Gli3 acts as a repressor downstream of Ihh in regulating two distinct steps of chondrocyte differentiation

Lydia Koziel, Manuela Wuelling, Sabine Schneider and Andrea Vortkamp*

Max-Planck-Institute for Molecular Genetics, Berlin, Germany and Department for Developmental Biology, Center for Medical Biotechnology, University Duisburg-Essen, Essen, Germany
*Author for correspondence (e-mail: andrea.vortkamp@uni-due.de)

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Summary

During endochondral ossification, the secreted growth factor Indian hedgehog (Ihh) regulates several differentiation steps. It interacts with a second secreted factor, parathyroid hormone-related protein (PTHrP), to regulate the onset of hypertrophic differentiation, and it regulates chondrocyte proliferation and ossification of the perichondrium independently of PTHrP. To investigate how the Ihh signal is translated in the different target tissues, we analyzed the role of the zinc- finger transcription factor Gli3, which acts downstream of hedgehog signals in other organs. Loss of *Gli3* in *Ihh* mutants restores chondrocyte proliferation and delays the accelerated onset of hypertrophic differentiation observed in *Ihh*$^{-/-}$ mutants. Furthermore the expression of the Ihh target genes patched (*Ptch)* and *PTHrP* is reactivated in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants. Gli3 seems thus to act as a strong repressor of Ihh signals in regulating chondrocyte differentiation. In addition, loss of *Gli3* in mice that overexpress *Ihh* in chondrocytes accelerates the onset of hypertrophic differentiation by reducing the domain and possibly the level of *PTHrP* expression.

Careful analysis of chondrocyte differentiation in *Gli3*$^{-/-}$ mutants revealed that Gli3 negatively regulates the differentiation of distal, low proliferating chondrocytes into columnar, high proliferating cells. Our results suggest a model in which the Ihh/Gli3 system regulates two distinct steps of chondrocyte differentiation: (1) the switch from distal into columnar chondrocytes is repressed by Gli3 in a PTHrP-independent mechanism; (2) the transition from proliferating into hypertrophic chondrocytes is regulated by Gli3-dependent expression of *PTHrP*. Furthermore, by regulating distal chondrocyte differentiation, Gli3 seems to position the domain of *PTHrP* expression.

Key words: Indian hedgehog (Ihh), Gli3, Gli2, Gli1, PTHrP (Pthlh), Chondrocyte, Cartilage, Bone

Introduction

Endochondral ossification is a multistep process, which starts with the formation of a cartilage template. Chondrocytes in the cartilage anlagen initially proliferate and undergo several steps of differentiation into terminal hypertrophic cells, which are subsequently replaced by bone. In the mouse embryonic long bones after E14.5, the spatial organization of distinct chondrocyte types reflects temporal development. From distal to central the following cell types can be distinguished: low proliferating, round chondrocytes expressing fibroblast growth factor receptor 1 (*Fgfr1*), high proliferating, columnar chondrocytes expressing *Fgfr3*, early hypertrophic chondrocytes expressing Indian hedgehog (*Ihh*) and *Fgfr3*, hypertrophic chondrocytes expressing procollagen type X α1 (*Col10a1*) and terminal hypertrophic chondrocytes expressing matrix metalloproteinase 13 (*Mmp13*) (Minina et al., 2005). A fibroblastic tissue, the perichondrium, surrounds the cartilage anlagen. In parallel to the onset of hypertrophic differentiation, cells in the perichondrium flanking the hypertrophic region differentiate into bone producing osteoblasts. Subsequently, blood vessels and osteoblasts from the newly formed bone start to invade the terminal hypertrophic region and to replace cartilage by bone and bone marrow (Erlebacher et al., 1995).

Ihh is one of the key regulators of endochondral ossification controlling at least three distinct differentiation steps. (1) To regulate the onset of hypertrophic differentiation, Ihh interacts with a second secreted growth factor, Parathyroid hormone related protein (PTHrP; Pthlh – Mouse Genome Informatics). Ihh signals to the distal periarticular chondrocytes to upregulate the expression of *PTHrP*. PTHrP in turn signals back to the proliferating chondrocytes and inhibits the differentiation of proliferating cells into the *Ihh* expressing early hypertrophic cell type. (2) In addition, Ihh regulates chondrocyte proliferation and (3) induces the ossification of the perichondrium in PTHrP independent mechanisms (Kronenberg, 2003).

Whereas the regulation of chondrocyte proliferation and the ossification process can be explained by Ihh acting on neighboring tissues, it has been an open question as to how the Ihh signal reaches the joint region to induce the expression of *PTHrP*. Initially, secondary factors have been hypothesized to mediate the Ihh signal. Recent analyses of mice carrying a hypomorphic allele of exostosin 1 (*Ext1*), a glycosyltransferase necessary for the synthesis of heparan sulfate (HS), have,

however, shown that HS negatively regulates the propagation of Ihh in the cartilage anlagen. In addition, these investigations strongly support a model in which Ihh acts as a long range morphogen, directly inducing the expression of *PTHrP* (Koziel et al., 2004).

To further understand Ihh signaling it is important to investigate how the signal is translated in the different skeletal target tissues. The molecular mechanism of hedgehog signaling has been best analyzed in *Drosophila*. In short, in the receiving cells, Hedgehog (Hh) is bound by a receptor complex consisting of the 12-transmembrane receptor Patched (Ptc) and the 7-transmembrane receptor Smoothened (Smo). Binding of Hh to Ptc releases the repression of Smo, which, via a complex signaling cascade, alters the activity of the zinc finger transcription factor Cubitus interruptus (Ci) (Lum and Beachy, 2004). Ci belongs to the Gli family of transcription factors. These proteins contain a domain of five conserved zinc fingers of the C2H2 type and a conserved C-terminal transactivation domain. In the absence of Hh signals the 155 kDa Ci protein is phosphorylated and proteolytically processed into a truncated N-terminal repressor protein of 75 kDa containing the zinc fingers, which inhibits the expression of Hh target genes. Upon Hh signaling, phosphorylation and thus proteolytic processing is blocked and full length Ci protein acts as a transcriptional activator of Hh target genes (Aza-Blanc et al., 1997; Chen et al., 1998; Jia et al., 2002; Methot and Basler, 2001; Price and Kalderon, 2002).

In vertebrates three Ci homologues have been identified: Gli1, Gli2 and Gli3. Biochemical investigations indicate that, similar to Ci, Gli2 and Gli3 can be proteolytically processed into a truncated repressor form, whereas Gli1 lacks the protein kinase A recognition site necessary for phosphorylation and subsequent cleavage. Gli1 is therefore likely to function exclusively as an activator (Aza-Blanc et al., 2000; Price and Kalderon, 2002; von Mering and Basler, 1999).

The role of Gli genes in vertebrates has been mainly analyzed in relation to sonic hedgehog (Shh) signaling. In the neural tube, Shh signaling from the floor plate controls the differentiation of six classes of neurons in a concentration-dependent manner. *Gli1* and *Gli2* are expressed in a gradient from ventral to dorsal flanking the expression domain of Shh. In contrast *Gli3* is expressed in an inverse gradient from dorsal to ventral. Gain- and loss-of-function experiments carried out in different laboratories have revealed complex, overlapping functions of these transcription factors: Gli3 seems to act mainly as a repressor of Shh target genes, whereas Gli2 and Gli1 act mainly as activators (Jacob and Briscoe, 2003). However, replacing *Gli2* with *Gli3* demonstrated that Gli3 can also act as an activator (Bai et al., 2004). Interestingly, Gli1 is not directly regulated by the Shh signaling cascade but requires activation by either Gli2 or Gli3 (Bai et al., 2002; Dai et al., 1999). In summary, Shh signaling in the neural tube is translated into gradients of decreasing Gli activator and increasing repressor activity from ventral to dorsal, thereby determining the graded differentiation of distinct neuronal cell types (Bai et al., 2004; Persson et al., 2002; Wijgerde et al., 2002).

The biological importance of Gli3 acting as a repressor has become strikingly evident by the analysis of $Shh^{-/-};Gli3^{-/-}$ double mutants. Limbs of *Shh* mutants lack anterior-posterior polarity and develop only one digit. Loss of *Gli3* converts the *Shh* phenotype into the polydactylous limb phenotype of $Gli3^{-/-}$ mutants (Litingtung et al., 2002; te Welscher et al., 2002). Shh seems thus to act mainly by opposing the repressive activities of Gli3.

Mutations in vertebrate *Gli* genes result in a range of different phenotypes, however the process of endochondral ossification is only mildly affected (Hui and Joyner, 1993; Mo et al., 1997; Park et al., 2000; Schimmang et al., 1992). Whereas no bone phenotype has been detected in $Gli1^{-/-}$ mutants, loss of *Gli2* or *Gli3* results in a slight reduction in bone length. Analysis of $Gli2^{-/-},Gli3^{+/-}$ compound mutants revealed a more severe phenotype indicating functional redundancy of *Gli2* and *Gli3* in controlling endochondral bone formation (Mo et al., 1997).

