Intraflagellar transport is essential for endochondral bone formation

Courtenay J. Haycraft¹*, Qihong Zhang¹*,†, Buer Song², Walker S. Jackson³,‡, Peter J. Detloff³, Rosa Serra¹ and Bradley K. Yoder¹,§

While cilia are present on most cells in the mammalian body, their functional importance has only recently been discovered. Cilia formation requires intraflagellar transport (IFT), and mutations disrupting the IFT process result in loss of cilia and mid-gestation lethality with developmental defects that include polydactyly and abnormal neural tube patterning. The early lethality in IFT mutants has hindered research efforts to study the role of this organelle at later developmental stages. Thus, to investigate the role of cilia during limb development, we generated a conditional allele of the IFT protein Ift88 (polaris). Using the Cre-lox system, we disrupted cilia on different cell populations within the developing limb. While deleting cilia in regions of the limb ectoderm had no overt effect on patterning, disruption in the mesenchyme resulted in extensive polydactyly with loss of anteroposterior digit patterning and shortening of the proximodistal axis. The digit patterning abnormalities were associated with aberrant Shh pathway activity, whereas defects in limb outgrowth were due in part to disruption of Ihh signaling during endochondral bone formation. In addition, the limbs of mesenchymal cilia mutants have ectopic domains of cells that resemble chondrocytes derived from the perichondrium, which is not typical of Indian hedgehog mutants. Overall these data provide evidence that IFT is essential for normal formation of the appendicular skeleton through disruption of multiple signaling pathways.

KEY WORDS: Cilia, Limb patterning, Hedgehog, Bone development, IFT, Mouse

INTRODUCTION

Cilia are microtubule-based organelles that are expressed on the surface of most cells in the mammalian body. Intraflagellar transport (IFT), the process by which cilia are formed and maintained, was first described in *Chlamydomonas*, and proteins required for IFT concentrate at the base of cilia, where they assemble into large protein complexes called IFT particles (Kozminski et al., 1995; Piperno and Mead, 1997). The IFT particles are trafficked along the axoneme by a heterotrimeric kinesin-II and a cytoplasmic dynein in the anterograde and retrograde directions, respectively. Cilia and flagella have diverse functions ranging from fluid and cell movement to mechanosensation and sensory perception (Davenport and Yoder, 2005; Scholey, 2003).

In mammals, Kif3a is a component of the kinesin-II motor protein complex required for cilia assembly while Ift88 (also known as Tg737 or polaris) is a core component of the IFT particle (Cole et al., 1998; Pazour et al., 2000; Taulman et al., 2001). Mice homozygous for mutations in Kif3a or any of the IFT proteins identified to date, including Ift88, die during mid-gestation and have randomization of the left-right body axis, neural tube closure and patterning defects, as well as polydactyly (Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 1998; Takeda et al., 1999). The severe phenotype of homozygous mutants and the expression of cilia on most cells throughout the body have complicated research directed at understanding the function of cilia on specific cell types or during distinct stages of development, as well as their role in normal tissue function in postnatal life.

Recent work from several groups has shown that disruption of IFT results in abnormal patterning of the developing murine limb and neural tube and that this is due to impaired sonic hedgehog (Shh) signal transduction (Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). Hedgehog signal transduction is both positively and negatively regulated, and disruption of this pathway leads to severe developmental defects (Huangfu and Anderson, 2006; Ingham and McMahon, 2001). In the absence of ligand, the pathway is repressed through the inhibition of the signal transducer smoothened (Smo) by the hedgehog receptor patched (Pch1). The Glioma family of transcription factors, Gli1, Gli2 and Gli3, are the main transducers of signaling. In the absence of ligand, Gli3 is proteolytically processed to generate a potent transcriptional repressor (Gli3R) of the pathway (Ding et al., 1999; Dunaeva et al., 2003; Stone et al., 1999; Wang et al., 2000). Whereas the major role of Gli3 appears to be repression of target gene transcription in the absence of ligand, Gli2 is predicted to act as the main transcriptional activator upon pathway induction (Bai and Ruiz i Altaba, 1999). Unlike Gli2 and Gli3, which are regulated post-translationally, Gli1 is predicted to act only as a transcriptional activator after pathway activation (Park et al., 2000).

