with a mineralized bone collar in which osteoclasts were expressing *Mmp9*. *Mmp9*-positive cells were also detected in the cartilage core, indicating the onset of endochondral ossification. In 3NA humeri, both core and flanking cells were still prehypertrophic. Indeed, they were moderately enlarged, were not mineralizing the cartilage matrix, were expressing *Ppr* strongly and *Col10a1* weakly, and were not expressing *Mmp13*. The perichondrium had not developed into a mineralized bone collar but contained *Mmp9*-expressing osteoclasts. Interestingly, chondrocytes expressing *Opn* were found adjacent to the bone collar and were thus terminally differentiating. By E15.0, the core of control humeri was ossifying (Fig. 3, C and D). It contained *Mmp9*-expressing osteoclasts/chondroclasts and cells expressing *Opn* and *Mmp13*, which were either terminal chondrocytes or mature osteoblasts. The core of 3NA humeri was still cartilaginous, but its cells were terminally differentiating as seen by *Ppr* inactivation and *Opn* and *Mmp13* activation. The cartilage matrix was mineralized only next to the bone collar and was starting to be invaded by bone. By E15.5, the 3NA humerus core was fully ossified (Fig. 4 A). Thus, 3NA chondrocytes were delayed and impaired in undergoing hypertrophy but terminally differentiating next to ossification fronts.

Defective columnar and hypertrophic zones in 3NA growth plates

To determine the roles of Sox5 and Sox6 in established growth plates, we compared the development of control and 3NA humeri from E13.5 until birth by measuring the elongation of their epiphyses, columnar and hypertrophic regions, and primary ossification centers (Fig. 4 A). The hypertrophic region was defined by enlarged cells, and thus contained late prehypertrophic, hypertrophic, and terminal chondrocytes. The columnar region contained metaphyseal chondroblasts and early prehypertrophic chondrocytes. Mutant and control humeri were similar at E13.5 (Fig. 4 B). From that time on, mutant epiphyses elongated at the same rate as controls. In contrast, the mutant columnar and hypertrophic zones became dramatically smaller than the controls, remaining only at 43% and 63% of the height of the respective control zones at birth. The mutant bone segments grew at 65–70% of the rate of controls. High magnification pictures of newborn humerus sections showed that the shortness of the mutant columnar region was not due to a



P0	control	3NA
Epiphyseal	60.0 ± 2.0	59.3 ± 2.3
Columnar	37.7 ± 2.1	17.7 ± 1.1
Hypertrophic	11.9 ± 1.3	9.0 ± 1.1

Figure 4. **Defective growth plates in 3NA humeri.** (A) Longitudinal sections in control and 3NA E15.5 humeri stained with Alcian blue. Four histological regions are recognized: E, epiphysis; C, columnar region; H, hypertrophic region; B, primary ossification center. (B) Relative growth of the histological regions in control and 3NA humerus proximal halves from E13.5 to birth (P0). Pictures of humerus sections were taken at identical magnification for all samples. The distance between the middle of the humerus and the proximal limit of each region was measured for two to four different mice per genotype and per age. The averages of these distances were calculated per mouse category and are presented relative to the length of the proximal half of newborn control humeri, which was assigned a value of 100. The curves connect the values measured for each region (indicated underneath the curves) from E13.5 or E15.5 to birth. (C) Longitudinal sections of control and 3NA humerus heads stained with Alcian blue. Brackets show the epiphysis (E), columnar (C), and hypertrophic (H) regions. Bottom left, high magnification pictures of cells in these regions. The table indicates the number of cell layers in each region for one representative embryo pair as the average with SD of measurements made in three nonconsecutive sections.

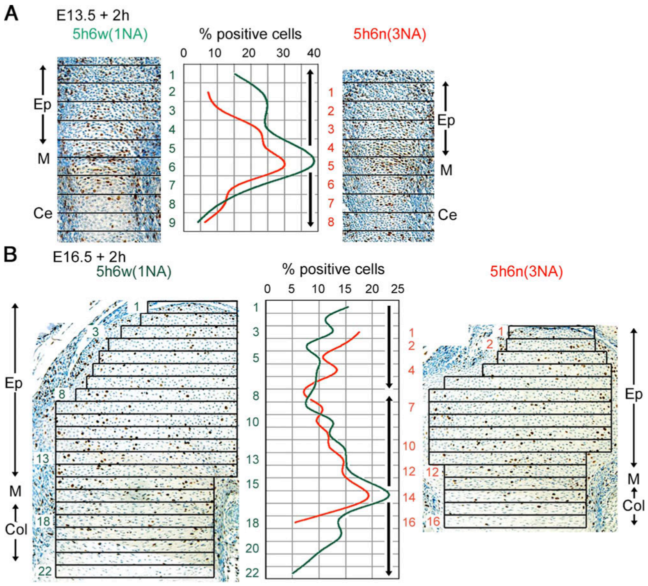


Figure 5. **Slow proliferation of 3NA chondroblasts.** (A and B) BrdU labeling and cell proliferation rates in control and 3NA humeri in E13.5 and E16.5 embryos. Pictures show longitudinal sections of humeri stained for BrdU (brown nuclei). The percentages of BrdU-positive chondroblasts were determined in the consecutive segments delineated with black lines and plotted in the graphs. The arrows in the graph indicate that the cells enlarging the distal half of epiphyses and elongating humeri shafts primarily arise from cells proliferating maximally at the top of metaphyses, and that cells in the proximal half of E16.5 epiphyses arise from cells highly proliferating in periarticular regions. Ep, epiphysis; M, metaphysis; Ce, center; Col, columnar zone.