To obtain insights into the function of the *Gli* transcription factors downstream of Ihh signaling, we investigated the role of Gli3 in regulating chondrocyte differentiation. Our analysis revealed that in the absence of Ihh signaling Gli3 acts as a strong repressor, negatively controlling chondrocyte proliferation and inhibiting the expression of the two Ihh target genes, *Ptch* (*Ptch1* – Mouse Genome Informatics) and *PTHrP*. Interestingly, loss of *Gli3* function in mice, which overexpress *Ihh* in chondrocytes, rescues the delayed onset of hypertrophic differentiation, strongly suggesting an activating role of Gli3 downstream of Ihh in activating PTHrP expression. Furthermore our analysis revealed Gli3 as key regulator of the differentiation from distal into columnar chondrocytes.

Materials and methods

Transgenic mice

Ihh and *Gli3-Xt^J* (*Gli3*) mutant mice were maintained as heterozygous stocks and crossed to generate different mutant combinations. *Col2a1-Ihh* embryos were obtained by intercrossing *Col2a1-Gal4* with *UAS-Ihh* mice (Long et al., 2001). For genotyping, PCR was performed on genomic tail DNA: *Gli3-Xt^J* mice (Xt^{J}5′-AAACCCGTGGCT-CAGCACAAG-3′; Xt^{J}5′-TACCCCAGCAGGAGACTCAGATTAG-3′; C3-5′-GTTGGCTGCTGCATGAAGACTGAC-3′; C3-5′-GGC-CCAAACATCTACCAACACATA-3′); *Ihh*-deficient mice (neo5′-TACCGGTGGATGTGGAATGTGTGCG-3′; fw5′-AGGAGGCAG-GGACATGGATAGGGTG-3′; rv5′-TGTTCTCCTCGTCCTTGAA-GA-3′); *Col2a1-Gal4* mice (fw5′-CTTCTATCGAACAAGCATG-CG-3′; rv5′-GCCAATCTATCTGTGACGGC-3′); *UAS-Ihh* mice (fw5′-GGGCGGGCGCTGGCGACGCTG-3′; rv5′-CGGGCTGCA-CGTGGCTG-3′). Wild-type mice (NMRI) were obtained from Charles River (Germany).

Histology

Paraformaldehyde-fixed tissue was embedded in paraffin wax and stained with Hematoxylin and Eosin (H&E), Toluidine Blue (TB) or with Safranin Weigert (SW) for histological analysis. To identify mineralized cartilage and bone, limbs were stained with 1% silver nitrate according to the method of van Kossa, and counterstained with nuclear fast red.

In situ hybridization analysis

Embryonic limbs were fixed overnight in 4% paraformaldehyde at 4°C and embedded in paraffin wax. Serial sections of 5 μm were processed for radioactive in situ hybridization using [^{33}P]UTP-labeled antisense riboprobes. Hybridization was carried out at 70°C in 50% formamide as previously described (Minina et al., 2002). Sections were counterstained with Toluidine Blue. Probes for in situ hybridization were as follows: *Col10a1* (Minina et al., 2001), *Ihh* (Bitgood and McMahon, 1995); *Ptch* (Goodrich et al., 1996), *Pthr1*

(Abou-Samra et al., 1994); *PTHrP* (Koziel et al., 2004), *Fgfr1* and *Fgfr3* (Minina et al., 2005), *Gli1 and Gli3* (Hui et al., 1994); *Gli2* (Niedermaier et al., 2005).

To analyze the size of expression domains, photographs of two different sections (of approximately 60 μm distance) per limb were taken at 50× magnification. All pictures were printed at the same resolution, the borders of the expression domains were defined by an independent investigator and measured in a double blind test (relative units). For all measurements, an unpaired two-tailed Student's *t*-test was performed (a P value ≤ 0.05 represents a significant difference). Original measurements are summarized in Table S1 in supplementary material.

BrdU labeling

Mice were sacrificed 2 hours after receiving an intra-peritoneal injection of 50 μg/g body weight of 5-bromo-2′-deoxyuridine (BrdU) (BrdU labeling and detection kit II; Roche). Limbs were fixed in 4% paraformaldehyde at 4°C and embedded in paraffin wax. Proliferating cells, in 5 μm sections, were detected by antibody staining performed according to the manufacturer's instructions.

Results

Expression pattern of the *Gli* genes in the developing bone

To gain insight into the function of Gli1, Gli2 and Gli3 acting downstream of Ihh, we analyzed their expression in relation to that of *Ihh* and *PTHrP* in the developing cartilage anlagen at embryonic stage 14.5 (E14.5) and E16.5. At both stages the three genes are expressed in the perichondrium surrounding the skeletal elements and in proliferating chondrocytes distal to the *Ihh* expression domain. At E16.5, *Gli1* is expressed uniformly in proliferating chondrocytes, whereas *Gli2* and *Gli3* expression is strongest in the distal ends of the skeletal elements, overlapping with the expression of *PTHrP*, and weaker towards the *Ihh* expression domain. In addition strong *Gli3* expression can be detected in the joint regions and in mesenchymal cells surrounding the carpals. Furthermore at E16.5 *Gli1*, *Gli2* and *Gli3* are expressed in the newly formed bone (Fig. 1). In summary, the overlapping but distinct expression domains of these transcription factors suggest individual and redundant functions in transducing the Ihh signal.

Gli3 acts as a repressor downstream of Ihh during endochondral ossification

Loss of any single *Gli* gene does not result in an obvious bone phenotype. As Gli3 has been shown to act as a strong repressor of Shh target genes, we decided to focus our analysis on this transcription factor. To determine, whether Gli3 similarly represses Ihh target genes during endochondral ossification, we examined the genetic interaction between Ihh and Gli3. *Ihh*-deficient mice are characterized by severely reduced chondrocyte proliferation, an accelerated onset of hypertrophic differentiation and lack of ossification in endochondral bones. Furthermore, hypertrophic differentiation is not initiated perpendicular to the longitudinal axis of the cartilage elements but starts in their center spreading into all directions. The resulting skeletal elements thus display a central, *Col10a1*-expressing hypertrophic region that is surrounded by non-hypertrophic cells expressing procollagen type II α1 (*Col2a1*). In addition, chondrocytes of *Ihh*-deficient mice fail to express

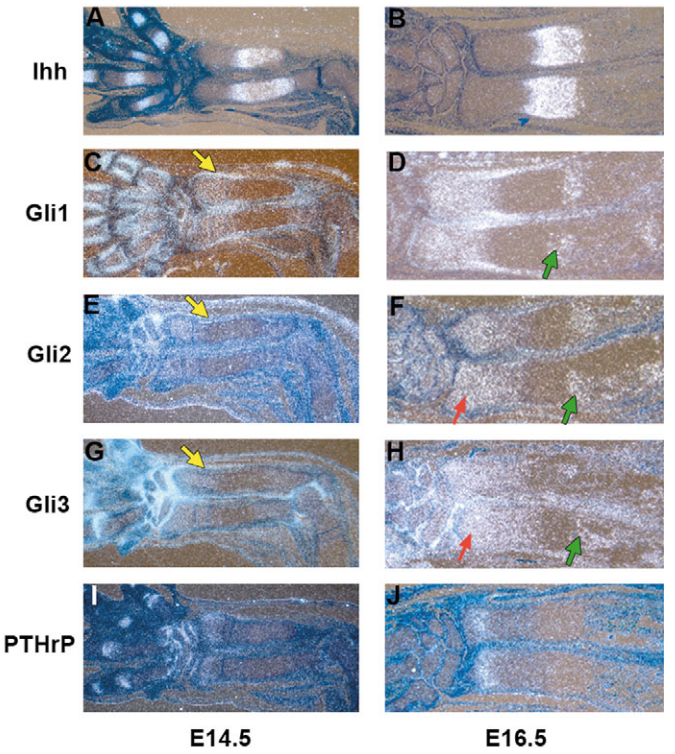

Fig. 1. Expression pattern of the *Gli* family of transcription factors. Sections of E14.5 (A,C,E,G,I) and E16.5 (B,D,F,H,J) wild-type limbs were hybridized with antisense riboprobes as indicated. At E14.5 *Gli1* (C), *Gli2* (E) and *Gli3* (G) are expressed in proliferating chondrocytes distal to the *Ihh* (A) expression domain. In the perichondrium low levels of *Gli2* and *Gli3* and strong levels of *Gli1* expression can be detected (yellow arrow, C,E,G). In elbow and carpal joints *Gli3* is strongly expressed and *Gli2* weakly expressed, overlapping with *PTHrP* (I) expression. At E16.5 *Gli1* is uniformly expressed in proliferating chondrocytes (D). By contrast there is a decreasing gradient of *Gli2* and *Gli3* expression from distal (red arrows F,H,) to central. All three *Gli* genes are additionally expressed in the chondro-osseus junction (green arrow, D,F,H) and in the perichondrium. Ulna is up and radius is down.

PTHrP and *Ptch* (Karp et al., 2000; St-Jacques et al., 1999). In contrast *Gli3*-deficient mice display only a mild endochondral ossification phenotype with a slight delay in the overall differentiation process (Mo et al., 1997).