Normal IFT function is required in the Shh signaling pathway, as Smo, the Gli transcription factors and Sufu have all been localized to the cilium axoneme (Corbit et al., 2005; Haycraft et al., 2005; May et al., 2005). While Sufu and the Gli proteins are found at the distal tip of cilia, Smo translocation to the cilium axoneme is induced in response to pathway activation (Corbit et al., 2005; Haycraft et al., 2005). In mice with congenital loss of Kif3a or Ift proteins required for anterograde trafficking, such as Ift88, the Shh signaling pathway remains inactive, despite the fact that the processing of Gli3 to the repressor form is severely impaired.

¹Department of Cell Biology, ²Department of Pathology and ³Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294-0005, USA.

*These authors contributed equally to this work.

¹Present address: Department of Pediatrics, University of Iowa, Iowa City, IA, USA

²Present address: Whitehead Institute for Biomedical Research, Cambridge, MA, USA

§Author for correspondence (e-mail: Byoder@uab.edu)

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(Pluck, 2002). In addition, exogenously expressed GlI2 is unable to activate signaling in cells lacking IFT (Haycraft et al., 2005). By contrast, GlI1 function is independent of IFT (Haycraft et al., 2005); however, its expression is dependent on hedgehog pathway activation and thus is also lost in IFT mutants.

During development and patterning of the mammalian limb, signaling molecules are secreted from three major signaling centers (Tickle, 2003). Cells on the apical ectodermal ridge (AER) secrete fibroblast growth factors (Fgfs) to promote proper proximodistal outgrowth of the limb, whereas the dorsal and ventral ectoderm secrete molecules including Wnts and Bmps essential for dorsoventral patterning. The formation of five digits is regulated by secretion of signaling molecules from mesenchymal cells in the zone of polarizing activity (ZPA). The main ligand secreted by cells in the ZPA is Shh, which acts to promote development of five patterned digits along the anteroposterior limb bud axis through inhibition of Gli3 proteolytic processing to generate Gli3R. This leads to the derepression of genes such as the Bmp antagonist gremlin in the anterior mesenchyme of the limb bud (Litingtung et al., 2002; te Welscher et al., 2002).

In addition to patterning of the digits in the mammalian limb, the hedgehog signaling pathway is also required for proper formation of other tissues, including the long bones of the appendicular skeleton (Razzaque et al., 2005; St-Jacques et al., 1999; Vortkamp et al., 1996). The long bones are formed by the aggregation and differentiation of cells from the lateral plate mesoderm to generate a cartilage template of the future bone, chondrocytes proliferate to contribute to the longitudinal growth of the bone. Prehypertrophic chondrocytes adjacent to the proliferating chondrocytes secrete Ihh, which is necessary for proper proliferation of the chondrocytes. The prehypertrophic chondrocytes undergo hypertrophic maturation and secrete extracellular matrix proteins, which are mineralized to form the trabecular bone. In Ihh null mice, the chondrocytes show reduced proliferation as well as premature hypertrophy, leading to severe shortening of the skeletal elements (Karp et al., 2000; St-Jacques et al., 1999). While Ihh signaling appears to directly regulate chondrocyte proliferation, its effects on hypertrophic differentiation are mediated through induction of PTH/Pr (Pithl – Mouse Genome Informatics) expression in the presumptive articular cartilage and perichondrium (Alvarez et al., 2002; Karp et al., 2000; Vortkamp et al., 1996).

Ihh secreted from prehypertrophic chondrocytes is also essential for formation of the bone collar from the perichondrium (Long et al., 2004; Razzaque et al., 2005; St-Jacques et al., 1999; Vortkamp et al., 1996). Mesenchymal cells forming the perichondrium surround the cartilage template and differentiate to generate osteoblasts, which form the bone collar and contribute to increased diametrical growth of the bone throughout postnatal life. Ihh signaling in the perichondrium leads to differentiation of osteoblasts through induction of canonical Wnt signaling (Hilton et al., 2005; Hu et al., 2005; Long et al., 2004).

