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Ext1-Dependent Heparan Sulfate Regulates the Range of Ihh Signaling during Endochondral Ossification

Lydia Koziel,[1] Melanie Kunath,[1] Olivia G. Kelly,[2] and Andrea Vortkamp[1,*]
[1]Otto-Warburg-Laboratory
Max-Planck-Institute for Molecular Genetics
Ihnestrasse 73
14195 Berlin
Germany
[2]Department of Molecular and Cell Biology
University of California, Berkeley
Berkeley, California 94720

Summary

Exostosin1 (Ext1) belongs to a family of glycosyltransferases necessary for the synthesis of the heparan sulfate (HS) chains of proteoglycans, which regulate signaling of several growth factors. Loss of *tout velu* (*ttv*), the homolog of *Ext1* in *Drosophila,* inhibits Hedgehog movement. In contrast, we show that reduced HS synthesis in mice carrying a hypomorphic mutation in *Ext1* results in an elevated range of Indian hedgehog (Ihh) signaling during embryonic chondrocyte differentiation. Our data suggest a dual function for HS: First, HS is necessary to bind Hedgehog in the extracellular space. Second, HS negatively regulates the range of Hedgehog signaling in a concentration-dependent manner. Additionally, our data indicate that Ihh acts as a long-range morphogen, directly activating the expression of *parathyroid hormone-like hormone*. Finally, we propose that the development of exostoses in the human Hereditary Multiple Exostoses syndrome can be attributed to activation of Ihh signaling.

Introduction

During embryonic development, the axial and appendicular skeleton and most of the facial bones are formed by a multistep process called endochondral ossification. This process begins with the condensation of mesenchymal cells that give rise to two cell types: chondrocytes, which form cartilage elements, and perichondrial cells that surround the cartilage anlagen. Starting from the center of the cartilage elements, chondrocytes undergo several steps of maturation—from proliferating chondrocytes to prehypertrophic, hypertrophic, and terminal hypertrophic cells, which are subsequently replaced by bone and bone marrow. As hypertrophic chondrocytes are continuously replaced by bone, the different steps of chondrocyte maturation must be tightly controlled to maintain a stable population of chondrocytes.

Several signaling molecules including Indian hedgehog (Ihh), Parathyroid hormone related hormone (Pthlh), Fibroblast growth factors (Fgfs), Bone morphogenetic proteins (Bmps), and Wnt proteins interact in a complex signaling network to regulate specific steps of this differentiation process (Kronenberg, 2003). It is, however, poorly understood how these signals reach their specific target tissues and how their respective ranges of action are regulated. The onset of hypertrophic differentiation, for example, is controlled by a negative feedback loop between Ihh and Pthlh. *Ihh*, which is expressed in chondrocytes undergoing hypertrophic differentiation (prehypertrophic chondrocytes), signals to the periarticular region to activate the expression of *Pthlh*. Pthlh, in turn, signals back to the proliferating chondrocytes, to inhibit the onset of hypertrophic differentiation (Kronenberg, 2003; Lanske et al., 1996; Vortkamp et al., 1996). It has not been resolved how the Ihh signal is propagated to the periarticular region to upregulate the expression of *Pthlh*. Ihh-dependent secondary signals have been hypothesized to act on the *Pthlh* promoter (Alvarez et al., 2002; Vortkamp et al., 1996; Zou et al., 1997). Alternatively, Ihh could act as a long-range signal that directly activates *Pthlh* expression.

Heparan sulfate proteoglycans (HSPGs) have been implicated in regulating the distribution and receptor binding of several members of Fgf, Wnt, Transforming growth factor β (Tgfβ), and Hedgehog families (Nybakken and Perrimon, 2002). HSPGs consist of extracellular core proteins, like glypicans and syndecans, carrying long heparan sulfate (HS) chains. The HS chains are synthesized in the golgi apparatus in a multistep enzymatic process. Initially, a tetrasaccharide linker is synthesized on conserved serine residues of the core protein. Elongation of the chain is catalyzed by a heteromeric complex of the glycosyltransferases, Exostosin1 (Ext1) and Exostosin2 (Ext2), which adds alternating units of N-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) (Lind et al., 1998; McCormick et al., 1998). Subsequent deacetylation, sulfation, and epimerization result in an ample spectrum of structural heterogenic HS chains. It has been shown that the sulfation pattern of HS chains is critical for binding specific signaling molecules (Esko and Selleck, 2002). Vice versa, Fgf, Tgfβ, and Hedgehog proteins bind to HS with conserved motifs characterized by clusters of basic amino acids (Cardin and Weintraub, 1989; Rubin et al., 2002).Genetic analyses have pointed to an important role for Ext1 in regulating Hedgehog transport. In mice, targeted deletion of *Ext1* leads to a complete lack of HS synthesis. Homozygous embryos fail to gastrulate and lack embryonic mesoderm and extraembryonic tissues. Although *Ihh* mRNA is expressed in mutant embryos, Ihh protein fails to associate with *Ext1*-deficient cells (Lin et al., 2000). In *Drosophila,* Hedgehog (hh) signals over several cell diameters in the wing imaginal disc. In contrast, in clones mutant for the *Ext1* homolog, *tout velu* (*ttv*), only cells directly flanking the source of hh activate hh target genes (Bellaiche et al., 1998; The et al., 1999). Thus, extracellular HS produced by ttv seems to be necessary to transport the hh signal.

In human, mutations in *EXT1* and *EXT2* lead to the autosomal dominant inherited syndrome, Hereditary Multiple Exostoses (HME) (Ahn et al., 1995; Stickens et

*Correspondence: vortkamp@molgen.mpg.de

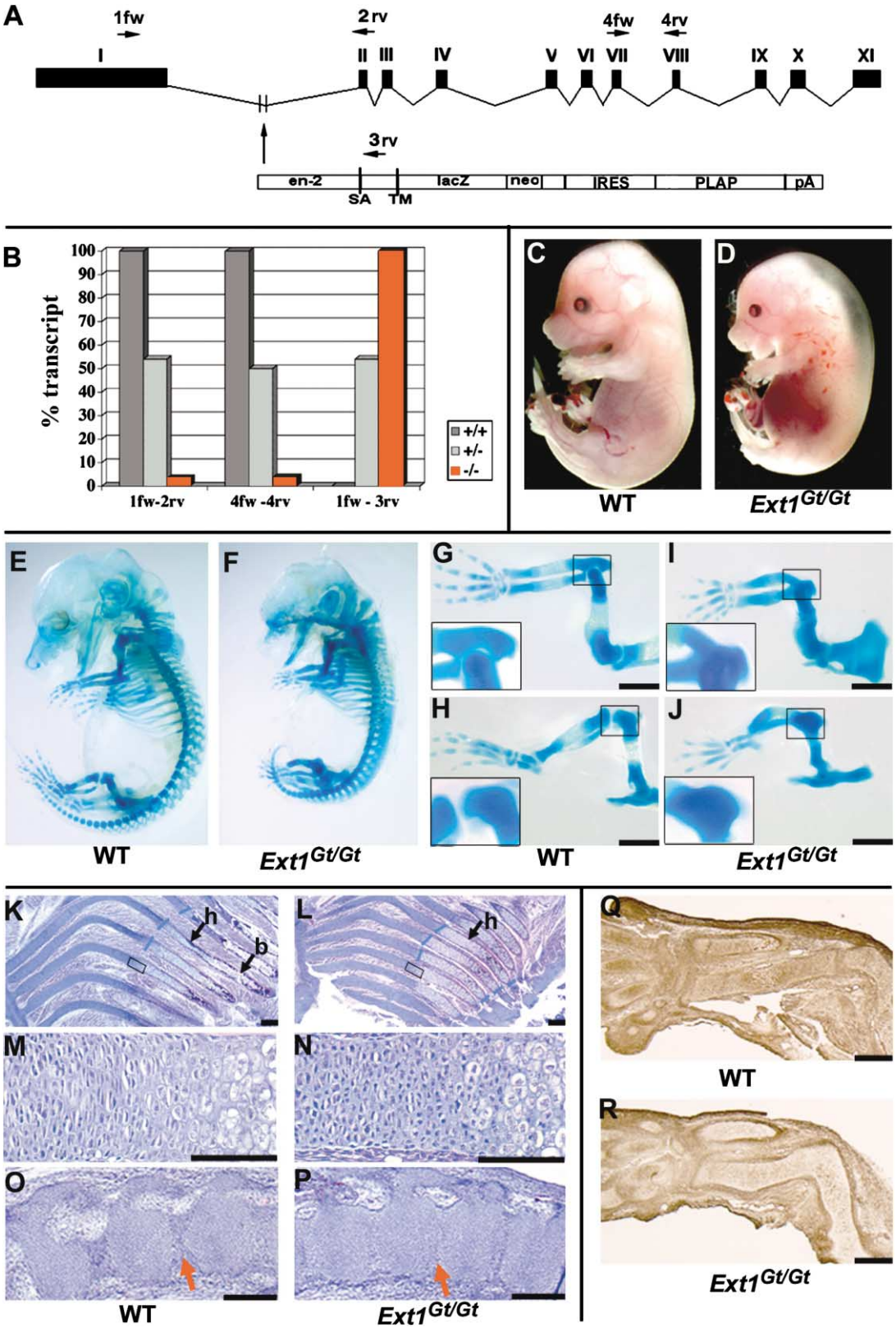

Figure 1. *Ext1*$^{Gt/Gt}$ Mice Display a Hypomorphic Allele of *Ext1*

(A) Schematic representation of the *Ext1* gene and the gene trap vector.