To investigate the role of Gli3 as a transcription factor downstream of Ihh signaling, we compared forelimbs of $Ihh^{-/-};Gli3^{-/-}$ double mutants to those of $Ihh^{-/-}$ mice at E14.5 and E16.5 (Fig. 2 and data not shown). Morphologically, $Ihh^{-/-};Gli3^{-/-}$ mice have considerably larger cartilage elements and a delay in the onset of hypertrophic differentiation compared to *Ihh*-deficient mutants (Fig. 2A-C). Interestingly, whereas $Ihh^{-/-}$ mice display no distinctive regions of columnar chondrocytes, morphology and orientation of proliferating chondrocytes are restored in double mutants, with small round cells in the distal ends and columnar cells towards the center of the cartilage anlagen (Fig. 2D-L).

To test if the increased size of the cartilage elements in $Ihh^{-/-};Gli3^{-/-}$ mutants results from an increased rate of chondrocyte proliferation we performed BrdU labeling in wild-type, $Ihh^{-/-}$ and $Ihh^{-/-};Gli3^{-/-}$ double mutant mice. Consistent

with previous investigations (St-Jacques et al., 1999), the proliferation rate in *Ihh*$^{-/-}$ mutants is severely reduced throughout the cartilage anlagen at E16.5. Strikingly, additional loss of *Gli3* dramatically increases the number of BrdU-positive chondrocytes (Fig. 2M-O).

On the molecular level, the expression of the hypertrophic marker *Col10a1*, which is expressed throughout the cartilage anlagen in *Ihh*$^{-/-}$ mice, is restricted to the center of the cartilage elements in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mice. The *Col10a1*-expressing hypertrophic cells are flanked by distinct domains of non-hypertrophic chondrocytes on either side. Remarkably, the onset of hypertrophic differentiation occurs perpendicular to the longitudinal axis of the bone (Fig. 3A-C). As Gli3 seems to act as a strong repressor downstream of Ihh in proliferating cells, we analyzed the expression of the Ihh target genes *Ptch* and *Gli1,* which are not expressed in *Ihh*$^{-/-}$ mutants. We found low, but significant, levels of *Ptch* expression throughout the proliferating chondrocytes in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (Fig. 3D-F). In contrast, *Gli1* is not expressed in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (Fig. 3G-I), suggesting that, as in the neural tube, activation of *Gli1* transcription depends on an activating Ihh signal.

The onset of hypertrophic differentiation is regulated by Ihh-dependent *PTHrP* expression. Consequently, no expression of *PTHrP* can be detected in *Ihh*$^{-/-}$ mutants. In contrast, in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants *PTHrP* is expressed in the perichondrium and the joint region (Fig. 3J-L). However, no *PTHrP* expression can be detected in the distal chondrocytes.

In summary loss of *Gli3* function rescues the chondrocyte phenotype of *Ihh*$^{-/-}$ mice to a significant degree, suggesting that Gli3 acts as a strong repressor of Ihh target genes.

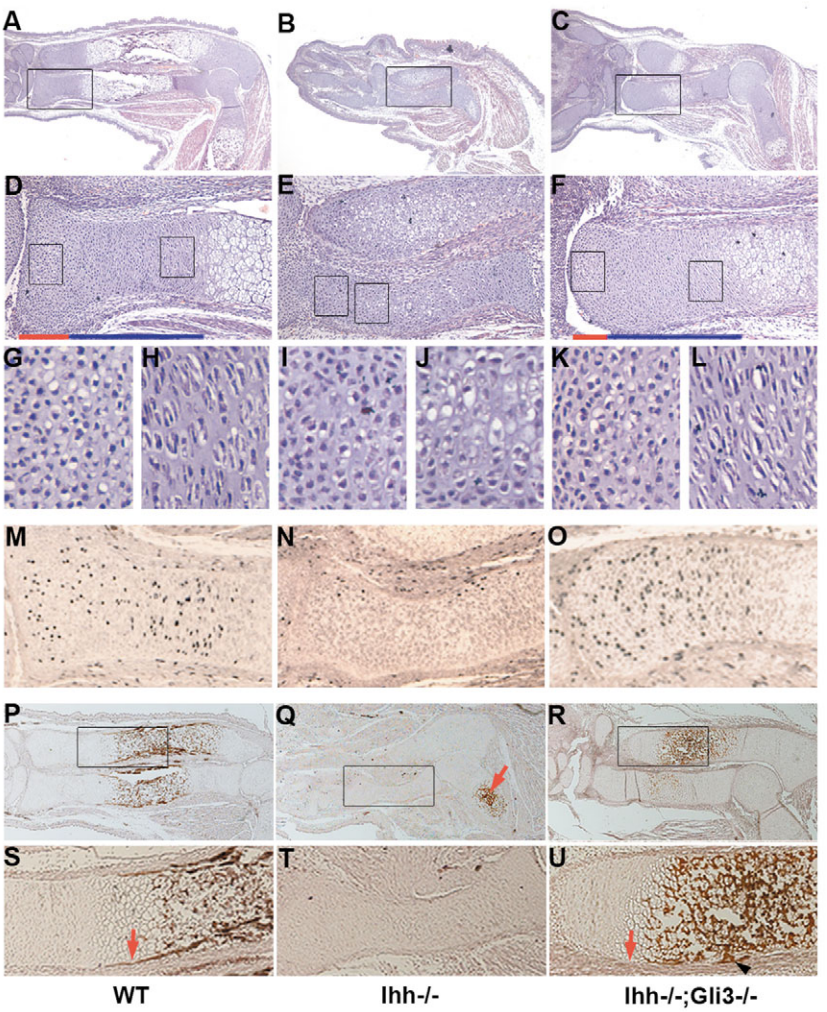

Fig. 2. Loss of *Gli3* in *Ihh*-deficient limbs rescues chondrocyte proliferation and the onset of hypertrophic differentiation, but not bone collar formation. (A-L) Hematoxylin and Eosin staining of E16.5 wild-type (A,D,G,H), *Ihh*$^{-/-}$ (B,E,I,J) and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ (C,F,K,L) limb sections. (A-C) Compared with *Ihh*$^{-/-}$ mutants (B) the size of the skeletal elements is increased and hypertrophic chondrocytes are restricted to the center of the cartilage anlagen in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (C). D-F are higher magnifications of the boxed regions in A-C; G-L show the chondrocyte morphology of distal (G,I,K) and central (H,J,L) regions indicated by the boxes in D-F. In *Ihh*$^{-/-}$ limbs no columnar chondrocytes can be detected, whereas wild-type and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ cartilage anlagen have a zone of distal (D,F, red bar) and columnar (D,F, blue bar) chondrocytes. (M-O) The chondrocyte proliferation rate in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ limbs (O) is similar to wild-type levels (M) and strongly increased compared to *Ihh*$^{-/-}$ limbs (N). (P-U) Van Kossa staining detects mineralization of chondrocytes of E16.5 wild-type (P), *Ihh*$^{-/-}$ (Q, red arrow in the humerus) and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ limbs (R). S-U are higher magnifications of the boxed regions in P-R. Wild-type limbs form a bone collar adjacent to hypertrophic chondrocytes (S, red arrow), which is missing in *Ihh*$^{-/-}$ (T) and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants (U, red arrow). In *Ihh*$^{-/-}$;*Gli3* $^{-/-}$ limbs, bone collar-like structures form in restricted regions (U, black arrowhead).

Loss of *Gli3* does not rescue bone collar formation in *Ihh*-deficient mice

Another important role of Ihh is the induction of the ossification process in the perichondrium (Long et al., 2004; Long et al., 2001; St-Jacques et al., 1999). In wild-type limbs the bone collar forms adjacent to the prehypertrophic and hypertrophic chondrocytes. We performed van Kossa staining to analyze matrix mineralization and bone collar formation in skeletal elements of *Ihh*$^{-/-}$ and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants. Although in *Ihh*$^{-/-}$ mutants hypertrophic differentiation is accelerated, perichondrial cells do not develop into osteoblasts. In *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants hypertrophic chondrocytes in the center of the skeletal elements are mineralized, however, no bone collar is formed surrounding the hypertrophic cells. Instead bone collar-like structures are found intermittently in areas adjacent to the mineralized region (Fig. 2P-U). Loss of *Gli3* is thus not sufficient to rescue perichondrial ossification.