To examine the role of cilia and IFT in the developing limb, we generated a new conditional mutant allele (Ift88fl) of the IFT protein Ift88. We flanked essential exons with loxp recombination sites to disrupt IFT in specific cell types in the mouse using transgenic strains expressing Cre recombinase. Following Cre expression, the level of Ift88 protein was severely reduced and cilia were no longer detected on cells. Surprisingly, in light of the reciprocal signaling events that occur between Shh-expressing cells of the ZPA, the AER and ectoderm, the loss of cilia and/or Ift88 on cells of the AER and ventral ectoderm did not result in major defects in limb outgrowth or in dorsoventral patterning. By contrast, mice lacking cilia and/or IFT in the mesenchyme due to expression of Cre recombinase under control of the Prxl (Prxl – Mouse Genome Informatics) limb enhancer developed multiple ectopic digits and showed a progressive loss of Shh signal transduction, preceded by expansion of gremlin expression in the anterior limb bud mesenchyme. Additionally, at later stages of limb development, endochondral bone formation in prxlcre;Ift88fl/fl conditional mutants was severely affected, and Ihh signaling was disrupted. The long bones displayed defects in chondrocyte differentiation and loss of the bone collar adjacent to the metaphysis. Surprisingly, ectopic chondrocyte-like cells were observed between the perichondrium and diaphysis. Overall, these results suggest that cilia and/or IFT are required in the mesenchyme at early stages of limb morphogenesis for Shh signaling to determine anteroposterior patterning of the digits and at later stages for proper endochondral bone formation.

MATERIALS AND METHODS

Mouse strains

All mice were maintained on a mixed genetic background. Transgenic prxlcre and msx2cre mice have been previously described, and both transgenic strains were genotyped by PCR using primers for the Cre coding region (Logan et al., 2002; Sun et al., 2000). The Kif3a conditional allele has been previously described (Lin et al., 2003). The standard Ift88fl/fl null allele has been previously described (Murcia et al., 2000).

Embryonic stem (ES) cells for targetting were derived from HPRT-deficient 129/Ola mice. Chimeric mice were generated from targeted ES cells by the UAB transgenic animal core facility. Germline transmission of the targeted allele was determined by coat color and resulting offspring were genotyped for the presence of the loxP-containing Ift88 allele (Ift88fl) using PCR. The null allele of Ift88 was generated using the prxlcre transgenic strain, which expresses Cre in the female germline. Ift88 conditional mice were genotyped by PCR using primers designed to amplify a region of genomic DNA flanking one of the loxP sites (wild-type and fl alleles) or spanning the region deleted upon Cre-mediated recombination (null allele; Ift88fl). Primer sequences are available on request. All mice were maintained in AAALAC certified mouse facilities at UAB with protocols approved by the IACUC. For staged embryos, noon of the day of the vaginal plug was designated as embryonic day (E) 0.5.

Immunofluorescence

Immunofluorescence on semi-thin frozen sections of limbs was performed on 15 μm-thick frozen sections as previously described (Haycraft et al., 2005; Taulman et al., 2001) using monoclonal antibodies to acetylated α-tubulin (Sigma Aldrich, St Louis, MO) or polyclonal antiserum to detect β-tubulin (Sigma Aldrich, St Louis, MO). Sections were fixed and frozen and sectioned as indicated for histology. Sections were incubated with 1 mg/ml hyaluronidase (Sigma Aldrich, St Louis, MO) at 37°C for 45 minutes before incubation with anti-aggrecaen antibodies (Millipore, Billerica, MA).

Histology and skeletal staining

Limbs were dissected from E18.5 embryos and fixed in 4% PFA in phosphate buffered saline (PBS) overnight at 4°C. Fixed tissues were washed with several changes of PBS and infiltrated with 30% sucrose in PBS overnight at 4°C. Sucrose-equilibrated samples were frozen in OCT and 15 μm sections were cut on a Leica CM1900 cryostat (Leica Microsystems GmbH, Wetzlar, Germany). Sections were fixed in 4% PFA and stained with Hematoxylin and Eosin Yellow (H&E) or Safranin O and Fast Green using standard protocols. For alkaline phosphatase staining, limbs from E18.5 wild-type and conditional mutant embryos were isolated, snap frozen in OCT, sectioned and fixed as described for immunofluorescence. Fixed sections were washed twice with Tris buffered saline (TBS), twice with NTMT (0.1 mol/l Tris pH 9.4, 0.1 mol/l NaCl, 0.05 mol/l MgCl2, 0.1% Tween-20) and incubated with BM Purple precipitating alkaline phosphatase...
substrate (Roche Diagnostics Corp., Indianapolis, IN) in the dark at room temperature for 10 minutes. After staining, sections were washed twice with TBS and nuclei were stained with Nuclear Fast Red (Sigma Aldrich, St Louis, MO). H&E- and alkaline phosphatase-stained sections were visualized on a Nikon TE2000 inverted microscope or a Nikon SMZ-800 dissecting scope and images were captured with a MicroPublisher 3.3 color digital camera (Q Imaging, Burnaby, BC).