(B) Quantification of *Ext1* transcripts. Primer pairs used for quantitative RT-PCR are indicated in (A). Heterozygous *Ext1*$^{Gt/+}$ mice express around 50% full-length wild-type (fw1-rv2, fw4-rv4) and 50% *Ext1* gene trap transcript (fw1-rv3). Homozygous *Ext1*$^{Gt/Gt}$ mice express 3% wild-type transcript (n = 2).

al., 1996). HME patients are characterized by reduced skeletal size and multiple, cartilage-capped, benign bone tumors (exostoses) that arise from the growth plate of endochondral bones. Interestingly, new exostoses only develop until the growth plate closes, implicating misregulation of chondrocyte differentiation as a likely cause for the development of exostoses. Correspondingly, *Ext1* and *Ext2* show elevated expression levels in the proliferating chondrocytes of endochondral bones in mice (Stickens et al., 2000).

The proposed relationship of ttv-dependent HS and hh transport in *Drosophila* in combination with the bone defects observed in HME patients implicate a role for Ext1 in regulating Ihh signaling. To test such an interaction, we have analyzed a mouse line carrying a hypomorphic allele of *Ext1* (*Ext1*$^{Gt/Gt}$) (Mitchell et al., 2001). In contrast to studies in *Drosophila* where loss of *ttv* function leads to an inhibition of hh movement, we found an elevated range of Ihh signaling in growth plates of *Ext1*$^{Gt/Gt}$ mutants, which express reduced amounts of HS. Furthermore, ectopic HS leads to a restricted range of the Ihh signal, implicating that HS shapes morphogen gradients in vivo. Furthermore, our results strongly suggest that Ihh acts as a long-range morphogen that directly induces the expression of *Pthlh*.

Results

Characterization of *Ext1*$^{Gt/Gt}$ Mice

To analyze the role of Ext1 during endochondral ossification, we investigated a mouse line mutant for *Ext1* (*Ext1*$^{Gt/Gt}$), which was generated in a gene trap screen (Mitchell et al., 2001). In *Ext1*$^{Gt/Gt}$ mice, the gene trap vector has inserted into the first intron, creating a truncated Ext1 protein fused to the β-geo reporter of the vector (Figure 1A). Homozygous *Ext1*$^{Gt/Gt}$ embryos survive until E14.5 at a nonmendelian ratio of 14%, and only 4% can be recovered at E16.5. *Ext1*$^{Gt/Gt}$ embryos are small and appear edematous (Figures 1C and 1D). Sections of mutant embryos at E14.5 reveal reduced cardiac muscles and a failure to septate the outflow tract and ventricular chambers (O.G.K. and W.C. Skarnes, unpublished data). These heart defects are a likely cause for the embryonic lethality. Alcian blue staining reveals a reduced skeleton size with fused vertebrae, shortened fore- and hindlimbs, fusions of elbow and knee joints, and occasionally syndactylies of digits (Figures 1E–1J). As targeted deletion of *Ext1* (*Ext1*$^{-/-}$) is lethal during gastrulation (Lin et al., 2000), the insertion of the gene trap vector in *Ext1*$^{Gt/Gt}$ mice seems to create a hypomorphic allele of *Ext1*.

Ext1$^{Gt/Gt}$ Mice Synthesize Reduced Amounts of HS

To test if HS synthesis is disturbed in *Ext1*$^{Gt/Gt}$ mice, we stained sections of E14.5 limbs with the anti-HS-antibody 3G10, which detects unsaturated glucuronate at the nonreducing ends of HS chains after digestion with heparatinase (David et al., 1992). Interestingly, reduced but significant amounts of HS can be detected in *Ext1*$^{Gt/Gt}$ mice (Figures 1Q and 1R). In contrast, no HS is synthesized in *Ext1*$^{-/-}$ mice (Lin et al., 2000), confirming that the gene trap insertion results in a hypomorphic allele of *Ext1*.

To test if expression of full-length protein contributes to the residual glycosyltransferase activity, we analyzed wild-type and mutant *Ext1* mRNA by quantitative RT-PCR (Figure 1B). Using primer pairs specific either for the wild-type (exon1/exon2 and exon7/exon8) or for the mutant allele (exon1/gene trap vector), we found that heterozygous *Ext1*$^{Gt/+}$ mice transcribe each allele in equal amounts of 50%. Interestingly, homozygous *Ext1*$^{Gt/Gt}$ mice transcribe about 3% of wild-type *Ext1* mRNA ($n = 2$). It is thus likely that alternative splicing around the gene trap vector results in low amounts of wild-type Ext1 protein in *Ext1*$^{Gt/Gt}$ mice.

Ext1$^{Gt/Gt}$ Mice Show Delayed Hypertrophic Differentiation

To investigate the role of Ext1 during bone development, we analyzed cartilage morphology after Safranin Weigert (SW) staining in radius and ulna of wild-type and mutant forelimbs. At E14.5, wild-type embryos display well-organized zones of proliferating and hypertrophic chondrocytes. In contrast, *Ext1*$^{Gt/Gt}$ mutants reveal joint fusions and a severe but highly variable delay in hypertrophic differentiation (Figures 2A and 2B). In situ hybridization revealed that *Ihh* is expressed in two domains of prehypertrophic chondrocytes, which flank a domain of *CollagenX* (*Col10a1*)-expressing hypertrophic chondrocytes in wild-type embryos. In contrast, limbs of *Ext1*$^{Gt/Gt}$ mutants show only weak expression of *Ihh* in the center of the cartilage elements and *Col10a1* is not expressed in most mutants at E14.5 (Figures 2A–2D). Impaired chondrocyte differentiation and a severely reduced region of Ihh-expressing cells can already be detected at E13.5 in *Ext1*$^{Gt/Gt}$ mutants (data not shown). In comparison to the majority of embryos at younger stages, *Ext1*$^{Gt/Gt}$ mutants at E15.5 and E16.5 display a milder, albeit more stable, phenotype, presumably because less-affected mutants survive longer. SW staining reveals short and broad skeletal elements with expanded zones of proliferating chondrocytes and severely delayed bone formation (Figures 2K and 2L). Proliferating chondrocytes next to the hypertrophic region,

(C–N) Phenotype of *Ext1*$^{Gt/Gt}$ mice. (C and D) E15.5 *Ext1*$^{Gt/Gt}$ mice are small and edematous. (E–J) Alcian blue staining of E15.5 *Ext1*$^{Gt/Gt}$ embryos reveals short skeletal elements and fused vertebrae. Fore- and hindlimbs show fusions in elbow and knee joints. (K–P) Hematoxylin/Eosin staining of sagittal sections of ribs and vertebrae. (K and L) Ribs of *Ext1*$^{Gt/Gt}$ mice show delayed endochondral ossification (blue bar, border of hypertrophic chondrocytes [h]; b, bone). (M and N) Higher magnification of insets in (K) and (L) show disorganized proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mice.

(O and P) Vertebrae are enlarged and individual elements are fused (red arrow).

(Q and R) Immunohistochemistry with the 3G10 antibody on limb sections of E14.5 wild-type mice detects high levels of ubiquitously distributed HS (brown staining), which are reduced in *Ext1*$^{Gt/Gt}$ mice.

Scale bars: 1 mm (G–J); 250 μm (K, L, O, P); 100 μm (M and N).