more compact cell organization or a failure of the cells to organize into columns (Fig. 4 C). The mutant region contained half as many cell layers as the control, corresponding to the height reduction. In contrast, the mutant hypertrophic zone contained 25% fewer cell layers but was 37% shorter than normal. Therefore, its reduced height was in part due to the smaller size of the cells. We measured that mutant hypertrophic cells were on average 83% as high as control cells in the four layers abutting the ossification front. Because endochondral bone forms by replacement of these layers and because 3NA bone was expanding at 65–70% of the normal rate, the 3NA bone grew slowly not only because hypertrophic chondrocytes were smaller but probably also because terminal chondrocytes died at a slower rate. Together with the absence of columnar and hypertrophic cells in 4NA elements (Smits et al., 2001), these data demonstrate that *Sox5* and *Sox6* ensure optimal growth of endochondral bones by specifically promoting the formation and maintenance of columnar and hypertrophic chondrocytes.

Reduced proliferation and precocious prehypertrophy of 3NA growth plate chondroblasts

The size of the columnar region is determined by the rate of cell proliferation within this zone and by the rate at which chondroblasts exit this zone to become hypertrophic. It may also depend on the rate at which epiphyseal chondroblasts enter this zone (Kobayashi et al., 2002). To measure the rate of chondroblast proliferation, we labeled proliferating cells in vivo with BrdU and counted the percentages of positive cells in small segments juxtaposed from the presumptive joint to the center of cartilage primordia or to the hypertrophic zone of growth plates (Fig. 5). We found that control chondroblasts proliferated at highly variable rates in different subregions of epiphyses and growth plates. They proliferated at the highest levels at the top of metaphyses both in E13.5 (Fig. 5 A) and in E16.5 humeri (Fig. 5 B). Interestingly, 3NA chondroblasts were consistently found to proliferate slower than control cells in this region. At E13.5, the rate of cell proliferation decreased from this peak toward the presumptive joint, where prechondrocytes were still differentiating, and toward the

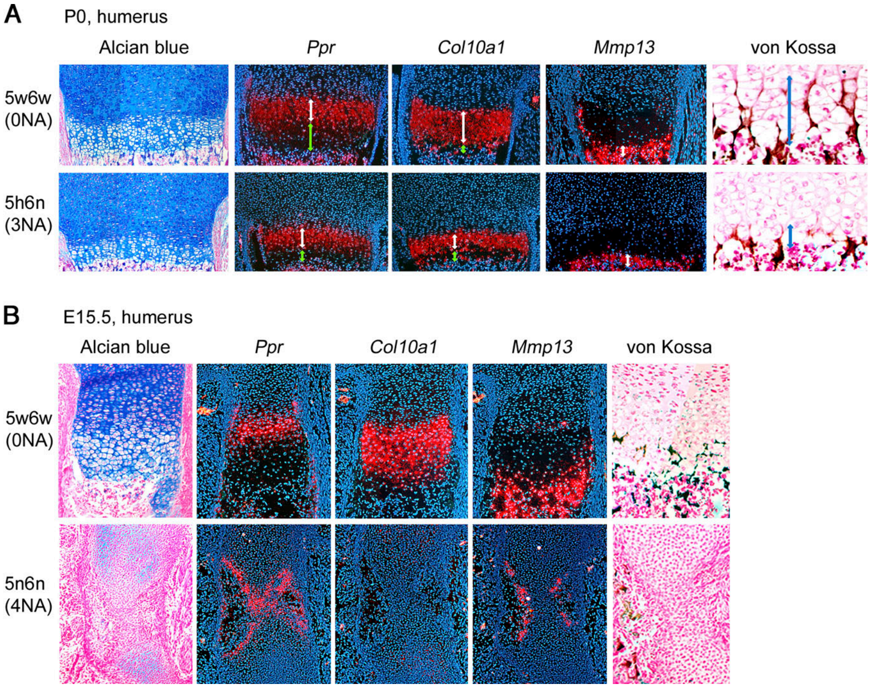


Figure 6. **Reduced hypertrophic zone in 3NA growth plates.** (A) Longitudinal sections through humeri of control and 3NA newborn mice. Consecutive sections were stained with Alcian blue or the von Kossa reagent, or hybridized with RNA probes, as indicated. Pictures focus on the lower part of growth plates and are aligned at the level of the ossification front. The von Kossa pictures show the last cell layers of the growth plates at high magnification. White arrows, expression domains of tested genes. Green arrows, cell layers between the gene expression domain and the ossification front. Blue arrows, layers of matrix-mineralizing chondrocytes. (B) Longitudinal sections through humeri of control and 4NA E15.5 fetuses. Consecutive sections were stained with Alcian blue or the von Kossa reagent, or hybridized with RNA probes, as indicated. Control pictures show the proximal growth plate of the humerus and mutant pictures show the entire humerus. The von Kossa pictures were taken at higher magnification.

center of the cartilage primordia, where chondroblasts were undergoing prehypertrophy. 3NA cells were proliferating at a lower rate than controls in epiphyses, likely reflecting their chondroblastic delay. At E16.5, chondroblasts were proliferating more slowly in the middle than in the periphery of epiphyses, but no major difference was seen between controls and mutants. The columnar zone was characterized by a progressive decline in cell proliferation, and this decline occurred twice as fast in 3NA humeri as in controls. Thus, 3NA chondroblasts formed shorter columnar zones because they failed to proliferate fast enough at the top of this zone and because they growth arrested faster than normal. The unaffected proliferation rate of 3NA epiphyseal chondroblasts was consistent with the normal growth rate of epiphyses, indicating that these cells unlikely contributed to the shortness of the columnar zone.