For skeletal analysis, staged embryos were stained with Alizarin Red and Alcian Blue to identify mineralized bone and cartilage, respectively, as previously described (McLeod, 1980). Postnatal mice were euthanized, and skeletal elements were stained with Alizarin Red as previously described (Selby, 1987).

**In situ hybridization**

For both whole-mount and radiouclide in situ analyses, staged embryos were dissected and fixed in 4% PFA in PBS overnight at 4°C followed by washing with PBS. Whole-mount in situ hybridization was performed with digoxigenin (DIG)-labeled antisense probes using standard protocols (Wilkinson, 1992). In situ hybridization was performed on 10 μm frozen sections as previously described (Pelton et al., 1990). Ihh, Ptch1, Gli1, PTHrP and gremlin probes have been previously described (Alvarez et al., 2001; Bitgood and McMahon, 1995; Goodrich et al., 1996; Hui et al., 1994; Lu et al., 2001).

**Western blotting**

Whole protein lysates from E11.5 limbs were generated by placing limb buds in RIPA buffer (150 mmol/l NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 1% SDS, 50 mmol/l Tris pH 8.0) followed by brief sonication. Equal volumes of wild-type and mutant lysate were separated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-Ift88 antiserum and goat-anti-rabbit IgG-HRP conjugate.

**RESULTS**

**Generation of a conditional allele of Ift88**

As Ift88 is expressed in most cell types throughout the mammalian body, and congenital loss of IFT proteins results in severe developmental defects and mid-gestation lethality, the analysis of cilia and/or IFT function in specific tissues and cell types has not been tenable (Murcia et al., 2000; Zhang et al., 2003). To circumvent these problems, we generated an allele of Ift88 containing loxP sites flanking exons 4-6 to allow disruption of Ift88 by Cre-mediated recombination (Fig. 1A). Removal of these three exons is predicted to result in a translational frame shift and loss of all Ift88 function. Mice were genotyped using DNA from tail biopsies and PCR primers designed to amplify a region of DNA containing one of the loxP sites or spanning the region deleted upon Cre-mediated recombination (Fig. 1B). To verify that the recombined allele results in loss of Ift88 function, we generated embryos carrying the deleted allele (Ift88\(^{\text{fl}}\), germline mutation derived from the floxed allele) and the previously described targeted congenital null mutation (Ift88\(^{\text{gal/2-3\beta-gal}}\)). At E11.5, embryos carrying both alleles (Ift88\(^{\text{fl}}\)/gal\(^{\text{2-3\beta-gal}}\)) showed phenotypes characteristic of Ift88\(^{\text{fl}}\)/gal\(^{\text{2-3\beta-gal}}\) embryos, including misalignment of the neural tube, failure of neural tube closure and cardiac sac ballooning (Fig. 1C and data not shown). The identical phenotype in the Ift88\(^{\text{fl}}\)/gal\(^{\text{2-3\beta-gal}}\) embryos confirmed that Cre-mediated deletion of the floxed allele results in a null mutation.

As prx1cre has been shown to be expressed specifically in the mesenchyme, but not the overlying ectoderm, of the developing limbs beginning at E9.5 (Logan et al., 2002), we analyzed the mesenchyme and ectoderm of wild-type and conditional mutants at E11.5 for the presence of cilia by immunofluorescence (Fig. 2A-C). In wild-type samples, cilia visualized by acetylated \(\alpha\)-tubulin and Ift88 immunostaining, are found on cells throughout the mesenchyme of the developing limb (Fig. 2A). By contrast, cilia were present on very few cells in prx1cre;Ift88fl/n mutant limb mesenchyme (Fig. 2B), while cilia on the cells of the overlying ectoderm in the same section were normal (Fig. 2C).

To verify that the loss of cilia and Ift88 immunostaining correlates with the loss of Ift88 protein, we isolated whole limb buds from E11.5 prx1cre;Ift88\(^{\text{fl}}\)/gal\(^{\text{2-3\beta-gal}}\) conditional mutants and compared the amount of Ift88 protein to that found in limb buds from a control littermate (left).
simultaneously blotted for showed a dramatic reduction in the amount of Ift88 expressed when frozen sections of E11.5 wild-type (A) and prx1cre;Ift88fl/n remaining in immunofluorescence data, the level of Ift88 protein was dramatically littermate by western blot analysis. In agreement with the immunofluorescence data, the level of Ift88 protein was dramatically reduced in conditional mutants (Fig. 2D). The low level of Ift88 remaining in prx1cre;Ift88fl/n conditional mutants is probably due to Ift88 expression in the limb bud ectoderm, where the Cre transgene is not expressed, and a small number of limb bud mesenchymal cells, where recombination has not occurred. The blot was simultaneously probed for β-tubulin to ensure similar loading of control and mutant samples.