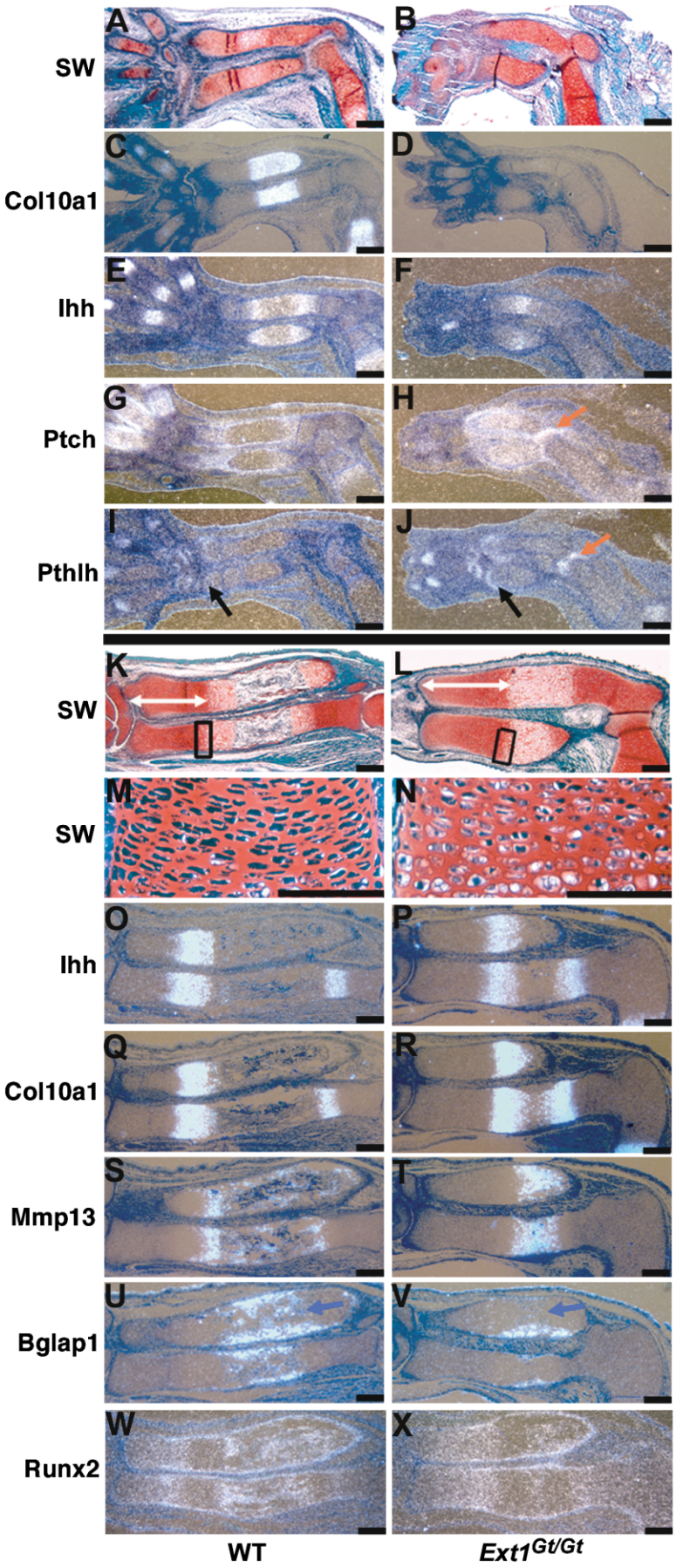

Figure 2. Hypertrophic Differentiation Is Delayed in *Ext1*$^{Gt/Gt}$ Mice

Sections of E14.5 (A–J) and E16.5 (K–X) wild-type and *Ext1*$^{Gt/Gt}$ mutant limbs were stained with Safranin Weigert (SW) or hybridized with antisense riboprobes as indicated. (E)–(J) and (O)–(V) display parallel sections.

(A and B) At E14.5, SW staining reveals severely delayed cartilage development. Whereas the zone of *Ihh* expression (E) has split into two domains flanking the expression domain of *Col10a1* in wild-type embryos (C), *Ext1*$^{Gt/Gt}$ mice display a small continuous *Ihh* expression domain (F) and do not express *Col10a1* (D). Nevertheless, *Ptch* expression is strongly upregulated throughout the cartilage anlagen (G and H). Similarly, the expression of *Pthlh* is upregulated in *Ext1*$^{Gt/Gt}$ mutants ([I] and [J], black arrow). Note the coexpression of strong *Pthlh* and *Ptch* expression in the elbow joint (red arrow). (K and L) At E16.5, *Ext1*$^{Gt/Gt}$ mice show an enlarged zone of proliferating chondrocytes (white arrow) and terminal hypertrophic chondrocytes have not been replaced by bone. (M and N) Higher magnification of proliferating chondrocytes marked in (K) and (L). (O and P) *Ext1*$^{Gt/Gt}$ mice show reduced expression of *Ihh*.

(Q–T) Central chondrocytes have differentiated into hypertrophic and terminal hypertrophic chondrocytes expressing *Col10a1* and *Mmp13,* respectively. (U and V) No bone formation can be detected in the center of the developing skeletal elements in *Ext1*$^{Gt/Gt}$ mutants as seen by the lack of *Bglap1* expression (blue arrow). (W and X) *Runx2* is expressed in normal pattern.

Scale bars: 100 μm (M and N); 250 μm (A–L, O–X).

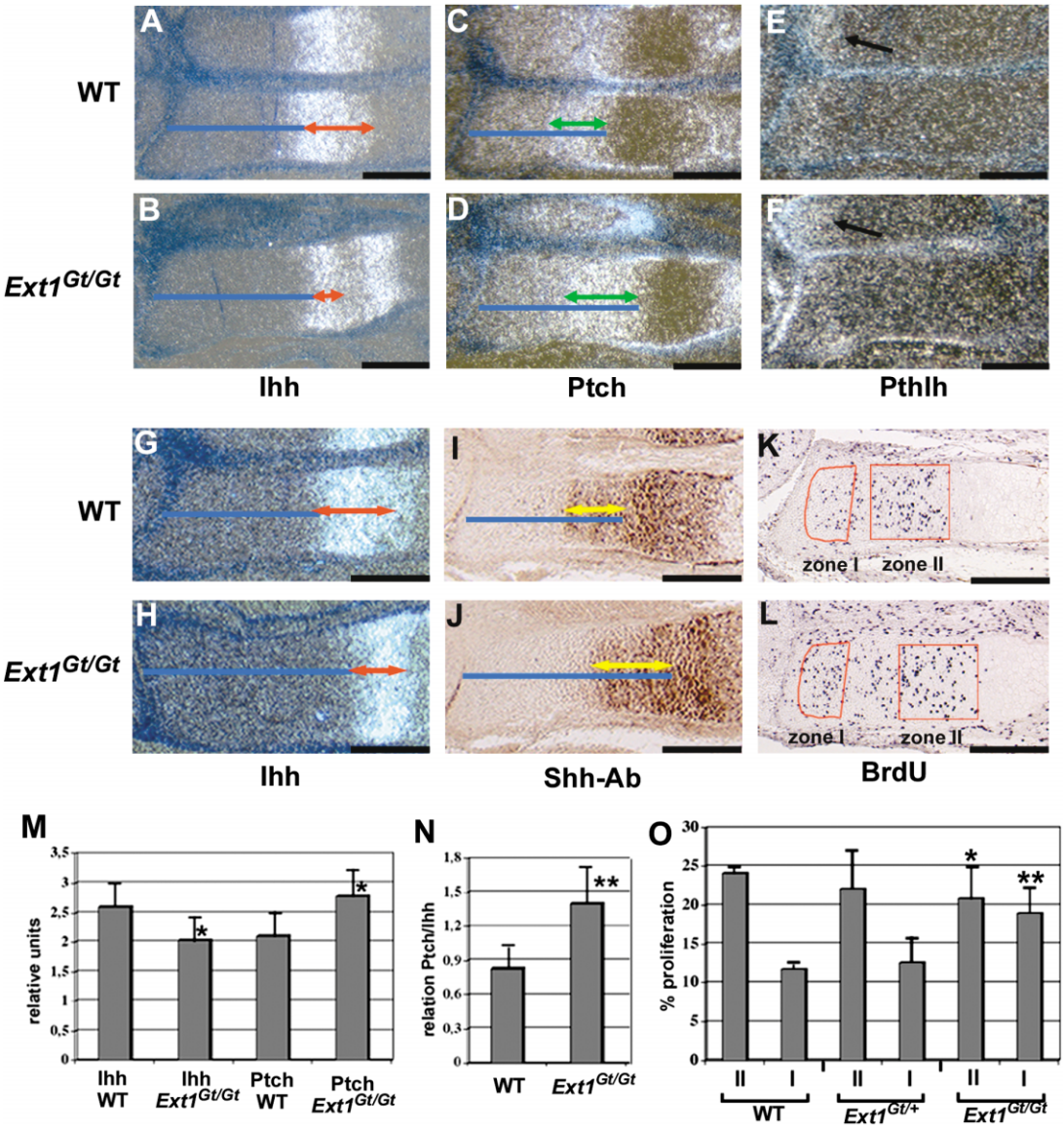

Figure 3. Ihh Signaling Is Increased in *Ext1*$^{Gt/Gt}$ Mice

(A–F) In situ hybridization on serial section of E15.5 wild-type and *Ext1*$^{Gt/Gt}$ mutant limbs revealed a reduced expression domain of *Ihh* ([A] and [B], red arrow) whereas the domain of strong *Ptch* expression is increased ([C] and [D], green arrow). (M) Relative sizes of *Ihh* and *Ptch* expression domains ($n = 14$, *: $p < 0.02$, unpaired student's t test). (N) The domain of *Ptch* expression in relation to the *Ihh* expression domain is increased by 60% ($n = 14$, **: $p < 0.01$, unpaired student's t test). (E and F) *Pthlh* expression is upregulated in *Ext1*$^{Gt/Gt}$ mice (black arrow). (G–J) ShhAb80 immunohistochemistry reveals an extended domain of detectable Ihh protein in proliferating chondrocytes ([I] and [J], yellow arrow). The distal border of the *Ihh* expression domain, which was identified by in situ hybridization with an *Ihh* riboprobe on controlateral limbs (G and H), was used as a reference (blue line) ($n = 4$).