Gli3 mutant mice display reduced zones of proliferating chondrocytes

Given our demonstration that Gli3 is an important effector molecule of Ihh in regulating chondrogenesis, we carefully analyzed chondrocyte differentiation in *Gli3*$^{-/-}$ mice using a panel of chondrocyte differentiation markers. At E14.5 and E16.5 the skeletal

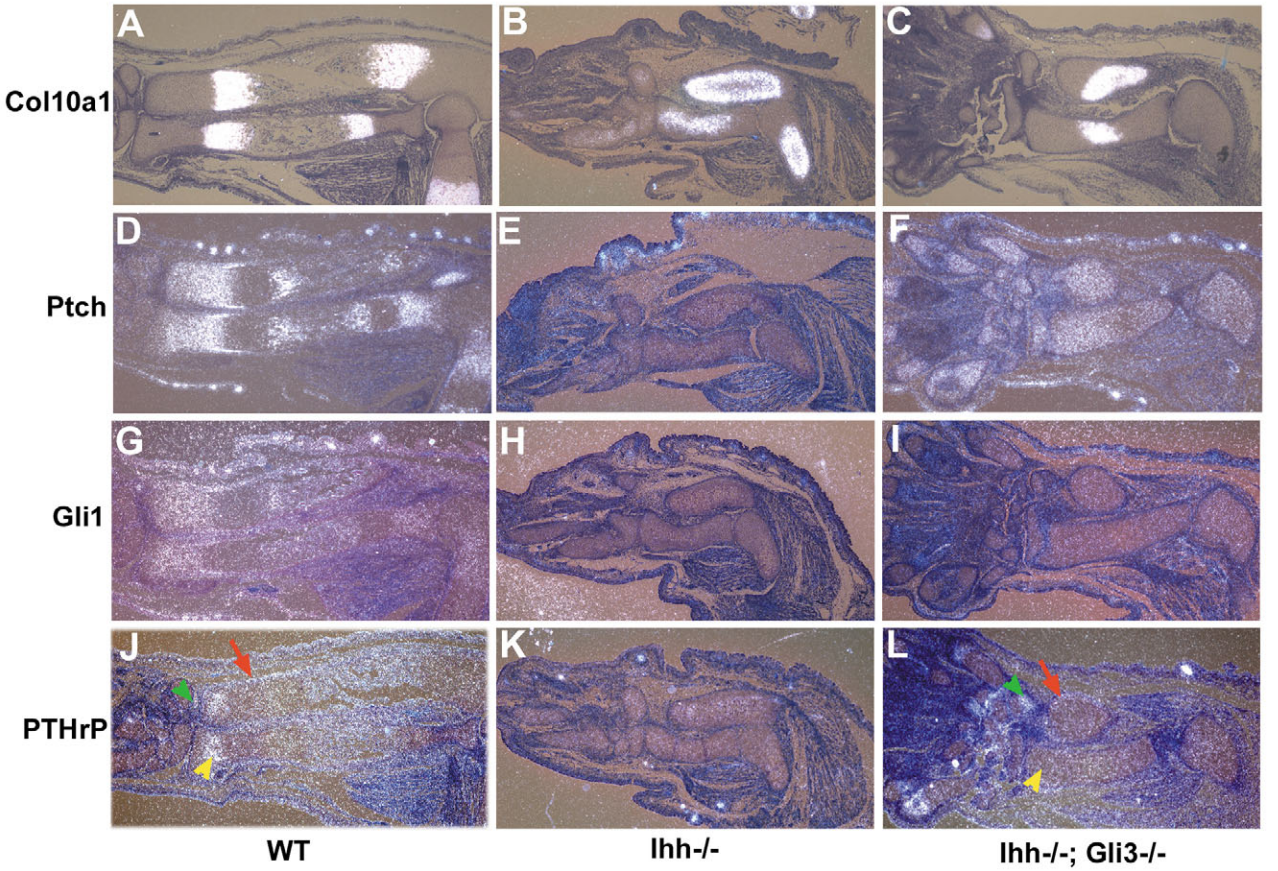

Fig. 3. Molecular characterization of *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ limbs. Serial sections of E16.5 wild-type (A,D,G,J), *Ihh*$^{-/-}$ (B,E,H,K) and *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ limbs (C,F,I,L) were hybridized with riboprobes as indicated. (B) In *Ihh*$^{-/-}$;*Gli3*$^{-/-}$-deficient mice *Col10a1* expression is restricted to the center of the skeletal elements, indicating a delayed onset of hypertrophic chondrocyte differentiation compared to *Ihh*$^{-/-}$ mice. (E,H,K) The Ihh target genes *Ptch*, *Gli1* and *PTHrP* are not expressed in *Ihh*$^{-/-}$ mice. (F,I,L) In *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ limbs the expression of *Ptch* and *PTHrP,* but not *Gli1,* is partially rescued. (F) *Ptch* is weakly expressed in proliferating chondrocytes. (J,L) *PTHrP* expression can be detected in the perichondrium (red arrow) and in the carpal joints (green arrowhead). No *PTHrP* expression can be detected in the distal chondrocytes (yellow arrowhead).

elements of *Gli3*$^{-/-}$ mutants are shorter than those of wild-type mice (92%, *n*=6, *P*=0,0036; Table S1A in supplementary material, and data not shown) and the zone of ossification in their center is reduced (Fig. 4A,B). Hybridization with *Ihh* and *Col10a1* revealed normal regions of hypertrophic cells in *Gli3*$^{-/-}$ limbs (Fig. 4A-

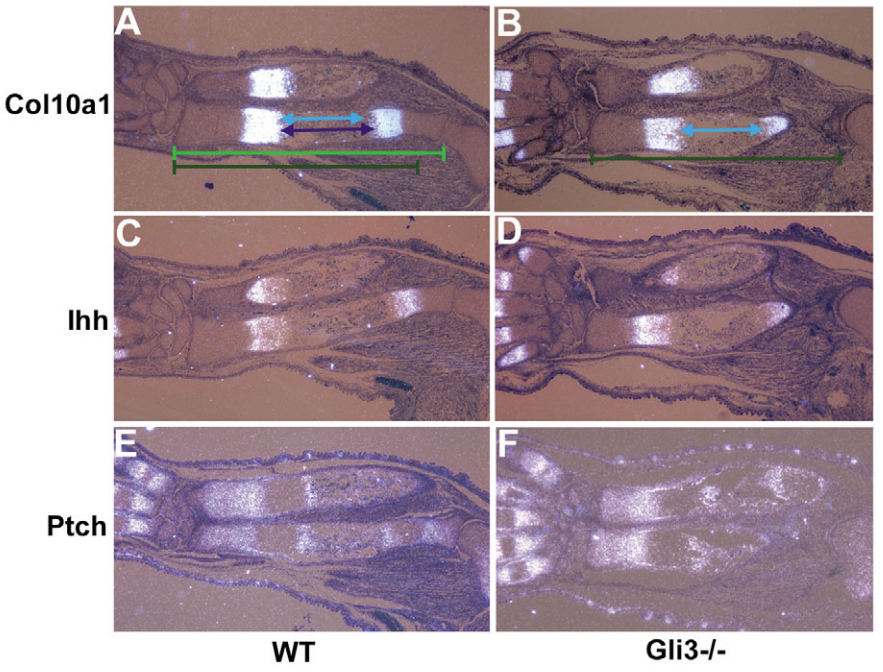

Fig. 4. *Gli3*$^{-/-}$ limbs are delayed in endochondral ossification. Serial sections of E16.5 wild-type (A,C,E,) and *Gli3*$^{-/-}$ (B,D,F) limbs were hybridized with riboprobes as indicated. (A-D) Expression of *Ihh* and *Col10a1* reveals normal regions of prehypertrophic and hypertrophic chondrocytes. (A,B) The overall size of the radius in wild-type mice (light-green bar) is reduced in *Gli3*$^{-/-}$ mutants (dark-green bar, *P*=0.0036), and the ossification is slightly delayed (compare dark- and light-blue double-headed arrows in wild-type and *Gli3*$^{-/-}$ limbs, respectively). (E,F) The expression of *Ptch* is unchanged in *Gli3*$^{-/-}$ limbs.

D). Similarly no alteration in the expression level and domain of *Ptch* and *Gli1*, the direct targets of Ihh signaling, could be detected (Fig. 4E,F and data not shown).

The onset of hypertrophic differentiation is controlled by the interaction between Ihh and PTHrP and can be monitored by measuring the distance between the distal border of the *Ihh* expression domain and the end of the skeletal element (Kronenberg, 2003). A reduced zone of proliferating chondrocytes indicates an accelerated onset of hypertrophic differentiation, whereas an increased region indicates a delay in this differentiation step. Interestingly, we found a reduced zone of proliferating chondrocytes in skeletal elements of *Gli3*$^{-/-}$ limbs, indicating an accelerated onset of hypertrophic differentiation (88%, n=6, P=0.00001; Fig. 5A-C). Importantly, this reduction in size is even significant if related to the overall size of the skeketal elements (92%, n=6, P=0.02; Table S1A in supplementary material).

As PTHrP is the effective regulator of hypertrophic chondrocyte differentiation downstream of Ihh, we next analyzed the expression of *PTHrP*. In wild-type mice *PTHrP* is expressed in the most distal chondrocytes at E14.5 whereas at E16.5 its expression domain has shifted towards the center of the cartilage anlagen. At this stage the most distally located chondrocytes have ceased to express *PTHrP* (Fig. 5D). In *Gli3*$^{-/-}$ mice we did not detect an obvious downregulation of the level of *PTHrP* expression. Instead we found that the shift of the expression domain has not occurred at E16.5 (Fig. 5E).