Loss of cilia and/or IFT in the AER and ventral ectoderm cells has no gross effect on limb patterning

As cilia are expressed on cells of the AER and ectoderm of the developing limb, we generated conditional mutants as described above using transgenic mice expressing Cre recombinase under the control of the Msx2 promoter (msx2cre). During early limb development, expression of Cre in this transgenic line is restricted to the AER and the ventral ectoderm (Sun et al., 2000). To verify that the msx2cre transgene is sufficient to disrupt cilia formation on the ventral ectoderm and AER of the limb bud, we performed immunofluorescence on frozen sections of E11.5 hindlimb buds. Cells in the dorsal ectoderm of msx2cre;Ift88fl/0n conditional mutants showed cilia when stained with antibodies for acetylated α-tubulin and Ift88 (Fig. 2E), whereas the ventral ectoderm of the same limb bud was nearly devoid of cilia (Fig. 2F). Despite the disruption of cilia on the ventral ectoderm, limbs of msx2cre;Ift88fl/0n mice showed no overt defects in outgrowth or patterning and were indistinguishable from their wild-type littermates (Fig. 3A-D). Identical results were seen with a conditional allele of the IFT kinesin-II subunit Kif3a, which functions along with Ift88 in anterograde IFT (data not shown). Although these data suggest that Ift88/IFT on the ectoderm has no function in limb outgrowth or patterning, we cannot exclude a role for IFT at stages before Cre expression in the AER or in the dorsal ectoderm, because the transgene is not expressed in these regions.

prx1cre;Ift88fl/0n mutants exhibit severe polydactyly

By contrast to the normal limb patterning observed in msx2cre;Ift88fl/0n mutants, prx1cre;Ift88fl/0n mutant mice developed eight non-patterned digits on each forelimb and a single extra preaxial digit on each hindlimb (Fig. 3E-H). The minimal effect observed in the hindlimbs is probably due to the previously reported weak expression of prx1cre in the hindlimb at early stages of limb development (Logan et al., 2002). In addition to the patterning defects in the autopod, all four limbs of prx1cre;Ift88fl/0n mutants were severely shortened along the proximodistal axis (Fig. 3I). Although the conditional mutants were smaller than wild-type littermates postnatally, the decreased length in the limbs was evident as early as E13.5, whereas the total size of the embryos was comparable to wild-type littermates (data not shown). An identical phenotype was seen using a conditional allele of the IFT kinesin-II subunit Kif3a (data not shown). Analysis of Alizarin Red-stained skeletons at postnatal day 11 indicate that all skeletal elements of the limb, including the humerus, radius and ulna, were formed properly in the conditional mutants (Fig. 3I), indicating that proximodistal patterning of the limb is not dependent on Ift88 function in the mesenchyme.

As congenital loss of Ift88 results in defects in Shh signaling during limb bud formation (Haycraft et al., 2005; Liu et al., 2005), we examined the level of Gli1 expression in the developing forelimbs of conditional prx1cre;Ift88fl/0n mutants by whole-mount in situ hybridization. Although prx1cre expression was detectable by E9.5, the expression of Gli1 appeared normal in the forelimbs at E10.5 (Fig. 4A,B); however, at E11.5, expression of Gli1 was nearly abolished in the conditional mutants (Fig. 4C,D). Small patches of cells were occasionally observed that retained Gli1 expression (arrowhead in Fig. 4D) and probably represent cells in which Ift88 expression was maintained due to inefficient Cre-mediated recombination.
By contrast to the normal expression of Gli1 at E10.5, expression of the Bmp antagonist gremlin was expanded into the anterior half of the developing limb bud of prx1cre;Ift88fl/n conditional mutants (Fig. 4E,F). At E11.5, gremlin expression was nearly absent in wild-type forelimbs, whereas conditional mutant forelimbs expressed significant levels of gremlin on the anterior side of the limb bud (Fig. 4G,H). These results suggest that while Shh signaling is retained for some time after loss of Ift88, processing of Gli3 to generate Gli3R is probably impaired before E10.5, resulting in de-repression of gremlin transcription in the anterior limb bud. In addition, as the Cre recombinase is expressed only in the developing mesenchyme, these results, along with the lack of a phenotype in msx2cre;Ift88fl/n conditional mutants, suggest that Ift88 function is required in the limb bud mesenchyme only to direct proper anteroposterior patterning.