Inability of chondrocytes to undergo overt hypertrophy in 3NA and 4NA long bone growth plates

Because chondrocytes no longer proliferate beyond early prehypertrophy, the number of cell layers in the hypertrophic region only reflects the time spent by the cells in this region. We determined if 3NA cells proceeded faster through late prehypertrophy, hypertrophy, or terminal differentiation by analyzing expression of stage-specific markers (Fig. 6 A). In newborn control humeri, *Ppr* is highly expressed in prehypertrophic chondrocytes and weakly expressed in hypertrophic and terminal cells. In 3NA humeri, its high expression domain was slightly reduced and its low expression domain strongly reduced. *Col10a1* is highly expressed in prehypertrophic and hypertrophic chondrocytes and turned off in terminal chondrocytes. Its expression domain was strongly reduced in mutants. The terminal chondrocyte marker *Mmp13* was similarly expressed in control and mutant growth plates. von Kossa staining revealed cartilage mineralization around several layers of lower hypertrophic and terminal chondrocytes in controls, but only around terminal chondrocytes in mutants. Thus, the shortening of the 3NA hypertrophic region was mostly due to reduction of the hypertrophic zone. In E15.5 4NA humeri (Fig. 6 B), *Mmp13* was expressed in cells lining the bone collar and *Ppr* was expressed in cells adjacent to them. There was no *Col10a1* expression and no cartilage matrix mineralization. Thus, 4NA cells were terminally differentiating immediately after reaching prehypertrophy. Thus, *Sox5* and *Sox6* promote chondrocyte hypertrophy and prevent terminal differentiation of prehypertrophic and hypertrophic cells.

Absence of growth plates in 3NA sternum and vertebrae

Control lumbar vertebrae featured short, bidirectional growth plates flanking the ossification centers of vertebral bodies and neural arches (Fig. 7 A). In contrast, growth plates were lacking in 3NA vertebrae and replaced with cartilage-mineralizing fully hypertrophic cells. Control sternums featured ossified sternebrae and intersternebral growth plates, whereas 3NA sternebrae and intersternebrae were both mostly filled with fully enlarged, matrix-mineralizing chondrocytes (Fig. 7 B). These cells were hypertrophic or terminally differentiated, expressing *Col10a1* and *Mmp13*, whereas the other chondrocytes were prehypertrophic, expressing *Ppr* and *Ihh* (Fig. 7 C). Thus, the abundance of hypertrophic chondrocytes in 3NA sternums contrasted with the reduction of hypertrophic zones in future long bones but indicated that 3NA chondrocytes were retaining the ability to undergo overt hypertrophy. Interestingly, 3NA sternum chondrocytes were maturing from the ventral to the dorsal side of the sternum, perpendicularly to the normal maturation gradient. The ventral side of the 3NA sternum was a nonmineralized perichondrium/periosteum, whereas the dorsal side was a continuous bone collar. Thus, terminal chondrocytes were developing against ossification fronts, as in long bones.

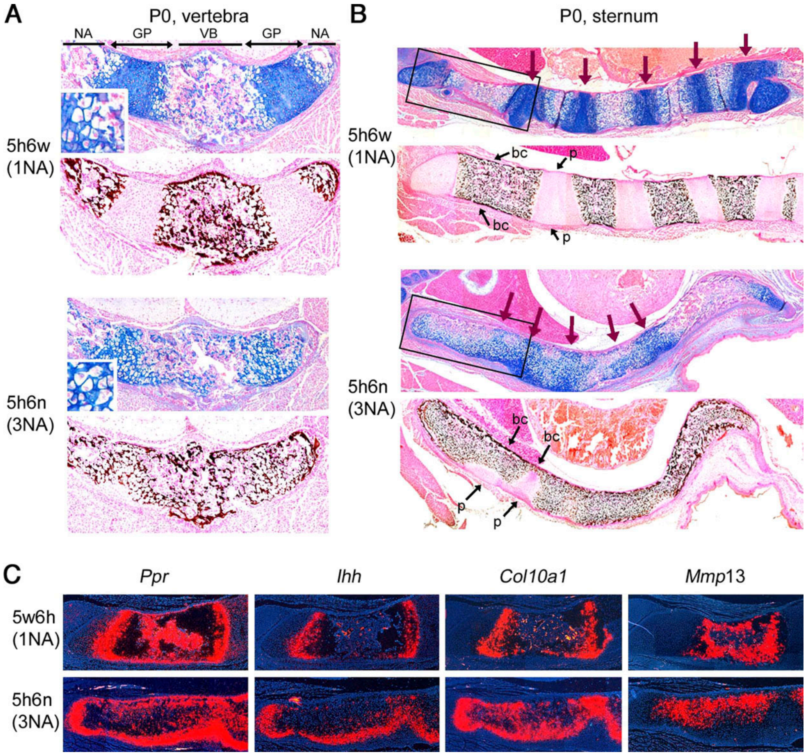


Figure 7. **Vertebrae and sternum of 3NA newborns.** (A) Transverse sections of lumbar vertebrae. The top section for each genotype was stained with Alcian blue and the bottom section with the von Kossa reagent. (insets) High magnification pictures of hypertrophic chondrocytes. NA, neural arch ossification center; GP, bidirectional growth plate; VB, vertebral body ossification center. (B) Mid-sagittal sections of the sternum. The mouse ventral side is at the bottom of the pictures. The top section for each genotype was stained with Alcian blue and the bottom with the von Kossa reagent. All sections were from different mice. Red arrows, intersternebrae. bc, bone collar; p, perichondrium/periosteum. Black boxes, regions of the sternums shown in C. (C) RNA in situ hybridization of sternum mid-sagittal sections consecutive to those stained with Alcian blue in B.