As the *PTHrP* expression

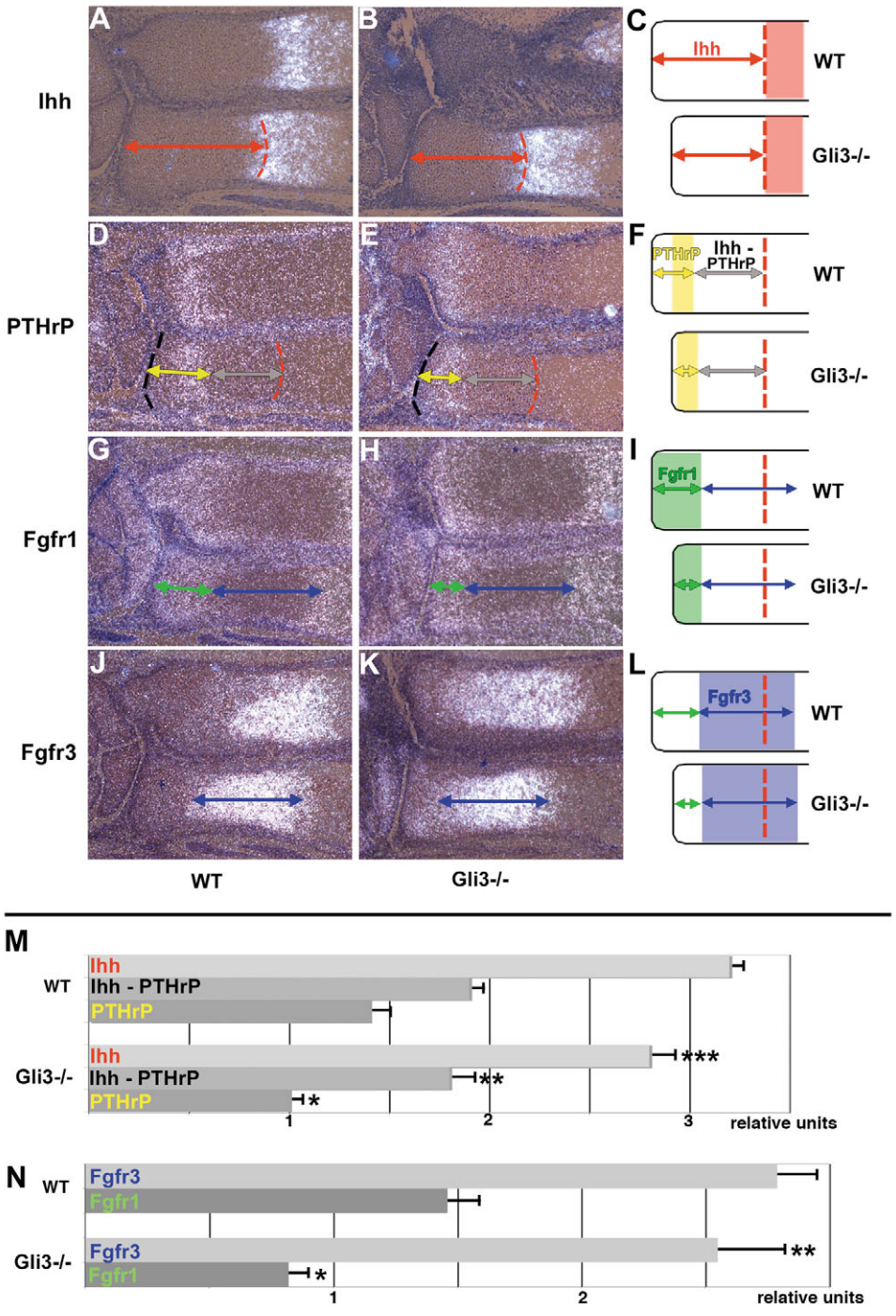

Fig. 5. Gli3 regulates distal chondrocyte differentiation. Two sets of serial sections, A,B,D,E and G,H,J,K, of E16.5 wild-type (A,D,G,J) and *Gli3*$^{-/-}$ (B,E,H,K) limbs were hybridized with riboprobes as indicated. Red dashed lines indicate the distal border of the *Ihh* expression domain. (C,F,I,L) Models of gene expression domains. (A-C) *Gli3*$^{-/-}$ mice display a reduced zone of proliferating chondrocytes compared to wild-type mice (red arrow). (D-F) *PTHrP* is expressed at similar levels in *Gli3*$^{-/-}$ and wild-type limbs, however its domain of expression is shifted towards the distal end of the cartilage anlagen in *Gli3*$^{-/-}$ limbs. (G-L) In *Gli3*$^{-/-}$ limbs the *Fgfr1* expression domain (G-I) is reduced (green arrow) and the *Fgfr3* expression domain (J-L) is shifted towards the end of the skeletal elements (blue arrow), indicating an accelerated differentiation of distal chondrocytes. (M) The zone of proliferating chondrocytes (red arrow in A-C) is shortened in *Gli3*$^{-/-}$ limbs (***P=0.00003, n=6). The *PTHrP*-expressing zone of distal chondrocytes (yellow arrow in D-F) is reduced in *Gli3*$^{-/-}$ limbs (*P=0.00008, n=6). The distance between the *Ihh* expression domain and the central end of the *PTHrP* expression domain (gray arrow in D-F) is of similar size in both, *Gli3*$^{-/-}$ and wild-type limbs (**P=0.304, n=6). (N) The zone of *Fgfr1*-expressing distal chondrocytes (green arrow in G-I) is reduced in *Gli3*$^{-/-}$ mice (*P=0.0002, n=5), whereas the zone of *Fgfr3* expression (blue arrow in J-L) is of similar size (**P=0.19, n=5).

domain is restricted to the most distal cells in *Gli3*$^{-/-}$ mice we reinvestigated the onset of hypertrophic differentiation by analyzing the distance between the central end of the *PTHrP* expression domain and the distal end of the *Ihh* expression domain. We found a similar distance in *Gli3*$^{-/-}$ and wild-type mice, indicating that the onset of hypertrophic differentiation is not accelerated if compared with the source of its regulator, PTHrP (Fig. 5M, *n*=6, *P*=0.3; Table S1A in supplementary material). By contrast, the distance between the central side of the *PTHrP* expression domain and the distal end of the skeletal elements is significantly reduced in *Gli3*$^{-/-}$ mutants compared to wild-type mice (Fig. 5M, 71%, *n*=6, *P*=0.0001; Table S1A in supplementary material).

Gli3 controls the transition from distal to columnar chondrocytes

We next asked if Gli3 specifically regulates the expression of *PTHrP* or if it acts as a general regulator of distal chondrocyte differentiation. The region of proliferating chondrocytes can be subdivided into a distal zone of low proliferating, round chondrocytes expressing *Fgfr1*, and a central zone of high proliferating, columnar chondrocytes, expressing *Fgfr3*. To characterize the differentiation of distal chondrocytes in *Gli3*$^{-/-}$ mutants, we analyzed the expression of *Fgfr1* and *Fgfr3* (Fig. 5G-L). Interestingly, the domain of *Fgfr1* expression is reduced in *Gli3*$^{-/-}$ mice (Fig. 5G-I, 56%, *n*=5, *P*=0.0002; Table S1B in supplementary material), whereas the expression domain of *Fgfr3* is of similar size but shifted towards the distal ends of the cartilage anlagen (Fig. 5J-L, *n*=5, *P*=0.19; Table S1B in supplementary material). As for the region of proliferating cells the reduction in the size of the *Fgfr1* expression domain is significant if compared to the overall size of the radius (*P*=0.0002; Table S1B in supplementary material).

In summary, the reduced expression domain of *Fgfr1* identifies Gli3 as a negative regulator of the differentiation of distal chondrocytes into columnar chondrocytes. Furthermore, as the level of *PTHrP* expression is not altered in *Gli3* mutants this regulation occurs independently of PTHrP. In contrast, the differentiation of columnar chondrocytes into hypertrophic chondrocytes seems to be regulated by the level of PTHrP. To support such a model, we analyzed which types of chondrocytes are present in *Ihh*-deficient mice. As *Fgfr3* is expressed in proliferating and in early hypertrophic cells it cannot be used to determine the size of the zone of columnar chondrocytes. We therefore analyzed the expression of *Fgfr1* in distal chondrocytes in relation to that of parathyroid hormone receptor 1 (*Pthr1*), which is expressed in prehypertrophic chondrocytes overlapping with the expression of *Ihh*. In wild-type limbs the expression domains of *Fgfr1* and *Pthr1* are clearly separated by columnar chondrocytes. Remarkably, in *Ihh*$^{-/-}$ limbs, which do not express *PTHrP*, a small stripe of *Fgfr1*-expressing distal chondrocytes is directly flanked by *Pthr1*-expressing prehypertrophic chondrocytes (Fig. 6A-D). We can, thus, conclude that the domain of columnar chondrocytes is significantly reduced or lost in *Ihh*$^{-/-}$ mutants. Furthermore, the remaining proliferating cells in these mutants represent distal chondrocytes. Correspondingly, no columnar chondrocytes could be detected by morphological analysis (Fig. 2H,J).

We next analyzed *Fgfr1* expression in *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants, in which *PTHrP* expression is restored, but *Gli3* function is lost. In these mutants *Fgfr1* expression is restricted to the most distal chondrocytes, as it is in *Gli3*$^{-/-}$ mutants. In contrast, the region of columnar chondrocytes between the *Fgfr1* and *Pthr1* expression domains is significantly expanded (Fig. 6E-H). In summary, these results strongly support a dual role for Gli3 in regulating distal chondrocyte differentiation and the onset of hypertrophic differentiation, by two independent mechanisms.