**Conditional mutants exhibit defects in endochondral bone formation**

As chondrocyte precursors of the long bones are present in the limb mesenchyme when Cre expression is active in prx1cre mice, and as the appendicular skeletal elements in the prx1cre;Ift88fl/n mutants show reduced length, we hypothesized that this phenotype was due to a defect in endochondral bone formation. To evaluate this possibility, we isolated the limbs from conditional mutants and
control littersmates at E18.5 and stained frozen sections with Hematoxylin and Eosin (H&E) to analyze the histology of the long bones. Sections from the tibia are shown, but similar results were seen for all long bones in the appendicular skeleton. While conditional mutants exhibited some variability in the severity of endochondral bone defects between litters, all prx1cre;Ift88fl/n and prx1cre;Kif3afl/n mutants showed a dramatic reduction in the overall length of the skeletal elements relative to controls (Fig. 5A,C,E), although the overall size of the embryos was unchanged. The growth region of the tibia in conditional mutants contained only a small disorganized area of round and flat proliferating chondrocytes, suggesting accelerated hypertrophic differentiation. Although vascularization did occur, it was delayed in conditional mutant embryos and hypertrophic cells persisted (Fig. 5D,F).

To confirm that cilia are disrupted on the chondrocytes, we performed immunofluorescence on frozen sections of wild-type and conditional mutant tibia at E18.5. Cilia were apparent on the chondrocytes in the growth region of the tibia in wild-type embryos (Fig. 6G,H). By contrast, few chondrocytes in the developing tibia of prx1cre;Ift88fl/n embryos (H) expressed a cilium.
(Fig. 5G). By contrast, only small cilia were occasionally observed on the chondrocytes of the developing tibia in prx1cre;Ift88fl/fl conditional mutants (Fig. 5H).

**Ihh signaling is disrupted in the bones of prx1cre;Ift88fl/fl** mutants

The loss of Shh signaling during early limb patterning in prx1cre;Ift88fl/fl conditional mutants prompted us to examine Ihh signaling in the long bones of conditional mutants. We analyzed the expression of Ihh and two downstream targets of the hedgehog pathway, Ptc1 and Gli1, in E18.5 conditional mutants by radioactive in situ hybridization. As seen for Shh in the autopod of Ift88 null embryos, Ihh expression was maintained in the prehypertrophic chondrocytes in the fibula of prx1cre;Ift88fl/fl conditional mutants although at reduced levels (Fig. 6C,D). A similar decrease in Ihh expression is seen in mice lacking Gli2 and may be due to the positive feedback loop required to maintain high levels of Ihh expression (Miao et al., 2004). Alternatively, the decrease in Ihh expression may reflect fewer prehypertrophic chondrocytes in conditional mutants. Despite expression of Ihh, no significant expression of Ptc1 or Gli1 was detected in the perichondrium flanking the prehypertrophic chondrocytes or the region of proliferating chondrocytes in the tibia of conditional mutants (Fig. 6H,L). These findings were evident in all skeletal elements in the developing limbs, suggesting that Ift88 is required for Ihh signaling during embryonic endochondral bone formation. By contrast to the loss of Ptc1 and Gli1 expression, PTHrP was expressed in the conditional mutants at E14.5 (Fig. 6O,P).

**Development of the bone collar is altered in conditional mutants**

The perichondrium consists of multipotent mesenchymal cells lining the outer edge of the bone anlagen. During development, Ihh secreted from prehypertrophic chondrocytes results in the activation of canonical Wnt signaling and subsequent differentiation of cells in the inner layer of the perichondrium to osteoblasts, thus forming the bone collar (Hu et al., 2005). By contrast to the uniform spindle-shaped appearance of perichondrial cells along the tibia in wild-type samples, the perichondrium in prx1cre;Ift88fl/fl conditional mutants was disorganized and cells did not adopt the characteristic flattened morphology (Fig. 7A-D). In addition to the loss of normal cell architecture, the perichondrium exhibited uneven thickness along the length of the bone (Fig. 7B). The most severe defects in perichondrial organization were observed flanking the diaphysis, where cells adjacent to the perichondrium resembled chondrocytes rather than osteoblasts (arrowheads in Fig. 7B). While it is unclear if these cells originated from the perichondrium or the growth plate of the developing bone, they appeared to be continuous with the perichondrium in some sections, suggesting that they may have originated there (arrowheads in Fig. 7B). The bone collar develops along the metaphysis of the bone anlagen and extends to the level of the prehypertrophic and hypertrophic chondrocytes in wild-type tibiae (Fig. 7C). In prx1cre;Ift88fl/fl mutant embryos, no bone collar was apparent adjacent to the corresponding region (Fig. 7D).