Changes in expression and activity of regulatory molecules in 3NA growth plates

Next, we determined whether or not the 3NA growth plate defects could be explained by changes in expression or activity of known regulatory molecules. Expression of *Sox9*, which may prevent chondrocyte hypertrophy, was unchanged in 3NA growth plates (unpublished data). Expression of *Runx2*, which can induce chondrocyte prehypertrophy, was strongly up-regulated in 3NA late chondroblasts, prehypertrophic chondrocytes, and periosteum cells in E13.5 and E14.5 humeri (Fig. 8 A). Thus, *Sox5* and *Sox6* may prevent prehypertrophy at least in part by down-regulating *Runx2*. Expression of *Pthrp*, which prevents chondroblast maturation, was similar in control and 3NA periarticular chondroblasts (Fig. 8 B). *Ihh*, which has major roles in the growth plate, including stimulation of chondroblast proliferation (St-Jacques et al., 1999), was expressed at similar levels in 3NA and control prehypertrophic chondrocytes (Fig. 8 C). However, more 3NA cells were expressing *Ihh* than control cells between E13.5 and E15.0 due to precocious prehypertrophy and delayed hypertrophy, and fewer 3NA cells were expressing *Ihh* than control cells later on due to reduction of the hypertrophic region. Interestingly, *Ptc1*, which encodes an Ihh receptor and is an Ihh transcriptional target, was up-regulated in 3NA chondroblasts, periosteum, and primary spongiosa between E13.5 and birth (Fig. 8 D). Moreover, although it was widely expressed in early cartilage primordia but restricted to columnar chondroblasts in established growth plates in control embryos, it remained widely expressed in 3NA fetal cartilages. Thus, these *Ptc1* data revealed an increase in Ihh signaling that was unexpected given the proliferation defect of 3NA chondroblasts. Hence, *Sox5* and *Sox6* unlikely control chondroblast proliferation by modulating Ihh expression or signaling. *Fgf18*, which is expressed in the perichondrium and signals through FGFR3 in chondrocytes (Liu et al., 2002; Ohbayashi et al., 2002), was expressed at a normal level in 3NA skeletal elements (unpublished data). *Fgfr3*, which is expressed in columnar chondroblasts and inhibits cell proliferation (Deng et al., 1996; Colvin et al., 1996), was found expressed in a positive gradient from the top to the bottom of the columnar zone in control growth plates. Thus, this gradient was opposite to the cell proliferation gradient. Interestingly, *Fgfr3* expression followed the same gradient in 3NA growth plates but was up-regulated earlier (Fig. 8 E). Therefore, *Sox5* and *Sox6* are likely to control chondroblast proliferation at least in part by delaying *Fgfr3* up-regulation. *Bmp2*, *Bmp4-7*, *Bmpr1a*, and *Bmpr1b* are expressed in discrete domains in the growth plate, perichondrium, and periosteum and interact with the Ihh and FGF signaling pathways. Of these genes, only expression of *Bmp6* was altered in 3NA elements (Fig. 8 F and not depicted). Its expression in developing joints was unchanged, but its expression in prehypertrophic chondrocytes was down-regulated. Expression of *Wnt5a* and *Wnt5b*, which control chondroblast proliferation and differentiation, was not altered in 3NA growth plates (unpublished data). As a result, *Sox5* and *Sox6* likely control growth plate chondrocytes at least in part through the key regulatory molecules RUNX2, IHH, FGFR3, and BMP6.

Discussion

This work has identified multiple, essential roles for *Sox5* and *Sox6* in cartilage growth plates and has allowed for a better understanding of the development and molecular control of these highly specialized structures. We have demonstrated that fetal chondroblasts proliferate at the highest level between epiphyses and metaphyses of future long bones. There-