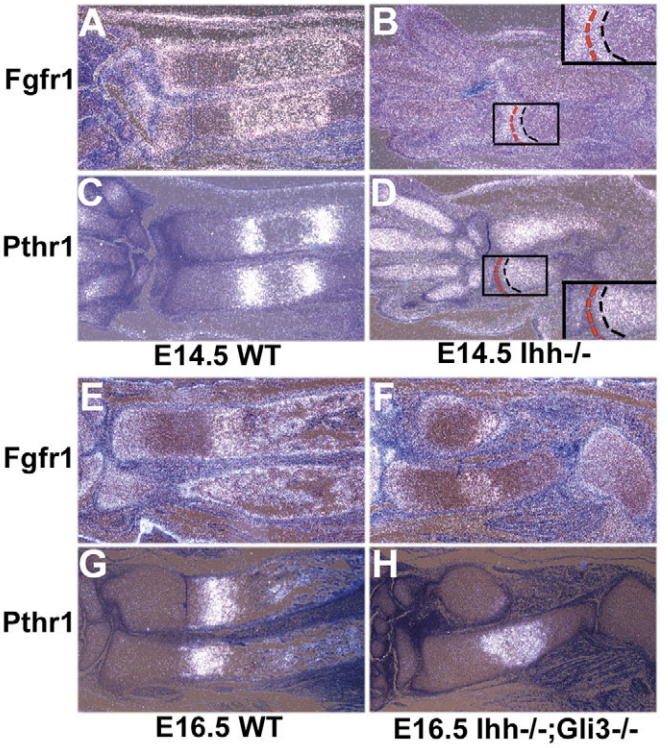

Fig. 6. (A-D) In *Ihh*$^{-/-}$ limbs distal chondrocytes differentiate directly into prehypertrophic chondrocytes. Sections of E14.5 wild-type (A,C) and *Ihh*$^{-/-}$ (B,D) limbs were hybridized with *Fgfr1* (A,B) and *Pthr1* (C,D) riboprobes. In *Ihh*$^{-/-}$ mutants *Fgfr1* is expressed in a stripe of distal chondrocytes, which is flanked by *Pthr1* expressing hypertrophic chondrocytes. Black dashed lines indicate the border between *Fgfr1* and *Pthr1* expression domains; red dashed lines indicate the end of the cartilage elements. (E-H) Loss of *Gli3* restores columnar chondrocyte differentiation in *Ihh*$^{-/-}$ mice. Sections of E16.5 wild-type (E,G) and *Gli3*$^{-/-}$;*Ihh*$^{-/-}$ (F,H) limbs were hybridized with riboprobes for *Fgfr1* (E,F) and *Pthr1* (G,H). In *Gli3*$^{-/-}$;*Ihh*$^{-/-}$ mutants a reduced zone of *Fgfr1* expressing distal chondrocytes is separated from the *Pthr1* expression domain by a population of columnar chondrocytes. B,D and F,H display serial sections.

Loss of *Gli3* rescues the delayed onset of hypertrophic differentiation in *Col2a1-Ihh* mice

Various studies of neural tube and early limb bud development have shown that Gli3 acts mainly as a repressor. However, high levels of hedgehog signaling stabilize full length Gli3, thereby inhibiting Gli3 repressor function (Bai et al., 2004; Wang et al., 2000). To test if Gli3 repressor function is similarly inhibited by Ihh we analyzed limbs of mice overexpressing Ihh under the *Col2a1* promoter (*Col2a1-Ihh* mice). This mouse line is characterized by an upregulation of *PTHrP* expression and consequently a delay in the onset of hypertrophic

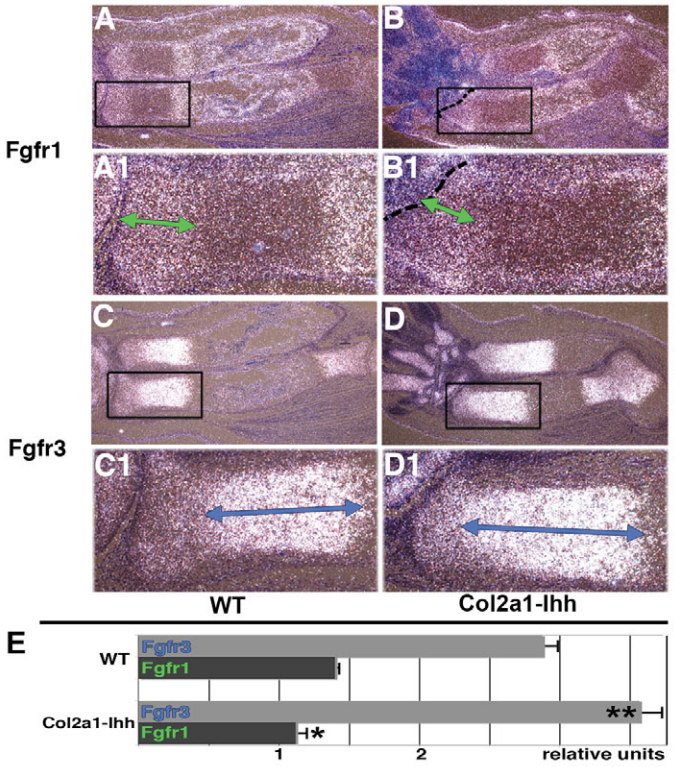

Fig. 7. Differentiation of distal chondrocytes is accelerated in *Col2a1-Ihh* mice. Sections of E16.5 wild-type (A,C) and *Col2a1-Ihh* (B,D) limbs were hybridized with *Fgfr1* (A,B) and *Fgfr3* (C,D) riboprobes. (A1-D1) are higher magnifications of boxed regions in A-D, the dashed line in B,B1 demarcates the end of the radius, which is partially fused with the carpal navicular lunare in *Col2a1-Ihh* mice. The domain of *Fgfr1* expression is reduced in *Col2a1-Ihh* mice (A,B, green arrow). The domain of *Fgfr3* expression is broadened and shifted towards the end of the skeletal elements in *Col2a1-Ihh* mice (C,D, blue arrow in C1,D1). (E) The *Fgfr1* expression domain (green arrow in A,B) is reduced in *Col2a1-Ihh* limbs (**P*=0.003, *n*=3) whereas the domain of Fgfr3 expression (blue arrow in C,D) is strongly expanded compared to that of wild-type limbs (***P*=0.007, *n*=3).

differentiation (Long et al., 2001). We first analyzed the expression domains of *Fgfr1* and *Fgfr3* in limbs of *Col2a1-Ihh* mice and found a reduced expression domain of *Fgfr1* compared to wild-type animals (Fig. 7A,B,E, *n*=3, *P*=0.003; Table S1C in supplementary material). Furthermore, the *Fgfr3* expression domain is increased and its distal border is shifted towards the end of the cartilage elements, indicating accelerated differentiation of distal chondrocytes into columnar chondrocytes (Fig. 7C-E, *n*=3, *P*=0.007). This result is consistent with the idea that overexpression of *Ihh* throughout the cartilage anlagen inhibits the repressing function of Gli3.

To further confirm this result, we analyzed *Col2a1-Ihh;Gli3*$^{-/-}$ double mutants. Interestingly, loss of *Gli3* in *Col2a1-Ihh* mutants significantly rescues the delayed hypertrophic differentiation as indicated by the reduced distance between the *Ihh* expression domain and the joint region (Fig. 8A-H). Analysis of *Fgfr1* expression revealed that the size of the *Fgfr1* expression domain is strongly reduced compared to that in the wild type and *Col2a1-Ihh* mutants (Fig. 8I-L), strongly supporting the idea that the delay in distal chondrocyte differentiation requires Gli3 repressor function. Interestingly the expression domain of *Fgfr3* is not only shifted towards the end of the cartilage elements in *Col2a1-Ihh;Gli3*$^{-/-}$ but also reduced in size compared to *Col2a1-Ihh* mutants, indicating an additional accelerated differentiation of columnar into hypertrophic chondrocytes (Fig. 8M-P).

We next analyzed the expression of *PTHrP*, which is upregulated in *Col2a1-Ihh* mutants, leading to the delayed differentiation of columnar chondrocytes into hypertrophic chondrocytes. Similar to the differentiation of distal chondrocytes, this upregulation of *PTHrP* expression could be attributed to the Ihh-dependent inactivation of the Gli3 repressor function. However, *Gli3*$^{-/-}$ mice, which do not express any Gli3 repressor, do not display upregulated levels of *PTHrP* expression. To investigate the activating potential of Gli3 we analyzed *PTHrP* expression in *Col2a1-Ihh;Gli3*$^{-/-}$ mice. Loss of *Gli3* in *Col2a1-Ihh* mice reduces the domain of *PTHrP* expression (Fig. 8T) as in *Gli3*$^{-/-}$ mutants. Furthermore, the expression level of *PTHrP*, which is upregulated in *Col2a1-Ihh* mice, seems to be reduced to a level similar to that in wild-type or *Gli3*$^{-/-}$ mice, implicating an activating role of Gli3 in regulating the expression of *PTHrP*. The reduced size of the expression domain in combination with reduced levels of *PTHrP* expression seems, thus, to be responsible for the accelerated onset of hypertrophic differentiation.

