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Analysis of Hedgehog Signaling in Mouse Intraflagellar Transport Mutants

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Abstract

Intraflagellar transport (IFT) has been studied for decades in model systems such as *Chlamydomonas* and *Caenorhabditis elegans*. More recently, IFT has been investigated using genetic approaches in mammals using the mouse as a model system. Through such studies, a new appreciation of the importance of IFT and cilia in mammalian signal transduction has emerged. Specifically, IFT has been shown to play a key role in controlling signaling by Sonic and Indian Hedgehog (Hh) ligands. The effects of mutations in IFT components on Sonic Hh signaling in the embryo are complex and differ depending on the nature of the components, alleles, and tissues examined. For this reason, we provide a basis for analyzing the phenotype as a guide for those investigators who study IFT in cell culture or use invertebrate systems and wish to extend their studies to include development of the mouse embryo. We provide an overview of Sonic Hh-dependent tissue patterning in the developing neural tube and limb buds, the two systems in which it has been studied most extensively, and we show examples of how this patterning is disrupted by mutations in mouse IFT components.

I. Introduction

Primary cilia are microtubule-based organelles found on nearly all mammalian cell types in interphase. As discussed elsewhere in this volume, assembly and disassembly of cilia and flagella is driven by intraflagellar transport (IFT), a bidirectional microtubule motor-driven transport process [for a review, see Pedersen and Rosenbaum (2008)]. IFT directs the anterograde transport of cargo-loaded protein complexes from the minus ends of axonemal microtubules at the base of cilia (the basal bodies) toward microtubule plus ends, where the cargo is used for ciliary assembly at their growing ends. IFT also mediates retrograde transport of protein complexes back to the cell body, allowing for recycling or degradation of the cargo and IFT machinery. IFT occurs through the movement of plus and minus end-directed motors, primarily kinesin II and cDynein1b. These motors function together with the IFT proteins, which are thought to act as adaptors linking cargo proteins to the motors. The IFT proteins have been biochemically classified as members of two large complexes, complex A and complex B (Cole *et al.*, 1998). Based on their mutant phenotypes in a variety of systems, complex B IFT proteins are believed to have a more crucial role in anterograde transport, whereas complex A IFT proteins are thought to act primarily in retrograde transport (Blacque *et al.*, 2006; Piperno *et al.*, 1998), although this distinction is probably an oversimplification.

A relationship between IFT and mammalian Hedgehog (Hh) signaling was first uncovered in 2003 through analysis of mouse mutations affecting embryonic development that proved to be mutations in IFT proteins (Huangfu *et al.*, 2003). Hedgehog is a secreted, lipid-

modified protein that signals to cells in most metazoans to control tissue patterning, growth, and differentiation during embryogenesis (Jiang and Hui, 2008). Mammalian genomes harbor three Hh ligands: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and desert hedgehog (Dhh). These signals primarily control transcriptional responses in the cells of target tissues by binding to a twelve-pass membrane receptor, Patched, thereby alleviating its repression of a seven-pass transmembrane protein called Smoothened (Smo, Fig. 1). Once activated, Smo regulates the Gli transcription factors (e.g., Gli1, Gli2, and Gli3 in mammals), which bind regulatory elements of target genes and regulate their expression. The Glis can act both as activators and as repressors of pathway targets, depending on the status of upstream Smo activity. For example, in the absence of Hh signals, Gli3 is proteolytically processed to a smaller transcriptional repressor form, Gli3Rep. Stimulation of the pathway blocks this processing and converts the Glis into transcriptional activators, collectively referred to as GliAct (Pan *et al.*, 2006; Wang *et al.*, 2000).

A growing body of evidence strongly suggests that IFT components function in mammalian Hh signaling in the context of primary cilia [reviewed in Eggenschwiler and Anderson (2007)]. This is supported by genetic data showing that at least five IFT proteins, subunits of both IFT motors, and a protein controlling the axonemal structure of the cilium (Arl13b), play key roles in Hh pathway regulation (Caspary *et al.*, 2007; Huangfu and Anderson, 2005; Huangfu *et al.*, 2003; Liu *et al.*, 2005; May *et al.*, 2005). Moreover, many established Hh-signaling components, such as Ptch1, Smo, SuFu, Gli1, Gli2, and Gli3, and several newly identified regulators, such as C2cd3, Tulp3, Ftm, and Evc, localize to primary cilia and/or basal bodies (Corbit *et al.*, 2005; Haycraft *et al.*, 2005; Hoover *et al.*, 2008; Norman *et al.*, 2009; Rohatgi *et al.*, 2007; Ruiz-Perez *et al.*, 2007; Vierkotten *et al.*, 2007). Interestingly, for at least two key signaling components, Ptch1 and Smo, localization to primary cilia is controlled by exposure to Hh ligands. In the absence of signaling, Ptch1 localizes to the cilium and Smo localizes to intracellular vesicles, whereas this pattern is reversed when Ptch1 binds Hh ligand (Corbit *et al.*, 2005; Rohatgi *et al.*, 2007).

The two systems in the developing mouse embryo where Shh signaling has been studied the most are the developing neural tube, which gives rise to the brain and spinal cord, and the limbs (Jiang and Hui, 2008). In both cases, Shh is produced by a small group of cells and acts as a long-range signal within the target tissues to control cell fate and tissue growth.