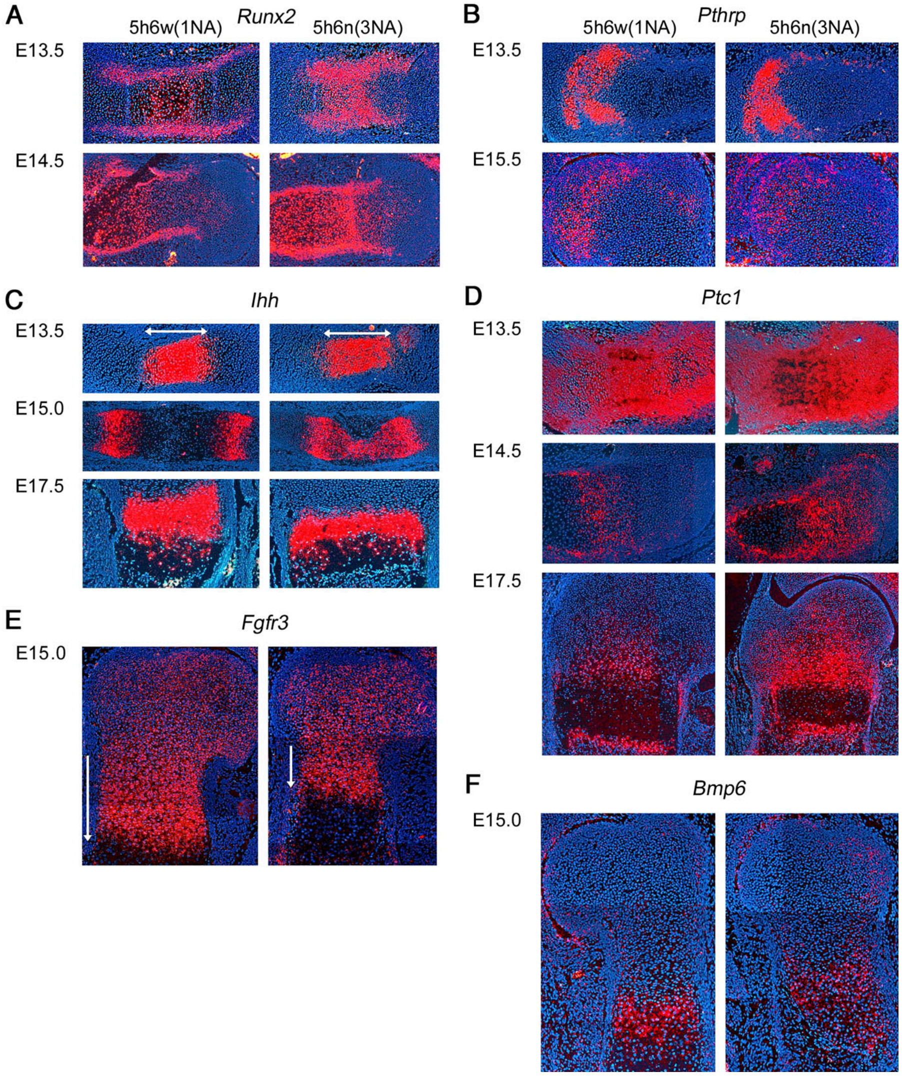


Figure 8. **Expression of regulatory genes in 3NA growth plates.** Longitudinal sections through humeri of control and 3NA embryos were hybridized at different ages with RNA probes for *Runx2* (A), *Pthrp* (B), *Ihh* (C), *Ptc1* (D), *Fgfr3* (E), and *Bmp6* (F), as indicated. Arrows, *Ihh* expression domain and positive gradient of *Fgfr3* expression in the columnar zone.

fore, we propose that these cells must be the main cellular source of growth plates. *Sox5* and *Sox6* are needed for the optimal proliferation of these cells. They are also needed to develop and maintain growth plate columnar zones by allowing chondroblasts arising from the source to decrease their proliferation rate at a slow pace and delay prehypertrophy. Although no longer expressed beyond chondrocyte prehypertrophy, *Sox5* and *Sox6* are required for chondrocyte hypertrophy and to prevent precocious terminal differentiation of prehypertrophic and hypertrophic chondrocytes. The observation that *Sox5/Sox6* mutant chondrocytes terminally differentiate only on contact with ossification fronts suggests an important role for ossification fronts in this last differentiation step. Finally, *Sox5* and *Sox6* were found to act in the growth plate at least in part through major regulatory factors.

Sox5/Sox6 3NA mice and *Sox9* mutant mice

This work would not have been possible using only 4NA mice because these mice die at ∼E16.5 with no true growth plates (Smits et al., 2001). 3NA embryos constituted a good complementary model because their generalized chondrodysplasia is mainly due to growth plate defects. Their chondrocytes are defective at many differentiation steps but do not arrest at any specific step and do complete their differentiation program before mouse death. Therefore, they have allowed us to study the roles of *Sox5* and *Sox6* at each of these steps. The two types of 3NA mice, *Sox5*$^{-/-}$/*Sox6*$^{+/-}$ and *Sox5*$^{+/-}$/*Sox6*$^{-/-}$ mice, exhibit virtually identical growth plate phenotypes, indicating that *Sox5* and *Sox6* are redundant in all steps of chondrocyte maturation. They are more affected than 2NA mice but less affected than 4NA mice, demonstrating that the expression dosage of *Sox5* and *Sox6* is critical for proper chondrogenesis. *Sox5/Sox6* 3NA mice resemble *Sox9*$^{+/-}$ mice (Bi et al., 2001) by the developmental delay and matrix deficiency of cartilage primordia as well as by ectopic mineralization and bending of endochondral elements. However, major differences can be noticed. The onset of cartilage mineralization is precocious in *Sox9*$^{+/-}$ fetuses but delayed in *Sox5/Sox6* 3NA fetuses; endochondral elements are short but not thin in *Sox5/Sox6* 3NA newborns and thin but not always short in *Sox9*$^{+/-}$ newborns; columnar zones are short in *Sox5/Sox6* 3NA fetuses but normal in *Sox9*$^{+/-}$ fetuses; and hypertrophic zones are short in *Sox5/Sox6* 3NA fetuses but long in *Sox9*$^{+/-}$ fetuses. These differences are unlikely due to the degree of severity of the phenotypes because *Sox5/Sox6* 2NA, 3NA, and 4NA mice have qualitatively identical defects. Thus, *Sox5*, *Sox6*, and *Sox9* must have distinct functions during chondrocyte maturation.