The loss of perichondrial architecture and bone collar development suggested a potential defect in osteoblast differentiation, prompting us to examine the presence of osteoblasts
in prx1cre;Ift88fl/n conditional mutants. Osteoblasts that generate the bone collar express specific markers, including alkaline phosphatase (AP), which can be easily detected using biochemical analyses. To determine if osteoblasts were present where the bone collar is normally located, we stained sections of the radius of wild-type and conditional mutant mice for AP activity using a colorimetric AP substrate. By contrast to the normal expression of AP adjacent to the proliferating chondrocytes in the radius of wild-type mice, no AP expression was observed in the perichondrium in the conditional mutant, whereas staining in the hypertrophic chondrocytes was unaffected (Fig. 7E,F).

**Ectopic chondrocytes develop in the perichondrium of prx1cre;Ift88fl/fl mice**

To determine the position of bone and cartilage matrix in the conditional mutants, we stained sections of the long bones with Safranin O and Fast Green. In wild-type tibiae, Safranin O staining was present along the growing epiphysis, whereas trabecular and cortical bone stained with Fast Green (Fig. 8A). Intriguingly, Safranin O staining was evident in regions of the diaphysis in conditional mutants (Fig. 8B), suggesting that some of the cells in the perichondrium of conditional mutants are differentiating along the chondrocyte lineage rather than the appropriate osteoblastic lineage. Despite the defects in perichondrial architecture and bone collar formation, some regions of prx1cre;Ift88 conditional mutant tibiae did contain mineralized bone (blue-green staining in Fig. 8B). To further investigate the ectopic perichondrial cells in conditional mutants, we performed immunofluorescence to determine the localization of the proteoglycan aggrecan. In wild-type tibiae, aggrecan is localized to the chondrocytes but is absent from the perichondrium and developing bone collar. By contrast, prx1cre;Ift88fl/fl mutant tibiae showed strong localization of aggrecan in the perichondrium (Fig. 8C,D).

Overall, these results suggest that prx1cre;Ift88 conditional mutant long bones share some features of Ihh;Gli3 mutants, including loss of the bone collar and restoration of PTHR expression relative to Ihh mutant mice (Hilton et al., 2005; St-Jacques et al., 1999). However, the development of ectopic chondrocytes along the diaphysis is not seen in Ihh;Gli3 mutants. Rather, this is characteristic of defects in canonical Wnt signaling (Hu et al., 2005).

**DISCUSSION**

Cilia are small microtubule-based organelles found on most cells in the mammalian body. The embryonic lethality and severe developmental defects associated with loss of cilia or IFT have hindered efforts to investigate the role of IFT in the development of specific tissues and postnatal tissue homeostasis (Davenport and Yoder, 2005; Scholey, 2003). We addressed this issue in the limb bud using the Cre-lox system and a conditional allele of the IFT gene Ift88. Whereas conditional loss of cilia on the ventral ectoderm and AER had no overt effect on limb patterning, loss of Ift88 in the limb mesenchyme resulted in extensive polydactyly on the forelimb and shortening of the long bones in all four limbs. In addition to abnormalities in chondrocyte differentiation in the prx1cre;Ift88fl/fl conditional mutants, the perichondrium was disorganized, ectopic cells that resemble chondrocytes developed along the diaphysis and the bone collar was not properly formed.

Although cilia are present on the ectodermal cells of the developing limb (Haycraft et al., 2005), the conditional loss of cilia on the ventral ectoderm and AER of the limb bud with msx2cre did not significantly affect limb patterning. This indicates that ciliary function is not required on these cell populations for normal limb development, although a role for cilia and/or IFT before AER formation or in the dorsal ectoderm cannot be excluded, as msx2cre is expressed in the ventral ectoderm and AER.