(K, L, and O) Chondrocyte proliferation is upregulated in periarticular chondrocytes in *Ext1*$^{Gt/Gt}$ mice. Proliferating cells were labeled with BrdU, detected by antibody staining, and analyzed in defined regions of wild-type, *Ext1*$^{Gt/+}$, and *Ext1*$^{Gt/Gt}$ mice at E15.5 ([K] and [L] and data not shown). (O) Proliferation rates in zone II are similar in all three genotypes ($n = 4$, *: $p > 0.05$ unpaired student's t test), whereas zone I of *Ext1*$^{Gt/Gt}$ mutants shows an increased proliferation rate of 19% compared to 12% in wild-type mice ($n = 4$, **: $p < 0.02$ unpaired student's t test). Scale bars: 250 μm.

which are normally organized in columns of flattened cells, appear round and disorganized in *Ext1*$^{Gt/Gt}$ mutants (Figures 2M and 2N). In E15.5 and E16.5 *Ext1*$^{Gt/Gt}$ mutants, the zone of *Ihh* expression is reduced (Figures 2O, 2P, 3A, and 3B). Markers for hypertrophic chondrocytes, *Col10a1* and *Matrix-metalloprotease-13* (*Mmp13*), are expressed in distinct domains similar to wild-type embryos (Figures 2Q–2T). However, the hypertrophic region is not separated by endochondral bone in *Ext1*$^{Gt/Gt}$ mutants as shown by lack of expression of the osteoblast marker *osteocalcin* (*Bglap1*) (Figures 2U and 2V). No significant alteration in the expression of either *Runx2* or *Runx3*, two positive regulators of chondrocyte and osteoblast differentiation, could be detected in *Ext1*$^{Gt/Gt}$ mutants (Figures 2W and 2X). Since *Bglap1* and *Runx2* expression is maintained in the periosteum in *Ext1*$^{Gt/Gt}$

mutants, the lack of endochondral bone is not due to impaired osteoblast differentiation, but rather reflects the delay in chondrocyte differentiation.

Similarly, Hematoxilin-Eosin staining of ribs and vertebrae of E15.5 embryos revealed disturbed chondrocyte and bone differentiation in *Ext1*$^{Gt/Gt}$ mice. Mutant ribs show disorganized proliferating chondrocytes and a failure to replace hypertrophic chondrocytes with endochondral bone. The cartilaginous vertebral bodies are fused, indicating increased chondrocyte proliferation (Figures 1K–1P). In contrast to *Ext1*$^{Gt/Gt}$ mice, we could not detect a skeletal phenotype in *Ext1*$^{Gt/+}$ embryos.

Pthlh and *Ptch* Are Upregulated in *Ext1*$^{Gt/Gt}$ Embryos

A reduced level of *Ihh* expression in *Ext1*$^{Gt/Gt}$ mice would be expected to accelerate the onset of hypertrophic differentiation. Instead, the expanded distance between the *Ihh* expression domain and the joint region in E16.5 mutant embryos indicates a severe delay in the onset of hypertrophic differentiation. Pthlh is the effective molecule downstream of Ihh signaling in regulating the onset of hypertrophic differentiation. Surprisingly, at all stages analyzed, *Pthlh* expression is upregulated in *Ext1*$^{Gt/Gt}$ mutants (Figures 2I and 2J and Figures 3E and 3F).

Upregulation of *Pthlh* expression despite reduced amounts of Ihh could indicate that other growth factors regulate *Pthlh* expression independent of Ihh. Alternatively, Ihh signaling might be potentiated by the reduced amounts of HS in *Ext1*$^{Gt/Gt}$ mutants. To test the latter, we examined the expression of *Patched* (*Ptch*), which is upregulated in all cells receiving a Hedgehog signal (Goodrich et al., 1996). In *Ext1*$^{Gt/Gt}$ mutant mice, *Ptch* expression is upregulated in most embryos at E14.5 (Figures 2G and 2H). Only strongly affected embryos expressing no or significantly reduced amounts of *Ihh* show reduced expression of *Ptch* compared to wild-type embryos (data not shown). Interestingly, in a subset of mutants, strong *Ptch* expression is detected overlapping with upregulated *Pthlh* expression (Figures 2H and 2J).

At E15.5 and E16.5, two domains of *Ptch* expression can be distinguished at the distal ends of ulna and radius of wild-type limbs: a domain of strong *Ptch* expression in chondrocytes adjacent to the *Ihh*-expressing cells, and a domain of weaker *Ptch* expression at the distal ends of the cartilage elements, which encompasses the *Pthlh* expressing periarticular cells. In *Ext1*$^{Gt/Gt}$ mutants, the region of strong *Ptch* expression is expanded toward the joint region (Figures 3C, 3D, and 3M). This difference is more significant if the domain of strong *Ptch* expression is related to the domain of *Ihh*-expressing cells (60% increase, Figure 3N). As *Ptch* expression can be used as readout for Ihh signaling, the broader domain of high *Ptch* expression strongly indicates an increased range of Ihh signaling in *Ext1*$^{Gt/Gt}$ mutants.

Ihh Distribution Is Extended in *Ext1*$^{Gt/Gt}$ Mice

The extended range of Ihh signaling in *Ext1*$^{Gt/Gt}$ mice suggests that reduced levels of HS alter the propagation of Ihh protein. To test this hypothesis, we analyzed the distribution of Ihh protein by immunohistochemistry using the Shh antibody, ShhAb80 (Yang et al., 1997), which crossreacts with Ihh protein (Gritli-Linde et al., 2001). At E15.5 and E16.5, ShhAb80 detects a gradient of Ihh protein that extends from the *Ihh*-expressing chondrocytes into the adjacent region of proliferating cells (Figures 3I and 3J). To analyze the distribution of Ihh protein in relation to its expression domain, we determined the distal border of *Ihh* mRNA expression in controlateral limbs. Using this border as start point, we measured the distance in which we could detect Ihh protein. We found extended domains of detectable Ihh protein in the proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mutants ($n = 4$) (Figures 3I and 3J). Similar to the expression of *Ptch*, this difference is more evident if the domain of Ihh protein is analyzed in relation to the *Ihh* expression domain. Together, these data strongly indicate that reduced amounts of HS in *Ext1*$^{Gt/Gt}$ embryos facilitate the distribution of Ihh protein.

Block of Ihh Signaling Rescues the Delayed Onset in Hypertrophic Differentiation in *Ext1*$^{Gt/Gt}$ Mice

To support the idea that increased Ihh signaling leads to the delayed onset of hypertrophic differentiation in *Ext1*$^{Gt/Gt}$ mice, we attempted to inhibit Ihh signaling. The alkaloid cyclopamine specifically inhibits the Ihh signaling pathway in limb explant cultures (Minina et al., 2001). Similar to wild-type limb explants, treatment of E15.5 *Ext1*$^{Gt/Gt}$ limbs with cyclopamine results in a block of *Ptch* and *Pthlh* expression (Figures 4E, 4F, 4K, and 4L). Furthermore, the onset of hypertrophic differentiation is accelerated, as indicated by a reduced distance between the *Ihh* expression domain and the joint region. As inhibition of Ihh signaling can rescue the delay in hypertrophic differentiation, increased Ihh signaling seems to be responsible for the *Ext1*$^{Gt/Gt}$ phenotype.

Chondrocyte Proliferation Is Increased in the Periarticular Region of *Ext1*$^{Gt/Gt}$ Mice

In addition to its role in regulating chondrocyte differentiation, Ihh signaling activates chondrocyte proliferation, independent of Pthlh (St-Jacques et al., 1999). The region of proliferating chondrocytes can be subdivided into a zone of low-proliferating, periarticular chondrocytes (zone I) and a zone of high-proliferating, columnar chondrocytes (zone II) (Long et al., 2001). We determined the proliferation rate in defined regions of each zone in radii of wild-type, *Ext1*$^{Gt/+}$, and *Ext1*$^{Gt/Gt}$ embryos at stage E15.5 (Figures 3K, 3L, and 3O). We found no statistically significant differences of chondrocyte proliferation in zone II (20%–24%). In contrast, the proliferation rate in zone I is increased from 12% in wild-type and *Ext1*$^{Gt/+}$ mice to 19% in *Ext1*$^{Gt/Gt}$ embryos. The increased proliferation rate in zone I of the *Ext1*$^{Gt/Gt}$ mutants is in accordance with an extended range of Ihh signaling.