Source chondroblasts

The presence of a major chondroblast proliferation peak between epiphyses and metaphyses in fetal long bones leads us to revisit the main cellular source of the growth plate. Most studies with fetal growth plates have assumed that epiphyseal chondroblasts (often referred to as reserve, resting, or periarticular chondrocytes) give rise to columnar chondroblasts. However, no definitive experimental evidence has validated this model. In contrast, our data strongly suggest that epiphyses elongate through cell proliferation mainly in the periarticular region and epiphyseal side of the major proliferation peak, and that columnar zones are generated and renewed mainly by cells originating in the metaphyseal side of this peak. Two pieces of data support our model. First, sternum growth plates develop and are maintained in the absence of epiphyses. They present in pairs oriented back-to-back and sharing a pool of intersternebral chondroblasts. This pool is the likely equivalent of the major proliferation peak in long bones. Second, long bone growth plates become postnatally restricted to the region of the major proliferation peak and metaphysis. They are physically separated from articular cartilage by the secondary ossification centers that develop in epiphyses. Therefore, their cellular source cannot be in articular cartilage. Consistent with our model, a study in rabbits has suggested that postnatal growth plates find their cell source just above the columnar zone, in a zone (called resting zone) that likely corresponds to the fetal chondroblast proliferation peak (Abad et al., 2002).

Epiphyseal chondroblasts

When 4NA fetuses die, their epiphyses are still largely precartilaginous. In contrast, epiphyseal chondroblasts differentiate with only a slight delay in 3NA embryos, and 3NA epiphyses are essentially normal at birth. These data indicate that epiphyseal chondroblasts require *Sox5* and *Sox6* for development, but only at a low dosage. PTHrP/PPR signaling prevents ectopic maturation of epiphyseal chondroblasts (Lanske et al., 1996; Chung et al., 1998; Kobayashi et al., 2002). Ectopic maturation of epiphyseal chondroblasts does not occur in 3NA cartilages, which is consistent with the normal expression of *Ppr* and *Pthrp* detected in these cartilages. Therefore, PTHrP/PPR signaling does not require high levels of *Sox5* and *Sox6*.

Columnar chondroblasts

3NA chondroblasts fail to proliferate at maximum levels at the top of the columnar zone and decrease their proliferation rate much faster than control cells. Thus, columnar chondroblasts require a high dosage of *Sox5* and *Sox6* to function optimally, in contrast to epiphyseal chondroblasts. Nevertheless, *Sox5* and *Sox6* appear expressed at similar levels from the joint area to the prehypertrophic zone (Lefebvre et al., 1998; unpublished data), which means that their expression levels do not directly dictate the cell proliferation changes. Ihh signaling stimulates chondroblast proliferation (St-Jacques et al., 1999). However, because Ihh levels are likely inversely proportional to the gradient of cell proliferation in the columnar zone, Ihh signaling probably attenuates rather than generates this gradient. Interestingly, *Ihh* expression is not substantially altered in 3NA cartilages, and Ihh signaling actually functions better in these cartilages than in control cartilages. This result was revealed by up-regulation and expansion of the expression domain of *Ptc1*. It has been shown that proteoglycans or glycosaminoglycans facilitate the movement of Ihh through tissues (Gritli-Linde et al., 2001). Therefore, it is possible that the 3NA cartilage matrix deficiency allows for a better diffusion of Ihh, and hence, for increased signaling. The other possibility, that 3NA chondroblasts are more responsive to Ihh signaling, is not supported by the fact that *Ptc1* is also up-regulated in 3NA perichondrium and bone cells, which do not express *Sox5* and *Sox6*.

Minina et al. (2001) have shown that BMP signaling is required in addition to Ihh signaling to maintain chondroblast proliferation in limb explants, but a definitive role for BMP signaling in columnar chondroblasts has not been demonstrated in vivo. Nevertheless, inhibition of early chondroblast proliferation in *Bmpr1b*$^{-/-}$ mice (Baur et al., 2000; Yi et al., 2000) and continued expression of *Bmpr1b* and several BMP genes in the growth plate and perichondrium strongly suggest that BMP signaling must contribute to columnar chondroblast proliferation. Expression of BMP and BMP receptor genes is not altered in 3NA cartilages, except for *Bmp6*, which is expressed in prehypertrophic chondrocytes. Although the exact role of *Bmp6* in growth plates has not been fully determined (Solloway et al., 1998), *Sox5* and *Sox6* appear to act upstream of BMP6 signaling, possibly in controlling chondroblast proliferation.

Fgfr3 and *Fgf18* negatively regulate chondroblast proliferation, acting at least in part by down-regulating Ihh expression and signaling (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998; Liu et al., 2002; Minina et al., 2002; Ohbayashi et al., 2002). *Fgf18* is expressed in the perichondrium, and its expression is unaffected in 3NA elements. *Fgfr3* is expressed in the columnar zone, and we found that its expression follows a positive gradient from the top to the bottom of this zone. This finding suggests that FGFR3 signaling generates the negative gradient of columnar chondroblast proliferation. Interestingly, 3NA chondroblasts up-regulate *Fgfr3* earlier than control cells in the columnar zone, strongly suggesting that *Sox5* and *Sox6* control columnar chondroblast proliferation at least in part by delaying *Fgfr3* up-regulation.

Chondrocyte prehypertrophy

In the cartilage primordia of future long bones, 3NA and 4NA chondroblasts activate the prehypertrophic marker *Ppr* later than control cells when considering the gestation time. However, when considering the time at which the cells become chondroblastic, 3NA chondroblasts activate *Ppr* faster than control cells. In the sternum primordium, activation of *Ppr* occurs so early and is so widespread that it is truly ectopic. Thus, *Sox5* and *Sox6* are needed to delay chondrocyte prehypertrophy. In seeking for the underlying mechanism, we found that *Sox5* and *Sox6* are needed to delay and down-regulate expression of the prehypertrophy inducer *Runx2*. However, their mode of action is likely indirect because *Runx2* is also up-regulated in the perichondrium where *Sox5*