Discussion

Ihh is one of the main regulators of endochondral ossification, acting on different target tissues. To understand how these tissues interpret the Ihh signal it is important to investigate the downstream transcription factors. Zinc finger transcription factors of the *Gli* family have been shown to act downstream of hedgehog signals in various systems. Investigations from numerous laboratories revealed that Gli3 acts as a strong repressor of Shh target genes. Furthermore loss of Gli3 rescues the *Shh* phenotype in many aspects, including in the neural tube and the early limb bud. Several lines of evidence indicate that Shh and Ihh are functionally equivalent and signal through the same signal transduction pathway: (1) expression of both genes leads to the upregulation of *Ptch* and *Gli* in target tissues (Marigo et al., 1996a; Marigo et al., 1996b); (2) similar to Shh, Ihh can induce anterior-posterior patterning effects in the developing limb, and Shh can replace Ihh in delaying chondrocyte differentiation (Pathi et al., 2001; Tavella et al., 2004; Vortkamp et al., 1996). It is thus to be expected that Ihh, like Shh, regulates the activity of the Gli transcription factors by inhibiting their repressor function and converting them into transcriptional activators.

Loss of *Gli3* partially rescues the *Ihh*$^{-/-}$ phenotype

During endochondral ossification, the three vertebrate homologues, *Gli1*, *Gli2* and *Gli3*, are expressed in overlapping domains in proliferating chondrocytes and in the developing bone (tissues that react to Ihh). Neither their expression pattern nor the deletion of single *Gli* genes identifies one of them as a main tranducer of the Ihh signal (Mo et al., 1997).

To obtain deeper insight into the interaction of *Gli* genes and Ihh we started to analyze the role of Gli3 in regulating Ihh

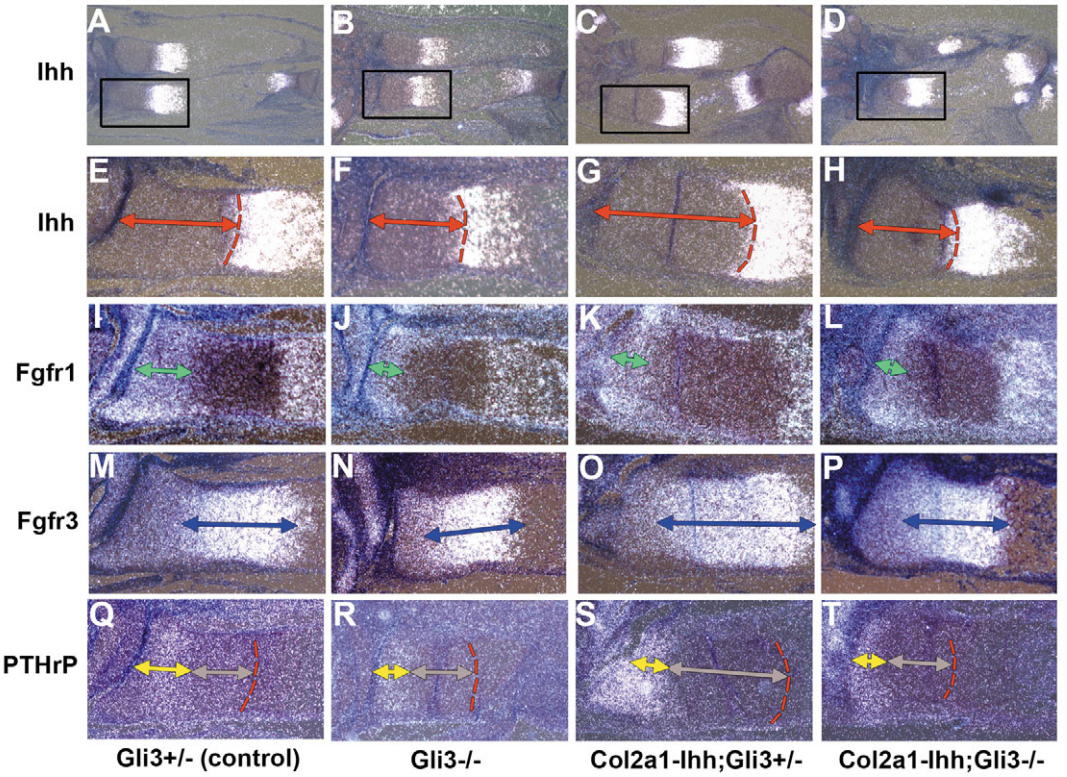

Fig. 8. Loss of Gli3 partially rescues the *Col2a1-Ihh* phenotype. Serial sections of E16.5 $Gli3^{+/-}$ (A,E,I,M,Q; control), $Gli3^{-/-}$ (B,F,J,N,R), *Col2a1-Ihh;*$Gli3^{+/-}$ (C,G,K,O,S) and *Col2a1-Ihh;*$Gli3^{-/-}$ (D,H,L,P,T) limbs of the same litter were hybridized with riboprobes as indicated. E-H are higher magnifications of the distal radii (boxed) in A-D. (E-H) The size of the zone of proliferating chondrocytes (red arrow) is reduced in $Gli3^{-/-}$ mice and strongly increased in *Col2a1-Ihh;*$Gli3^{+/-}$ mice. Compared to *Col2a1-Ihh;*$Gli3^{+/-}$ mice, the zone of proliferating chondrocytes is decreased in double transgenic *Col2a1-Ihh;*$Gli3^{-/-}$ mice. (I-L) The size of the domain of distal chondrocytes, demarcated by the *Fgfr1* expression domain (green arrow), is slightly reduced in *Col2a1-Ihh;*$Gli3^{+/-}$ and strongly reduced in $Gli3^{-/-}$ and *Col2a1-Ihh;*$Gli3^{-/-}$ mice. (M-P) The *Fgfr3* expression domain is expanded in *Col2a1-Ihh;*$Gli3^{+/-}$ limbs, whereas in double transgenic *Col2a1-Ihh;*$Gli3^{-/-}$ mice the *Fgfr3* expression domain is of similar size to that in wild-type and $Gli3^{-/-}$ limbs. (Q-T) The upregulated level of *PTHrP* expression in *Col2a1-Ihh;*$Gli3^{+/-}$ mice seems to be downregulated in *Col2a1-Ihh;*$Gli3^{-/-}$ mice to similar levels as in control or $Gli3^{-/-}$ limbs.

target genes, focusing on the role of Ihh in controlling chondrocyte differentiation. Despite the fact that *Gli3*-deficient mice display only a mild bone phenotype our investigations demonstrated that loss of *Gli3* in *Ihh* mutants rescues the $Ihh^{-/-}$ phenotype to a significant degree: the region of hypertrophic cells, which represents most of the chondrocytes in $Ihh^{-/-}$ mutants, is clearly restricted to a central region, flanked by non-hypertrophic, proliferating cells, on either end. Interestingly, in these phenotypically rescued limbs, chondrocyte proliferation is upregulated, the expression of *Ptch* and *PTHrP* is restored and hypertrophic differentiation of chondrocytes is delayed. It can thus be concluded that in the absence of Ihh signaling, Gli3 acts as a strong repressor of chondrocyte proliferation and of at least the expression of *Ptch* and *PTHrP*. Furthermore, similar to Shh signaling, the activating role of Ihh in these processes seems to be mainly mediated by inhibiting the repressor function of Gli3.

Surprisingly, although *PTHrP* expression is clearly induced by loss of *Gli3*, distal chondrocytes of $Ihh^{-/-}$;$Gli3^{-/-}$ double mutants seem to express no, or only low levels of, *PTHrP*. Strong expression of *PTHrP* is instead found in the joint region outside the cartilage elements and in the perichondrium, contributing to the delayed onset of hypertrophic differentiation. Similarly, ectopic expression of *PTHrP* in the joint region outside the distal chondrocytes has been found after retroviral overexpression of Ihh in chick embryos (Vortkamp et al., 1996). Interestingly, neither in these experimental settings nor in *Col2a1-Ihh* mice columnar chondrocytes is *PTHrP* expressed. Two conclusions can be drawn from these observations. (1) Only less differentiated cells of the joint region and distal chondrocytes are competent to express *PTHrP*, whereas columnar chondrocytes have lost this competence. (2) *Gli3* seems to be an important inhibitor of *PTHrP* expression in the future joints in wild-type embryos.

Despite the dramatic rescue of many aspects of the $Ihh^{-/-}$ phenotype, loss of *Gli3* does not fully convert the $Ihh^{-/-}$ phenotype: the perichondrium seems to ossify only in isolated, restricted areas and *PTHrP* expression is not restored to normal levels in the distal chondrocytes. Whether these processes require an activating function of Gli3 or are regulated by another member of the *Gli* family will be the subject of future studies. In the proliferating chondrocytes, *Gli2* expression overlaps with that of *Gli3* and both genes are expressed more strongly in the distal regions. Remaining Gli2 repressor activity might thus downregulate the expression of *PTHrP* in

Ihh$^{-/-}$;*Gli3*$^{-/-}$ double mutants. Alternatively, activating functions of either Gli2 or Gli3 might be required for full activation of *PTHrP* expression.