Ectopic HS Restrict Ihh Signaling

Our results so far suggest that reduced amounts of HS in *Ext1*$^{Gt/Gt}$ mice lead to an increased range of Ihh signaling. Consequently, increased HS concentrations should restrict Ihh signaling and accelerate the onset of hypertrophic differentiation. To test this hypothesis, we treated limb explants of E15.5 wild-type embryos in culture

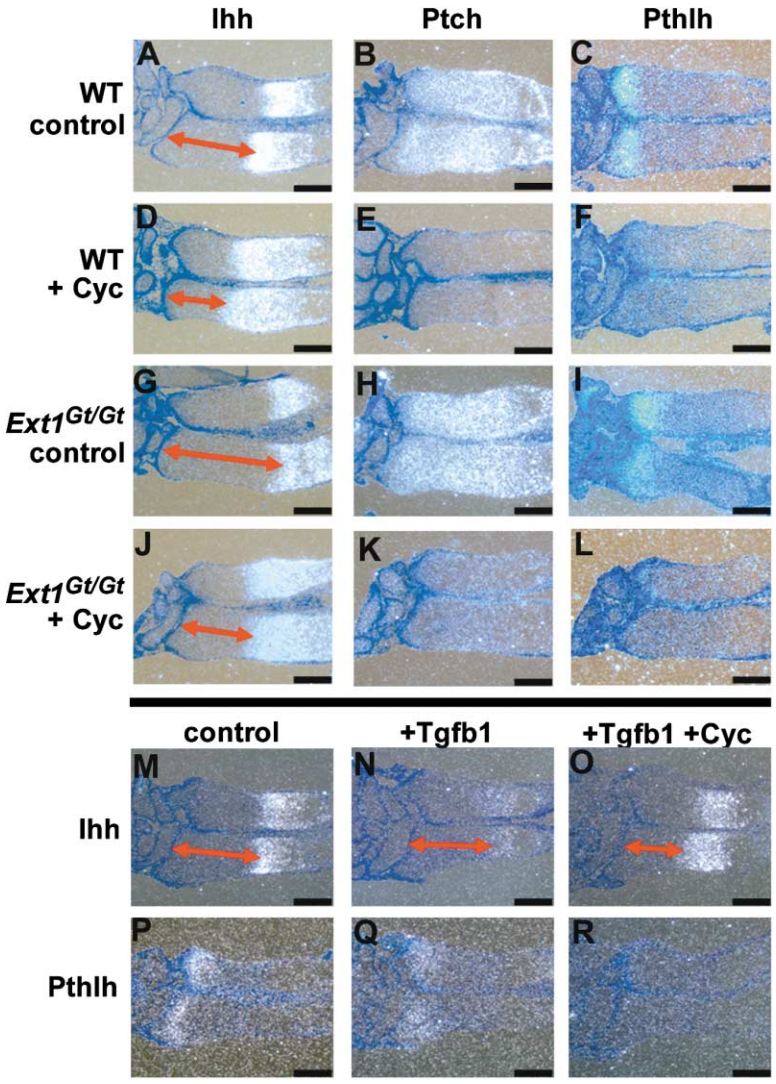

Figure 4. Ihh Acts Downstream of Ext1

(A–L) Forelimbs of E15.5 wild-type (A–F) and *Ext1*$^{Gt/Gt}$ (G–L) mouse embryos were cultured for 2 days with control medium (A–C and G–I) or medium supplemented with cyclopamine (D–F and J–L). Serial sections were hybridized with antisense riboprobes as indicated. Wild-type and *Ext1*$^{Gt/Gt}$ limbs show accelerated hypertrophic differentiation, which leads to a reduced distance between the *Ihh* expression domain and the joint region ([A], [D], [G], and [J], red arrow). Neither *Ptch* nor *Pthlh* expression can be detected after treatment with cyclopamine.

(M–R) Tgfβ cannot activate *Pthlh* independent of Ihh. Forelimbs of E15.5 wild-type mice were cultured for 2 days in control medium (M and P) or treated with Tgfβ1 (N and Q) or Tgfβ1 and cyclopamine (O and R) and hybridized with riboprobes as indicated. Treatment with Tgfβ1 leads to a slightly accelerated onset of hypertrophic differentiation ([M]–[O], red arrow). Tgfβ1 cannot activate *Pthlh* expression independent of Ihh signaling (O–R). Scale bars: 250 μm.

with ectopic HS, heparin, or chondroitin sulfate (CS) for 2 days (Figure 5). Treatment of limbs with 1 μg/ml, 10 μg/ml, or 100 μg/ml HS leads to an accelerated onset of hypertrophic differentiation in a dose-dependent manner (Figures 5A, 5D, and 5G). To explore the range of Ihh signaling after treatment with HS, we analyzed the expression of *Ptch*. Strikingly, in limbs treated with high concentrations of HS, *Ptch* expression is restricted to a narrow stripe of chondrocytes directly adjacent to the *Ihh* expression domain and to the flanking perichondrium/periosteum (Figures 5B, 5E, and 5H). No expression of *Ptch* could be detected in distal chondrocytes. Similar results were obtained after treatment with heparin (Figures 5J–5L). Interestingly, heparin, which is more highly sulfated, is more effective in restricting the Ihh signal, indicating that not only the amount of HS but also the degree of sulfation might be critical to determine the range of the Ihh signal. In contrast, treatment with CS does not inhibit *Ptch* expression, supporting the specificity of HS for binding Ihh.

We furthermore treated limb explants of E15.5 *Ext1*$^{Gt/Gt}$ mice with HS, heparin, and CS. As in wild-type limbs, we observed a dose-dependent acceleration of the onset of hypertrophic differentiation with HS and heparin, whereas CS has no effect (Figures 5P–5U). Similar to cyclopamine, HS supplementation thus rescues the *Ext1*$^{Gt/Gt}$ phenotype.

To explore the mechanism by which HS regulates Ihh signaling, we treated limb explants of mice overexpressing *Ihh* under the *Collagen2a1* (*Col2a1*) promoter (*Col2a1-Ihh*) (Long et al., 2001) with heparin. As in untreated controls, we detected an upregulation of *Ptch* expression throughout the proliferating chondrocytes (data not shown). Therefore, HS seems to regulate Ihh signaling by restricting the distribution rather than by inhibiting the reception of the Ihh signal.

Regulation of *Pthlh* Expression

As explained above, it has not yet been resolved whether Ihh directly or indirectly regulates *Pthlh* expression. After HS treatment, cells adjacent to the *Ihh* expression domain still react to Ihh signals by upregulating *Ptch* expression and should thus be able to produce secondary signals. Surprisingly, we found severely reduced expression of *Pthlh* in the periarticular region after treatment

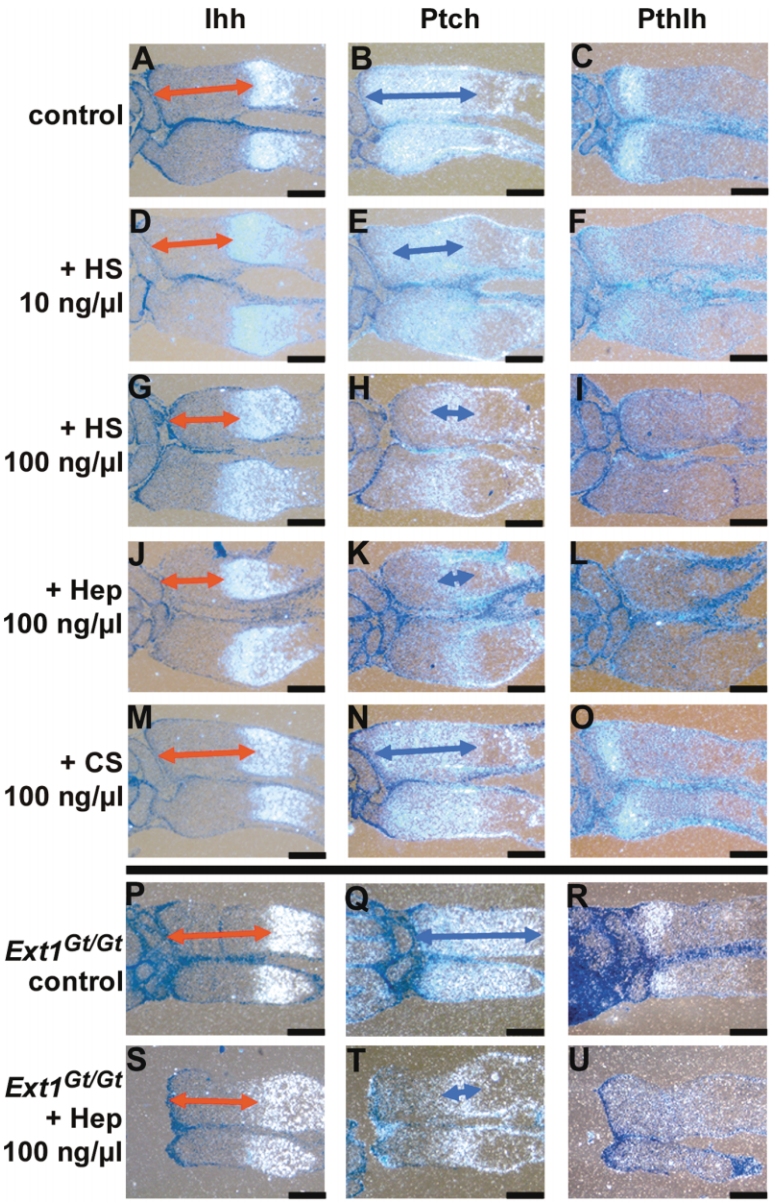

Figure 5. Ectopic HS Restrict Ihh Signaling

Forelimbs of E15.5 wild-type (A–O) or *Ext1*$^{Gt/Gt}$ (P–U) mouse embryos were cultured for 2 days in control medium (A–C, P–R) or in medium supplemented with HS (D–I), heparin (Hep) (J–L, S–U), or chondroitin sulfate (CS) (M–O). Serial sections were hybridized with antisense riboprobes as indicated. Treatment of wild-type and *Ext1*$^{Gt/Gt}$ limbs with HS and heparin leads to a reduced distance between the Ihh expression domain and the joint region (red arrow). HS and heparin restrict *Ptch* expression (blue arrow) to the *Ihh* expression domain in a concentration-dependent manner (B, E, H, and K). Similarly, *Pthlh* expression is reduced in a concentration-dependent manner by HS and heparin (C, F, I, and L). (M–O) Treatment with CS does not effect *Ptch* (N) or *Pthlh* expression (O). The expression of *Ptch* (Q and T) and *Pthlh* (R and U) is reduced in *Ext1*$^{Gt/Gt}$ mutant limbs after heparin treatment. Scale bars: 250 μm.

with heparin or HS in a dose-dependent manner, implicating a direct regulation of *Pthlh* expression by the Ihh signal (Figures 5C, 5F, 5I, and 5L). In contrast, treatment with CS does not alter the expression of *Pthlh*. Similar results were obtained after treatment of *Ext1*$^{Gt/Gt}$ mutant limbs.