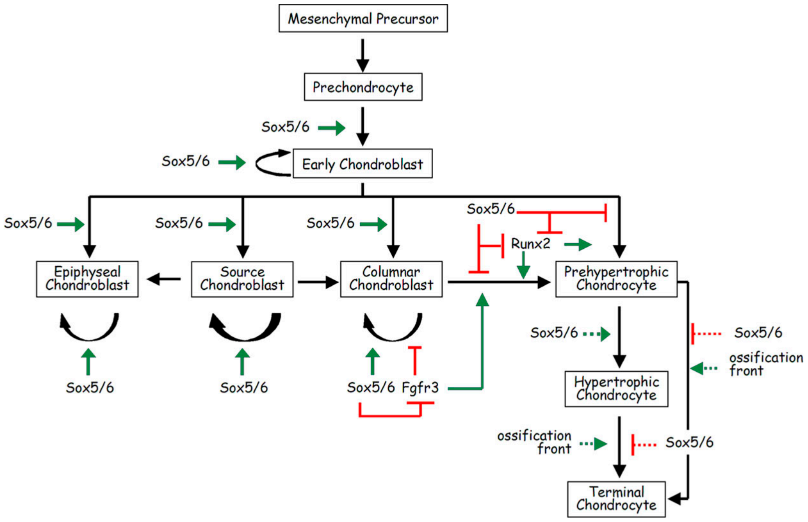


Figure 9. **Proposed model for the roles of *Sox5* and *Sox6* in chondrocytes.** *Sox5* and *Sox6* are expressed in prechondrocytes, chondroblasts, and early prehypertrophic chondrocytes such that their mode of action at these stages must be direct or indirect (green and red solid lines). They are no longer expressed in late prehypertrophic, hypertrophic, and terminal chondrocytes, and therefore their mode of action at these stages must be indirect (red and green dashed lines). *Sox5* and *Sox6* act to promote (green arrows) the differentiation of prechondrocytes into early chondroblasts and the subsequent differentiation of early chondroblasts into epiphyseal, source, or columnar chondroblasts. *Sox5* and *Sox6* also stimulate proliferation of all chondroblasts (curved arrows indicate cell proliferation and their thickness reflects proliferation rates). *Sox5* and *Sox6* maintain columnar chondroblasts proliferating and delay (red bars) prehypertrophic differentiation of early and columnar chondroblasts at least in part by delaying *Fgfr3* up-regulation and by down-regulating *Runx2*. Finally, *Sox5* and *Sox6* are needed to allow induction of chondrocyte hypertrophy and to delay terminal differentiation of prehypertrophic and hypertrophic chondrocytes induced by ossification fronts.

and *Sox6* are not expressed. Significant changes in expression of *Pthrp* and *Ppr*, which delay prehypertrophy, are not detected in 3NA growth plates, indicating that *Sox5* and *Sox6* unlikely act upstream of PPR signaling. FGF signaling accelerates the onset and the pace of prehypertrophic chondrocyte differentiation in limb explants in vitro and BMP signaling antagonizes the effect of FGF signaling (Minina et al., 2002). Because *Sox5* and *Sox6* might control chondroblast proliferation at least in part by delaying up-regulation of *Fgfr3* in columnar chondroblasts, they may similarly delay prehypertrophy and may decrease the effect of FGF signaling by up-regulating *Bmp6*.

Chondrocyte hypertrophy and terminal differentiation

Chondrocytes become hypertrophic with a significant delay in 3NA cartilage primordia, and then form short hypertrophic zones because they terminally differentiate prematurely. In 4NA embryos, chondrocytes skip hypertrophy as seen namely by failure to express *Col10a1*, the most specific marker of hypertrophic chondrocytes, and directly differentiate from early prehypertrophy to the terminal stage. This critical role of *Sox5* and *Sox6* in allowing chondrocytes to undergo hypertrophy and in delaying terminal differentiation appears opposed to that of *Sox9*, which may prevent chondrocyte hypertrophy and accelerate terminal differentiation (Bi et al., 2001). *Sox9* is expressed at a normal level in 3NA and 4NA chondroblasts and is correctly turned off in 3NA hypertrophic cells (Smits et al., 2001; unpublished data). Therefore, it is not involved in the phenotype of *Sox5*/*Sox6* mutants. Because *Sox5*, *Sox6* and *Sox9* are no longer expressed in hypertrophic chondrocytes, their actions on these cells must be indirect. *Runx2* is needed for chondrocyte prehypertrophy and may still be needed for hypertrophy because it was proposed to directly activate *Col10a1* (Zheng et al., 2003). Therefore, its up-regulation in 3NA cells is at odds with the impairment of hypertrophy, unless *Runx2* promotes prehypertrophy but delays hypertrophy, or cooperates with another yet unknown factor to specify chondrocyte hypertrophy. The signaling pathways that promote chondrocyte hypertrophy are still unknown, but the possibility that chondroblasts depend on *Sox5* and *Sox6* to express signaling molecules mediating hypertrophy of neighboring chondrocytes is not supported by observations made in *Sox9*/*Col2-Cre* mutants (Akiyama et al., 2002). In these mutants, cells in the core of cartilage primordia appear to undergo normal differentiation up to and including hypertrophy, whereas all other cartilage cells remain prechondrocytic and fail to activate *Sox5* and *Sox6*. This suggests that chondrocyte hypertrophy is controlled cell autonomously. A mechanism whereby *Sox5* and *Sox6* might be required cell autonomously but indirectly for chondrocyte hypertrophy might be that chondroblasts need to surround themselves with a proper cartilage matrix, a function promoted by *Sox5* and *Sox6,* to be able to undergo hypertrophy.