Gli3 regulates chondrocyte differentiation in PTHrP dependent and independent mechanisms

Although endochondral long bones of *Gli3* mutant mice develop similarly to those of wild-type mice, our analysis of chondrocyte differentiation revealed a reduced domain of proliferating chondrocytes, indicating an accelerated onset of hypertrophic differentiation. Surprisingly, the expression levels of *PTHrP*, the main regulator of differentiation from proliferating into hypertrophic chondrocytes, and its receptor *Pthr1* (data not shown) seem to be normal in *Gli3*$^{-/-}$ mutants, indicating that the accelerated differentiation is induced by a PTHrP-independent mechanism. Instead loss of *Gli3* leads to a shift in the expression domain of *PTHrP* towards the distal ends of the cartilage elements at E16.5. Furthermore the region of *Fgfr1*-expressing distal chondrocytes is reduced in size in these mice. Thus, Gli3 seems to act as a negative regulator of an early step of chondrocyte differentiation, i.e. the transition from distal into columnar cells, thereby positioning the *PTHrP* expression domain. The reduced zone of proliferating chondrocytes in *Gli3*$^{-/-}$ mice can therefore be attributed to an accelerated differentiation of distal into columnar cells. In summary, we can conclude that Gli3 regulates two steps of chondrocyte differentiation: the transition from distal into columnar cells in a PTHrP-independent mechanism, and the transition of columnar into hypertrophic cells by regulating *PTHrP* expression.

Successive roles for Gli3 and PTHrP in determining the switch from distal into columnar chondrocytes and from columnar into hypertrophic chondrocytes, respectively, are supported by the investigation of various combinations of *Ihh* and *Gli3* alleles. In *Ihh*$^{-/-}$ mice, the remaining non-hypertrophic cells are distal, *Fgfr1*-positive chondrocytes, which are presumably maintained by the strong Gli3 repressor function. As *PTHrP* expression is absent, these distal cells differentiate directly into *Pthr1*-expressing hypertrophic chondrocytes. Because of the lack of a positive marker we cannot completely exclude the possibility of a short columnar phase. However, no chondrocyte columns can be detected morphologically. Loss of *Gli3* in *Ihh*$^{-/-}$ mutants restores the expression of *PTHrP* and subsequently delays the onset of hypertrophic differentiation. As Gli3 function is lost the differentiation of distal into columnar chondrocytes is accelerated and the majority of proliferating chondrocytes are of the columnar type. In *Col2a1-Ihh* mice the repressor function of Gli3 is antagonized by overexpression of Ihh. Accordingly, the differentiation of distal into columnar chondrocytes is accelerated, leading to a shortened zone of distal chondrocytes. In parallel, upregulated *PTHrP* expression leads to a delayed onset of hypertrophic differentiation. Complete loss of *Gli3* in these mutants (*Col2a1-Ihh;Gli3*$^{-/-}$) results in a further acceleration of distal chondrocyte differentiation. Consequently the reduced domain of *PTHrP* expression, in combination with a possibly reduced *PTHrP* expression level, accelerates the transition from columnar to hypertrophic cells.

Is activation by Gli3 required for *PTHrP* expression?

Whereas the differentiation of distal into columnar chondrocytes seems to be regulated by Gli3 repressor activity, the regulation of *PTHrP* expression by Gli3 might be more complex. Loss of *Gli3* in *Ihh*$^{-/-}$ mice restores the expression of *PTHrP*, clearly demonstrating that Gli3 negatively regulates *PTHrP* expression. However, in *Gli3*$^{-/-}$ mice, *PTHrP* expression is not upregulated compared to wild-type mice whereas *Col2a1-Ihh* mice express significantly upregulated levels of *PTHrP*. Therefore, high expression of *PTHrP* in chondrocytes seems to require the release of a potential repression by Gli2 or an activation by either Gli3 or Gli2. Interestingly, loss of *Gli3* in *Col2a1-Ihh* mutants not only leads to a reduction in the size of the *PTHrP* expression domain but, in addition, seems to downregulate the level of *PTHrP* expression. As the function of Gli2, acting as a repressor or an activator, should not be affected by loss of *Gli3*, this experiment suggests that upregulation of *PTHrP* expression in these limbs requires activation by Gli3. We can not, however, determine by this experiment whether Gli3 directly activates the *PTHrP* promotor or if it acts as a repressor of other inhibitory transcription factors.

It is difficult to determine if *PTHrP* expression requires activation by Gli3 in wild-type limbs. The activating function of Gli3 might be minimal in wild-type limbs and might only be induced by ectopic overexpression of *Ihh*. However, the normal level of *PTHrP* expression in *Gli3*$^{-/-}$ mice is at least in agreement with a simultaneous loss of a Gli3 activator and repressor function. Furthermore, the role of Gli2 in this process remains obscure, as overexpression of Ihh should convert Gli2 into a strong activator. Loss of repression by Gli3 should thus allow Gli2 to activate *PTHrP* expression, again supporting an activating role for Gli3. Further studies addressing the role of Gli2 are obviously required to fully understand the regulation of PTHrP.

Conclusions

Our study has identified several important roles for Gli3 in regulating chondrocyte differentiation. (1) The rescue of the *Ihh*$^{-/-}$ phenotype revealed that Gli3 acts as a strong repressor of Ihh target genes. The activating activity of Ihh is therefore mainly mediated by inhibiting the Gli3 repressor function. (2) Gli3 controls two distinct steps of chondrocyte differentiation: the switch from distal into columnar chondrocytes in a PTHrP-independent manner and the switch from columnar into hypertrophic chondrocytes by regulation of *PTHrP* expression. (3) *PTHrP* expression is regulated by Gli3 repressor, and possibly activator, activity.

PTHrP-dependent and -independent regulation of chondrocyte differentiation has been proposed previously from the analysis of mice carrying hypomorphic and null alleles of *Pthr1*. Based on cell morphology, Kobayashi et al. predicted that PTHrP regulates the switch from columnar into hypertrophic cells, whereas Ihh accelerates the differentiation from distal into columnar cells (Kobayashi et al., 2002; Kobayashi et al., 2005). Our analysis has, for the first time, used *Fgfr1* and *Fgfr3* as markers to define the domains of distal and columnar chondrocytes. In addition, we identified Gli3 as a regulator of both differentiation steps. Combining the investigations of both groups we would thus propose a model in which Gli3 repressor function delays distal chondrocyte differentiation. Inactivation of the Gli3 repressor function by Ihh from the prehypertrophic region would induce

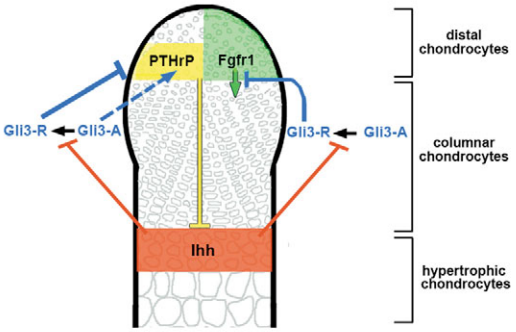

Fig. 9. Gli3 regulates two steps of chondrocyte differentiation: the transition from (1) distal into columnar and from (2) columnar into hypertrophic chondrocytes. Left half: Gli3 regulates the onset of hypertrophic differentiation by regulating *PTHrP* (yellow) expression. In distal chondrocytes Gli3 acts as a strong repressor of *PTHrP* expression. Ihh signaling inhibits the repressor function of Gli3 (Gli3-R) and possibly activates *PTHrP* expression by Gli3-mediated activation (Gli3-A). As columnar chondrocytes loose the competence to express *PTHrP* its expression domain is restricted to distal chondrocytes. In the most distal cells, which do not receive sufficient Ihh, the Gli3 repressor cannot be inactivated and therefore inhibits the expression of *PTHrP,* resulting in a stripe of *PTHrP* expression. Right half: Gli3 negatively regulates the differentiation of *Fgfr1* expressing, distal (green) chondrocytes into columnar cells independent of PTHrP. Ihh antagonizes the repressor function of Gli3 and promotes this differentiation process.

the differentiation from distal into columnar chondrocytes. The distance from the *Ihh* expression domain at which the differentiation occurs would be determined by the level of Ihh signaling: more distal if the Ihh signal is high and more central if the signal is low. In addition Ihh-dependent inactivation of the Gli3 repressor function would determine the level of *PTHrP* expression and thus the transition from columnar into hypertrophic chondrocytes (Fig. 9).

Another important aspect of our studies is the observation that with expansion of the domain of distal chondrocytes the region of *PTHrP* expression moves into the cartilage anlagen. Such a delocalization would be necessary to maintain the interaction of Ihh and PTHrP at later stages of bone development when a secondary ossification center develops in the most distal cells. The strong repression of *PTHrP* by Gli3 in these chondrocytes suggests a mechanism of how the *PTHrP* expression domain could be moved: Ihh induces the expression of *PTHrP* in distal cells and the differentiation of distal into columnar chondrocytes by antagonizing Gli3 repression at a certain distance from its expression domain. With increasing bone length, the Ihh signal is, however, not strong enough to release Gli3 repressor activity in the most distal cells. Continuous proliferation of the distal cells might consequently release more and more cells from the influence of Ihh and increase the population of future epiphysial cells without disrupting the Ihh/PTHrP interaction.

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Supplementary material

Supplementary material available online at http://dev.biologists.org/cgi/content/full/132/23/5249/DC1

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