Bmps and Tgfβs have long been hypothesized to act as secondary signals downstream of Ihh to induce the expression of *Pthlh* (Alvarez et al., 2002; Zou et al., 1997). Previously, we have excluded Bmps from acting as such mediators (Minina et al., 2001). To further support a direct regulation, we have treated limb explants of E15.5 and E16.5 embryos with Tgfβ1. We detected no significant alteration in *Pthlh* expression and a slight acceleration of hypertrophic differentiation. Importantly, cotreatment of limb explants with cyclopamine and Tgfβ1 could not rescue the expression of *Pthlh*, which is lost after cyclopamine treatment (Figures 4D, 4F, and 4M–4R). We can thus exclude Tgfβ1 as a secondary signal inducing *Pthlh* expression downstream of Ihh. Together, these data strongly suggest that Ihh directly activates the expression of *Pthlh* independent of secondary factors.

Activated Fgf Signaling Does Not Rescue the Delay in Differentiation of *Ext1*$^{Gt/Gt}$ Mice

In vertebrates, Fgf signaling is dependent on an interaction between Fgf receptor, Fgf ligand, and HS (Esko and Selleck, 2002). The analysis of *Fgfr3*$^{ach/+}$ mice, which express a constitutively activated *Fgf receptor 3* (*Fgfr3*) under the *Col2a1* promoter, has shown that Fgf signaling reduces chondrocyte proliferation and accelerates the onset as well as the process of hypertrophic differentiation (Minina et al., 2002; Naski et al., 1998). Disturbed Fgf signaling might thus contribute to the *Ext1*$^{Gt/Gt}$ phenotype. To test if *Ext1*$^{Gt/Gt}$ chondrocytes can respond to Fgf signals similar to wild-type chondrocytes, we treated

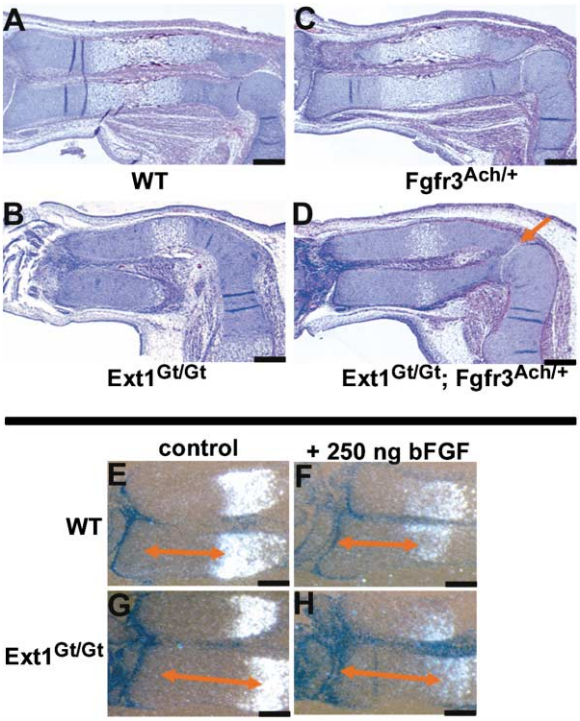

Figure 6. Fgf Signaling in *Ext1*$^{Gt/Gt}$ Mutants

(A–D) Hematoxylin/Eosin staining of E15.5 wild-type (A), *Ext1*$^{Gt/Gt}$ (B), *Fgfr3*$^{Ach+}$ (C), and *Ext1*$^{Gt/Gt}$;*Fgfr3*$^{Ach+}$ (D) limb sections. *Ext1*$^{Gt/Gt}$; *Fgfr3*$^{Ach/+}$ mutants display a similar delay in hypertrophic differentiation as *Ext1*$^{Gt/Gt}$ mutants; however, fusions of elbow joints are partially rescued (red arrow in [D]). (E–H) Forelimbs of E15.5 wild-type (E and F) or *Ext1*$^{Gt/Gt}$ (G and H) mouse embryos were cultured for 2 days in control medium or medium supplemented with Fgf2. Serial sections were hybridized with an antisense riboprobe for *Ihh*. Limbs of wild-type and *Ext1*$^{Gt/Gt}$ embryos react to Fgf treatment with reduced expression of *Ihh* and a subsequent accelerated onset of hypertrophic differentiation (red arrows). Scale bars: 250 μm.

E15.5 *Ext1*$^{Gt/Gt}$ limb explants in culture with Fgf2 (Figures 6E–6H). In both wild-type and *Ext1*$^{Gt/Gt}$ explants, treatment with Fgf2 leads to reduced *Ihh* expression and a subsequent acceleration of the onset of hypertrophic differentiation, indicating that receptor binding is not disturbed in *Ext1*$^{Gt/Gt}$ mutants. We further examined ligand-independent activation of Fgf signaling in *Ext1*$^{Gt/Gt}$; *Fgfr3*$^{ach/+}$ compound mutants. Interestingly, one allele of *Fgfr3*^{ach} does not rescue the delayed onset of hypertrophic differentiation in *Ext1*$^{Gt/Gt}$ mutants at E15.5 and E16.5 (Figures 6A–6D). However, elbow and knee joints, which are always fused in *Ext1*$^{Gt/Gt}$ mutants, are partially rescued by activated Fgf signaling in *Ext1*$^{Gt/Gt}$;*Fgfr3*$^{Ach/+}$ mice (Figure 6D, red arrow). In summary, Fgf signaling does not seem to be significantly disturbed in *Ext1*$^{Gt/Gt}$ mice.

Discussion

Reduced HS Synthesis in *Ext1*$^{Gt/Gt}$ Mice

HSPGs are main structural components of the ECM in cartilage. In addition, they play important roles in regulating signal propagation of various growth factors. To analyze the role of HSPGs in regulating chondrocyte differentiation, we have investigated mice carrying a gene trap insertion in *Ext1*, a glycosyltransferase necessary for the synthesis of HS.

In contrast to *Ext1*$^{-/-}$ mice, which die during gastrulation (Lin et al., 2000), *Ext1*$^{Gt/Gt}$ mice survive until midgestation, revealing a residual function of the mutant *Ext1* allele. Correspondingly, reduced amounts of HS can be detected in *Ext1*$^{Gt/Gt}$ mutant embryos by immunohistochemistry, whereas no HS is synthesized in *Ext1*$^{-/-}$ mice. Similarly, a parallel study revealed that embryonic fibroblasts from *Ext1*$^{Gt/Gt}$ mice produce about 18% HS compared to wild-type cells. The lesser amounts of HS are primarily due to shortened rather than to decreased numbers of HS chains. Interestingly, the sulfation pattern of the mutant HS seems to be normal, suggesting that interactions with growth factors are not perturbed in general (Yamada et al., 2004).

The residual glycosyltransferase activity in *Ext1*$^{Gt/Gt}$ mice could theoretically be contained in the truncated Ext1 fusion protein. It has been shown, however, that nonsense and missense mutations scattered throughout the *EXT1* gene lead to similar HME phenotypes in humans (Zak et al., 2002). Furthermore, a murine cell line carrying a splice mutation, which leads to a truncation of the Ext1 protein shortly behind exon1, lacks any HS polymerase activity (McCormick et al., 2000). It is therefore not likely that the truncated *Ext1*$^{Gt/Gt}$ protein, consisting only of the polypeptide encoded by exon1, would maintain sufficient glycosyltransferase activity for the synthesis of HS in *Ext1*$^{Gt/Gt}$ mice. On the other hand, functional Ext1 protein could be generated by alternative splicing around the gene trap vector. By quantitative RT-PCR, we detected about 3% full-length *Ext1* transcripts in homozygous *Ext1*$^{Gt/Gt}$ mice. Low amounts of wild-type Ext1 protein might thus produce sufficient amounts of HS to allow survival until E16.5. Different levels of alternative splicing in mutant mice may then contribute to the variability of the *Ext1*$^{Gt/Gt}$ phenotype.