Despite hypertrophy impairment, 3NA and 4NA chondrocytes undergo terminal differentiation, a step that is not yet fully understood at the regulatory level. Several works have shown a block of chondrocyte differentiation at the mineralizing stage, for instance upon inactivation of *Mmp9* (Vu et al., 1998), *Vegf* (Gerber et al., 1999), *Runx2* (Komori et al., 1997; Otto et al., 1997), or *Osx* (Nakashima et al., 2002), but these works have not demonstrated whether chondrocytes are blocked at the hypertrophic or terminal stage. We have shown that 3NA and 4NA chondrocytes terminally differentiate only on contact with ossification fronts, strongly suggesting that chondrocyte terminal differentiation is induced at least in part by ossification fronts.

3NA cells undergo terminal differentiation even if they do not reach full hypertrophy, and 4NA cells do so immediately after reaching prehypertrophy, indicating that hypertrophy is a dispensable step in the chondrocyte pathway. Thus, *Sox5* and *Sox6* allow chondrocytes to add hypertrophy in their pathway, a step determinant for the proper elongation of endochondral elements. Here, they may also function by allowing cells to accumulate extracellular matrix and thereby protect themselves against bone invasion. In agreement with this model, 3NA sternum chondrocytes may be able to become hypertrophic because they undergo prehypertrophy so massively that ossification fronts cannot invade them all at once.

Conclusions

This work has demonstrated essential, redundant roles for *Sox5* and *Sox6* in chondrocyte differentiation and proliferation (Fig. 9) beyond their critical role in promoting differentiation of prechondrocytes into early chondroblasts (Smits et al., 2001). *Sox5* and *Sox6* ensure the development of early chondroblasts into epiphyseal, source, and columnar chondroblasts and delay chondrocyte prehypertrophy. They stimulate the proliferation of source chondroblasts, keep columnar chondroblasts proliferating, and prevent precocious prehypertrophy at least in part by delaying up-regulation of *Fgfr3* and by down-regulating *Runx2*. They are also needed to allow development of hypertrophic chondrocytes and to prevent precocious terminal differentiation of prehypertrophic and hypertrophic chondrocytes. Because they are no longer expressed in chondrocytes beyond prehypertrophy, their actions in hypertrophic and terminal chondrocytes must be indirect, possibly extracellular matrix mediated. Thus, *Sox5* and *Sox6* are required for the establishment of cartilage growth plates and thereby for the proper and timely development of endochondral bones.

Materials and methods

Generation of *Sox5*/*Sox6* mutant mice

The generation and genotyping of *Sox5*/*Sox6* mutant mice was previously described (Smits et al., 2001). Mice were 129/SvEv × C57BL/6 hybrids, and skeletal phenotypes were fully penetrant. 3 and 4NA mice were generated by crossing *Sox5*/*Sox6* double heterozygous males with single or double heterozygous females. All experiments were repeated with two or more pairs of control and mutant littermates.

Skeleton staining, histology analysis, and RNA in situ hybridization

Whole-mount staining of embryos, skeletal preparations, and histology were performed as described previously (Smits et al., 2001). Samples from control and mutant littermates were embedded in the same paraffin blocks to minimize experimental variation. Sections stained with Alcian blue or with the von Kossa reagent were counterstained with nuclear fast red. The *Bmp6* probe was an 893-bp EcoRI–SacI fragment of the mouse cDNA (a gift from Y. Furuta, University of Texas, Houston, TX), the *Mmp13* probe was the first 699 nucleotides of the mouse cDNA, the *Opn* probe was a 1.3-kb EcoRI fragment of the rat cDNA (a gift from K. Nakashima and B. de Crombrugghe, University of Texas, Houston, TX), and the *Ptc1* probe was the full-length mouse cDNA (a gift from R.L. Johnson, University of Texas, Houston, TX). The *Agc1* and *Col10a1* probes (gifts from E. Vuorio, University of Turku, Turku, Finland), the *Fgfr3* probe (a gift from C.X. Deng, National Institutes of Health, Bethesda, MD), the *Ihh* probe (a gift from A.P. McMahon, Harvard University, Cambridge, MA), the *Mat1* probe (a gift from A. Aszódi, Max-Planck Institute, Martinsried, Germany), the *Mmp9* probe (a gift from Z. Werb, University of California, San Francisco, San Francisco, CA), the *Ppr* probe (a gift from H.M. Kronenberg, Massachusetts General Hospital, Boston, MA), and the *Runx2* probe (a gift from G. Karsenty, Baylor College of Medicine, Houston, TX) were as described previously (Smits et al., 2001).

Cell proliferation assay

Pregnant mice were injected with BrdU reagent (Zymed Laboratories; 10 μl/g of mouse) and killed 2 h later. BrdU-labeled nuclei were detected by immunostaining (Zymed Laboratories), and sections were counterstained with hematoxylin, as described previously (Smits and Lefebvre, 2003). Cell proliferation rates were determined by counting the percentages of BrdU-positive cells in consecutive segments distributed from the periarticular to the hypertrophic zone of growth plates. Reproducible data were obtained with three sets of control and mutant littermates.

Image acquisition and manipulation

Samples were visualized on a microscope (model BX50; Olympus) equipped with Uplanapo 10×/0.40 and Uplanapo 20×/0.70; ∞/0.17 lenses (Olympus). Images were captured with a digital camera (model DMC2; Polaroid) using accompanying software. In situ hybridization images were taken under dark field using a red filter for RNA signals and under blue fluorescence for nuclei stained with Hoechst 33258 dye. Figures were composed using Adobe Photoshop 7.0.

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