Despite the fact that Gli3 expression appears normal at E10.5 in the prx1cre conditional cilia mutants, gremlin, which is normally restricted to the posterior region of the limb, is expanded anteriorly. This expanded domain of gremlin is also observed in the Gli3 and Shh;Gli3 double mutants (Litingtung et al., 2002; te Welscher et al., 2002). These data suggest that by E10.5, loss of IFT or the cilia has already impaired the formation of Gli3R in the anterior, leading to de-repression of gremlin. This is in agreement with previous work demonstrating that Ift88 is essential for efficient processing of Gli3 (Haycraft et al., 2005; Liu et al., 2005).

In addition to a role in Shh signaling, our data indicate that cilia are required for normal Ihh signaling activity based on the loss of Ptc1 and Gli1 expression in the long bones of prx1cre;Ift88fl/fl conditional cilia mutants. However, the phenotype of the conditional mutants is distinct from that seen in mice with congenital loss of Ihh, which exhibit premature hypertrophic differentiation due to loss of PTHR expression in the presumptive articular cartilage and perichondrium, decreased chondrocyte proliferation and loss of vascularization of the long bones (St-Jacques et al., 1999). Recent work has shown that congenital loss of Gli3 in the developing bones of Ihh null mice can restore the formation of proliferating chondrocytes in Ihh null mutants, although at reduced levels, as well as restore PTHR expression in both the presumptive articular cartilage and perichondrium and vascularization of the bones (Hilton et al., 2005; Koziel et al., 2005). While prx1cre;Ift88fl/fl conditional
mutants share characteristic phenotypes with *Ihh* null mutants, including accelerated chondrocyte hypertrophy and loss of proper bone collar development. *PTHR1* is expressed in conditional mutants potentially through disruption of Gli3R formation as seen for *gremlin* in the developing limb. Additionally, vascularization occurs in the conditional mutants, although it is delayed.

Osteoblast differentiation and development of the bone collar also requires canonical Wnt signaling downstream of *Ihh*. In agreement with this, conditional loss of β-catenin in the developing perichondrium results in defective differentiation of the bone collar (Hilton et al., 2005; Hu et al., 2005). While undifferentiated cells accumulated between the perichondrium and diaphysis of *Ihh*;*Gli3* double mutants and in mice with conditional loss of β-catenin, the ectopic cells observed in *Ihh*;*Gli3* mutants expressed markers of preosteoblasts. By contrast, mice with conditional loss of β-catenin in the developing skeleton exhibited ectopic chondrocyte differentiation in the perichondrium. A similar accumulation of cells between the perichondrium and diaphysis was seen in *prxlcre:*Iftr88<sup>fl/+</sup> conditional mutants. The ectopic cells in conditional mutants expressed aggrecan and morphologically resembled chondrocytes, suggesting that they are similar to the ectopic chondrocytes observed due to loss of canonical Wnt signaling.

Taken together, the endochondral bone phenotype seen in *prxlcre:*Iftr88<sup>fl/+</sup> conditional mutants has similarities with several mouse models, including *Ihh, Ihh*;*Gli3* double mutants and mice with conditional disruption of β-catenin in the skeleton, but the phenotype is not identical to any one mouse model. This suggests that the role of IFT and cilia during endochondral bone development is not restricted to the known role for IFT in hedgehog signaling and may include a role for cilia or IFT in additional signaling pathways such as canonical Wnt signaling. Indeed, previous reports have pointed to a role for cilia or centrosomes in canonical and noncanonical Wnt signaling (Cano et al., 2004; Ross et al., 2005; Simons et al., 2005).

Overall, the generation of a conditional allele of the IFT protein Iftr88 provides a valuable resource to allow the study of IFT function in specific cell types during development, as well as normal tissue homeostasis. Using the Cre-lox system we have uncovered a role for Iftr88 in Ihh signal transduction, similar to that previously reported in Shh signaling during limb and neural tube patterning (Haycraft et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005) in addition to a possible role in multiple signaling pathways required for endochondral bone formation. Together these data demonstrate an essential requirement for cilia or IFT function in normal patterning of the autopod during early stages of limb development in addition to a requirement during endochondral bone formation in the appendicular skeleton at later stages of limb morphogenesis. Analysis of ciliary function during long bone development was previously untenable due to the early lethality of IFT null mutants. The use of these conditional cilia-IFT mutant alleles in combination with additional strains expressing Cre recombinase under the control of alternative and inducible promoters will allow a more thorough analysis of the functional importance of IFT and cilia throughout development and during postnatal life.

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References


DEVELOPMENT