Ext1-Dependent HS Regulates Ihh Signaling

The expanded zone of proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mice, which reflects a delay in the onset of hypertrophic differentiation, resembles that of mice overexpressing Ihh under the *Col2a1* promoter. In contrast to *Col2a1-Ihh* mice, we found reduced expression of *Ihh* in *Ext1*$^{Gt/Gt}$ mice. Nevertheless, *Pthlh* expression is upregulated, indicating either Ihh-independent regulation of *Pthlh* expression or increased Ihh signaling. To differentiate between these possibilities, we have investigated Ihh signaling at different levels: First, analysis of *Ptch* expression, a direct target of Ihh signaling (Goodrich et al., 1996), revealed an extended domain of strong *Ptch* expression in proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mice. Second, by immunohistochemistry we detected a broader domain of Ihh protein in the proliferating chondrocytes of mutant mice. Third, chondrocyte proliferation is upregulated in periarticular chondrocytes similar to mice overexpressing either *Ihh* or an activated form of the Hedgehog receptor *Smoothened* (*Smo*) (Long et al., 2001) under the *Col2a1*-promotor. Fourth, inhibition of Ihh signaling with cyclopamine in *Ext1*$^{Gt/Gt}$ limb explants results in a rescue of the *Ext1*$^{Gt/Gt}$ phenotype. We thus conclude that the range of Ihh signaling is extended

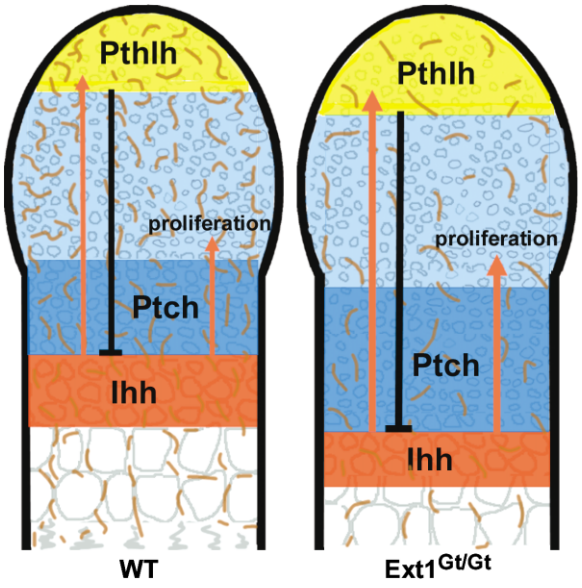

Figure 7. Ext1-Dependent HS Regulates Ihh Signaling

Ihh, expressed in prehypertrophic chondrocytes (red), travels through the proliferating chondrocytes to directly activate the expression of *Pthlh* (yellow). Ihh signaling induces strong *Ptch* expression (dark blue) in columnar chondrocytes flanking the *Ihh* expression domain and weaker *Ptch* expression in distal chondrocytes including the periarticular, *Pthlh*-expressing cells (light blue and yellow). HS (brown) negatively regulates the propagation of the Ihh signal. Reduced levels of HS in *Ext1*$^{Gt/Gt}$ mutants facilitate Ihh transport and lead to an increased domain of strong *Ptch* expression and an upregulation of *Pthlh* expression. Pthlh in turn delays the onset of hypertrophic differentiation.

toward the distal regions of the cartilage anlagen. As *Pthlh* expression is lost after cyclopamine treatment, we can furthermore exclude an Ihh-independent regulation of *Pthlh* expression in *Ext1*$^{Gt/Gt}$ mice. In summary, our results strongly indicate that upregulation of *Pthlh* expression and the resulting delay in hypertrophic differentiation is due to increased Ihh signaling (Figure 7). In accordance with these data, a slightly increased distribution of Ihh protein was found in heterozygous *Ext1*$^{+/-}$ mice, which express about 50% HS (M.J. Hilton et al., unpublished data). In contrast, we could not detect a phenotype in mice heterozygous for the hypomorphic *Ext1*$^{Gt/+}$ allele. Together, these data suggest that the distribution of Ihh in the growth plate is dependent on the concentration of HS.

A functional link between HS and Hedgehog signaling was first implicated by analyses of the *Drosophila* mutant *ttv* (Bellaiche et al., 1998; The et al., 1999). Recently, two other members of the *Ext* family have been identified in *Drosophila*: *Sister of ttv* (*sotv*), a homolog of *Ext2,* and *brother of ttv* (*botv*), the fly *Ext-like 3* homolog, a more distantly related member of the Ext family (Takei et al., 2004). Mutations in either gene lead to loss or severely reduced amounts of HS. Consequently, in clones of mutant cells in the imaginal discs, hh target genes are activated in cells directly flanking the *hh* expression domain but not in cells located several cell diameters away from the source of hh expression (Han et al., 2004; The et al., 1999). These results indicate a role for ttv-dependent HS in transporting hh in the extracellular space.

Based on the *Drosophila* studies, it might be expected that the reduced levels of HS in *Ext1*$^{Gt/Gt}$ mice would result in decreased Ihh signaling and, hence, loss of *Pthlh* expression and accelerated hypertrophic differentiation. In contrast, our results clearly demonstrate that Ihh signaling is increased, not decreased. As *Ext1*$^{Gt/Gt}$ mice produce lower amounts of HS, we conclude that HS restricts Ihh propagation in mice, thereby negatively regulating Ihh signaling. This hypothesis is supported by treatment of wild-type limb explants with HS, which leads to a concentration-dependent restriction of *Ptch* expression to cells flanking the *Ihh* expression domain. The role of HS in regulating Ihh signaling in mice seems thus to be in contrast to its proposed function in regulating hh in *Drosophila*. This discrepancy could implicate a different role of HS in regulating Hedgehog signaling in vertebrates and flies. Given the conservation of the Hedgehog signaling pathway, however, it is more likely that the difference reflects the different alleles investigated, null in *Drosophila* and hypomorphic in mice. We thus propose a dual function for HS in controlling Hedgehog signals: First, HS is necessary to bind Hedgehog molecules in the extra cellular space, thereby facilitating transport of the Hedgehog signal from cell to cell. Total loss of HS as in *ttv* mutants or *Ext1*$^{-/-}$ mice would then result in loss of biological available Hedgehog protein and consequently in a loss of Hedgehog signaling. Second, increasing concentrations of HS sequester increasing amounts of Hedgehog molecules, thereby restricting Hedgehog activity in a concentration-dependent manner to the source of its expression domain. Fine-tuning the levels of HS would thus provide an important mechanism to regulate the range of Hedgehog acting as a morphogen.

Similar mechanisms have been proposed for the regulation of dpp and wg signaling by HSPGs in *Drosophila*. Overexpression of *dally* or *dally-like*, the two *glypican* homologs in *Drosophila*, restricts wg and dpp proteins to the site of their expression (Baeg et al., 2001; Fujise et al., 2003). These data suggest that an excess of HSPGs negatively regulates the distribution of different growth factors.

In addition to regulating protein distribution, HS mediates binding of FGFs to their receptors (Esko and Selleck, 2002). It has still to be resolved if HS is required for binding of Ihh to its receptor. In *Drosophila*, clones of cells with different defects in the HS synthesis pathway still activate Hedgehog target genes in one row of cells flanking the source of hh (Han et al., 2004). Further studies are needed to determine whether binding of hh to its receptor in these clones is dependent on HS from neighboring wild-type cells. In our limb explant studies, addition of HS fails to affect the upregulation of Ihh target genes in limbs overexpressing Ihh. These data support a role of HS in regulating the distribution rather than the reception of Ihh signals.

Direct Regulation of *Pthlh* by Ihh

As previously explained, it has not been resolved if Ihh signals directly act on the Pthlh promoter or if secondary

signals like Bmps or Tgfβs are needed (Alvarez et al., 2002; Zou et al., 1997). Recent experiments have excluded Bmps from mediating the Ihh signal (Minina et al., 2001). Similarly, we show here that Tgfβ cannot induce *Pthlh* expression in an Ihh-independent way in midgestation embryos, a result that differs from previous studies (Alvarez et al., 2002) and might reflect stage-specific differences. Instead, the data presented in this study, in combination with experiments from other laboratories, strongly implicate a direct role of Ihh in regulating *Pthlh* expression (Figure 7). First, we and others (Gritli-Linde et al., 2001) have shown by immunohistochemistry that Ihh can travel over long distances in the developing cartilage anlagen. Second, we detected weak but significant expression of *Ptch* at the distal ends of the skeletal elements including those chondrocytes that express *Pthlh*. In addition, in some $Ext1^{Gt/Gt}$ embryos, *Ptch* expression is strongly upregulated in cells expressing *Pthlh* (Figures 2H and 2J), supporting a direct regulation. Third, treatment of limb explants with ectopic HS restricts *Ptch* expression to cells directly flanking the *Ihh* expression domain. These cells are the most likely source to express a secondary signal. However, no *Pthlh* expression can be detected in periarticular cells. Although we cannot completely exclude that HS restricts the distribution of a hypothetical secondary signal similar to that of Ihh, our results are most parsimonious with Ihh acting as a long-range signal that directly activates *Pthlh* expression. Fourth, the function of *Smo* has recently been disrupted in *Col2a1*-expressing chondrocytes (*Col2a1-Smo*^{C}) (Long et al., 2001). Unexpectedly, chondrocyte differentiation is not affected in these mutants. In wild-type embryos, *Pthlh* is expressed in *Col2a1*-expressing periarticular chondrocytes (Figures 3E and 3F). In contrast, in *Col2a1-Smo*^{C} mice, cells in the joint region, which do not express *Col2a1*, highly express *Pthlh,* whereas no expression can be detected in the most distal chondrocytes (Figure 5 in Long et al., 2001). The shift of *Pthlh* expression to cells outside the *Col2a1* expression domain in *Col2a1-Smo*^{C} mutants strongly supports a direct activation by Ihh.

HS and Fgf Signaling

Our results indicate that potentiation of Ihh signaling might be the main cause for the $Ext1^{Gt/Gt}$ phenotype. It is, however, possible that other signaling systems are also affected. As HS stabilizes the FGF ligand-receptor complex (Esko and Selleck, 2002), reduced Fgf signaling might contribute to the delay in hypertrophic differentiation in $Ext1^{Gt/Gt}$ mice. However, the response of $Ext1^{Gt/Gt}$ limbs treated with Fgf in culture indicates that reduced amounts of HS in these mutants do not significantly alter binding of Fgfs to their receptors. In addition, ligand-independent activation of Fgf signaling in compound $Ext1^{Gt/Gt}$;$Fgfr^{Ach/+}$ mutants cannot rescue the $Ext1^{Gt/Gt}$ phenotype. It is thus unlikely that loss of Fgf signaling is a major cause for the delayed differentiation at the investigated stages. We did, however, observe a reduced degree of elbow fusions in $Ext1^{Gt/Gt}$;$Fgfr3^{ach/+}$ mutants compared to $Ext1^{Gt/Gt}$ mice. Fgf-dependent inhibition of chondrocyte proliferation might thus prevent secondary joint fusions. Together, these results correlate well with earlier studies placing Fgf signaling upstream to that of Ihh in regulating the onset of hypertrophic differentiation and in parallel in regulating chondrocyte proliferation (Minina et al., 2002).

It has recently been shown that mutations in *ttv*, *sotv*, and *botv* affect wg and dpp signals in addition to hh (Takei et al., 2004). Similarly, tissue-specific deletion of *Ext1* using the *nestin* promoter leads to distinct neuronal defects reminiscent of disrupted Fgf, Wnt, and Slit signaling (Inatani et al., 2003). Signaling through these molecules might thus be affected at later stages or in different organs in $Ext1^{Gt/Gt}$ mice.

Ihh Signaling and the Development of Exostoses

Although mutations in *EXT* genes have been linked to HME, it is poorly understood how they lead to the formation of exostoses. *Ext1* has been classified as a tumor suppressor gene and somatic Loss of Heterozygosity (LOH) or secondary mutations in an *EXT* homolog have been hypothesized to give rise to the isolated exostoses. However, mutation analysis in exostoses tissues of 16 HME patients have detected only one case of LOH, giving limited support for the second hit model (Hall et al., 2002). The reduced levels of HS in $Ext1^{Gt/Gt}$ and $Ext1^{+/-}$ mice (Lin et al., 2000) suggest that HME patients produce reduced levels of HS, making haploinsufficiency of *Ext1* or *Ext2* the most likely cause for the development of exostoses.

Our results suggest that reduced amounts of HS potentiate Ihh signaling, resulting in delayed hypertrophic differentiation and increased chondrocyte proliferation (Figure 7). In mice, it has recently been shown that activated signaling through the receptor of Pthlh, Pthr1, which acts downstream of Ihh, results in local enchondroma-like lesions, characterized by overproliferation and delayed chondrocyte differentiation (Hopyan et al., 2002). Activated Ihh signaling in HME patients might similarly result in clusters of chondrocytes that overproliferate. Groups of proliferating cells in close contact to the perichondrium might then be able to escape the overall regulation of growth plate differentiation by breaking through the perichondrium, thereby inducing the development of an exostosis. Disturbed chondrocyte orientation as observed in $Ext1^{Gt/Gt}$ mice might facilitate such a mechanism.

Experimental Procedures

Transgenic Mice

The insertion site of the gene trap vector (Leighton et al., 2001) in the 234 kb-long first intron of the $Ext1^{Gt/Gt}$ mice (official designation, $Ext1^{Gt(pGT2TMpfs)064Wcs}$) (Mitchell et al., 2001) was localized 48 kb downstream of Exon1 by inverse PCR. Genomic DNA isolated from liver of $Ext1^{Gt/+}$ mice was digested with BamHI and recircularized with T4 DNA ligase. Two rounds of PCR were performed with nested primer pairs located in the pGT2TMpfs vector: (1) Inv-fw1: 5′-TGCTTCTGATGAGGTGGTCC-3′ and Inv-rv1: 5′-TACATAGTTGGC AGTGTTTGGG-3′; (2) Inv-fw2: 5′-GCCAGAGACTCAGTGAAGCCT-3′ and Inv-rv2: 5′-GGGTCTCAAAGTCAGGGTCAC-3′. PCR fragments were cloned in pCR4-TOPO (Invitrogen) and sequenced. The insertion side maps at 14.449.442 of the contig NT_039621.1 (NCBI). Primers *Ext1*fw: 5′-CACATCAGGTGCCTCACAAC-3′; *Ext1*rv: 5′-CTCCCAGCACTTTTCCTGAC-3′ and 5′pgto: 5′-TACATAGTTGG CAGTGTTTGGG-3′ were designed to detect a 0.6 kb wild-type and a 0.8 kb mutant band. $Fgfr^{ach/+}$ mice were genotyped as in Naski et

al. (1998). Wild-type mice (NMRI) were derived from Charles River (Germany).

Quantitative RT-PCR

Total RNA was isolated from limbs of E14.5 *Ext1*$^{Gt/Gt}$, *Ext1*$^{Gt/+}$, and wild-type embryos using Trizol Reagent (Invitrogen). cDNA was generated by reverse transcription using random primers (First strand kit, Amersham Biosciences). Quantification of *Ext1* mRNA expression was performed with the ABI Prism 7700 Sequence Detection System using SYBR Green I (Applied Biosystems). Primers were selected with Primer Express Software (Applied Biosystems) as follows: 1fw-2:5′-CTGGCAAAAGCACAAGGATTC-3′ and 2rv: 5′-TTGTGCAGCATTTCCCGATA-3′; 1fw-3: 5′-TGGCAAAGACTGGCAAAAGC-3′ and 3Gtrv: 5′-GTTTTCGGGACCTGGGACTT-3′; 4fw: 5′-GCCGGTTTCTGCCCTATGA-3′ and 4rv: 5′-TACGGTGAAGGCAAAATCCA-3′. cDNAs were normalized against transcript levels of *Hprt* (*Hprt*fw: 5′-GCTCGAGATGTCATGAAGGAGAT-3′ and *Hprt*rv 5′-AAAGAACTTATAGCCCCCCTTGA-3′).

Organ Cultures of Embryonic Limb Explants

Forelimbs of E15.5 and E16.5 mouse embryos were cultured as described in Minina et al. (2001). Cultures were supplemented with 10 μM cyclopamine (Incardona et al., 1998), 250 ng/ml Fgf2 (Sigma), 1 μg/ml, 10 μg/ml, or 100 μg/ml HS sodium salt; 1 μg/ml, 10 μg/ml, or 100 μg/ml heparin sodium salt (Sigma); or 10 μg/ml or 100 μg/ml CS sodium salt (Sigma). Limbs were cultured for 2 days. Each treatment was repeated at least five times.

In Situ Hybridization Analysis

Embryonic limbs or limb explants after culture were fixed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin. Serial sections of 5 μm were processed for radioactive in situ hybridization using [P^{33}]-UTP labeled antisense riboprobes (Vortkamp et al., 1996). Probes for in situ hybridization were as follows: *Col10a1* (Jacenko et al., 1993), *Ihh* (Bitgood and McMahon, 1995), *Ptch* (Goodrich et al., 1996), *Bglap1* (Celeste et al., 1986), *Runx2* (Stricker et al., 2002), and *Mmp13* (Yamagiwa et al., 1999). A 423 bp mouse *Pthlh*-fragment was amplified by RT-PCR from embryonic limbs using primers: fw5′-GCTGCCGCCAAGACTAATTAG-3′, rv5′-GTAGACTAGCGCCTCTAGGTG-3′ and cloned into pCR-2.1-TOPO (Invitrogen).

BrdU Labeling

Mice were sacrificed 2 hr after receiving an intraperitoneal injection of 31 μg/g body weight 5-bromo-2′-deoxy-uridine (BrdU) (BrdU labeling and detection kit II, Roche). Cultured limb explants were treated with 31 μg/ml for 2 hr before harvesting. Limbs were fixed in 4% paraformaldehyde at 4°C and embedded in paraffin. Proliferating cells on 6 μm sections were detected by antibody staining according to the manufacturer. BrdU-positive and -negative nuclei were counted in periarticular and columnar chondrocytes in zones of equal size ($n = 4$, eight sections each).

Immunohistochemistry

Immunohistochemistry with ShhAb80 (Bumcrot et al., 1995) and 3G10 (biotinylated antibody) (David et al., 1992) (Seikagaku Corporation) was performed as described in Gritli-Linde et al. (2001). Before incubation with 3G10, limb sections were overlayed with 250 mU heparinase III (Sigma) in digestion buffer for 4 hr. Chromogenic signals were enhanced with the Tyramide Signal Amplification Kit (NEN Life Science products) according to the manufacturer's instructions.

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