During neural tube patterning, Shh is initially produced by the notochord that lies ventral to the neural tube and it forms a ventral (high) to dorsal (low) gradient within the neural tissue. Cells of the neural tube adopt distinct identities as a function of the Shh concentration to which they are exposed. The most ventral cells within the neural tube are specified to become the floor plate, which acts as a secondary source of Shh ligands, whereas cells positioned further away adopt other fates such as motor neurons or interneurons. Shh acts by inducing and repressing the expression of a number of transcription factors, such as members of the homeobox and bHLH families, within proliferating progenitor cells. In turn, these transcription factors act in a combinatorial fashion to direct distinct neural differentiation programs.

In the limb buds, Shh is used to control anterior–posterior (i.e., thumb to pinky) digit identity and to control limb bud growth (McGlinn and Tabin, 2006). Shh is produced by a group of mesenchymal cells in the posterior limb bud. This region is termed the zone of polarizing activity because of its ability to organize pattern throughout the rest of the tissue. In the limb bud, cells in proximity to the source of Shh (or cells which experience Shh signaling for longer periods of time) express different sets of genes and adopt different digit identities than those further away experiencing little or no Shh signaling. Shh controls differential gene expression in the limb primarily through inhibiting the formation of Gli3

repressor, Gli3Rep, although the Shh-dependent gene expression profile is also controlled by the GliAct forms (te Welscher *et al.*, 2002; Vokes *et al.*, 2008).

II. Rationale

The regulation of gene expression by Shh signaling in the mouse embryo is quite complex. For example, some targets are induced by weak Shh signaling, yet are repressed by higher levels, whereas others are only induced by intermediate or high levels. In addition, targets can be positively regulated by the activator forms of the Gli transcription factors (GliAct), negatively regulated by the Gli repressor forms (Gli3Rep, in particular), or they may be regulated by both modes. Finally, some of the genes encoding components of the Shh signal transduction apparatus, such as *Shh*, *Gli1*, *Gli3*, and *Ptch1*, are also direct or indirect transcriptional targets of the pathway. Such regulation is thought to provide feedback within the system. Here we outline these expression patterns and present examples of how they are affected in mouse mutants with defects in IFT components.

A. Mutations

The effects of IFT mutations on Shh signaling vary widely depending on the component and the nature of the mutations. Here we show three examples to illustrate these effects:

Kif3a^{-/-}—Null mutations in components required for anterograde IFT typically disrupt ciliogenesis entirely (e.g., Fig. 2D, H, L) and lead to lethality around embryonic day 9.5 (E9.5) (Marszalek *et al.*, 1999; Murcia *et al.*, 2000). This allows for an analysis of early Shh-dependent neural tube patterning, but largely precludes the study of limb patterning as limb development has just begun at this stage. An example presented below (*Kif3a^{tm1Gsn}*) is a null mutation in *Kif3a*, encoding a subunit of kinesin II, but other mutants, such as *Ift88* null homozygotes, show similar phenotypes (Marszalek *et al.*, 1999; Murcia *et al.*, 2000). In such mutants, the Gli activators are rendered mostly or entirely nonfunctional (Haycraft *et al.*, 2005). However, Shh targets are not entirely suppressed in these embryos because formation of Gli3Rep is also disrupted, leading to partial derepression of target genes normally responding to weak Shh signaling (Huangfu and Anderson, 2005; Liu *et al.*, 2005).

Ift88^{fxo/-}—Hypomorphic mutations that partially disrupt anterograde IFT, such as *Ift88^{fxo}*, reduce the frequency of ciliogenesis and/or result in shorter cilia (Fig. 2C, Liu *et al.*, 2005; Taulman *et al.*, 2001). In such mutants, Gli activator function is reduced but not abolished and Gli3 repressor formation is impaired (Liu *et al.*, 2005). These mutants survive to later embryonic stages (beyond E10.5), allowing analysis of Shh signaling in the limbs, as well as the neural tube. Here we show as examples the limb and neural tube-patterning phenotypes of *Ift88^{fxo}/Ift88^{null}* compound heterozygotes.

Ift122^{sopb}—Mutations in IFT components can also have qualitatively distinct effects on ciliogenesis and Hh signaling. For example, components of IFT complex A, such as the mouse Ift139 homolog THM1/aln, appear to have a more important role in retrograde IFT in comparison to anterograde IFT (Tran *et al.*, 2008). Here we provide another example of a mutation in a complex A IFT protein, a null allele of *Ift122* (*Ift122^{sopb}*). *Ift122^{sopb}* mutants harbor a missense mutation in the start codon preventing protein translation (J. Qin and J. T. E., in preparation). In *Ift122^{sopb}* mutants, cilia are generated at, or near, normal frequencies, but the tips of their cilia are swollen and show accumulation of IFT components and other ciliary proteins (Fig. 1B, F, J and data not shown). In these mutants, Gli2Act is constitutively active independently of Shh, leading to an overall hyperactivation of the pathway (J. Qin and J.T.E., in preparation). Such mutants typically survive until E12.5, allowing for neural tube and limb-patterning analysis.

B. Neural Tube Patterning

Patterning of cell fates along the dorsal–ventral axis of the developing mouse neural tube is governed to a large extent by graded activity of the Shh pathway. Shh signaling represses the expression of genes marking dorsal neural fates and induces the expression of markers for ventral fates. Because Shh acts as a morphogen, each marker is induced over a distinct range of Shh signaling. The Shh-dependent gene expression patterns in the neural tube presented below are summarized in Fig. 3.

Figure 4 shows an analysis of such markers in different mutant neural tubes as examined by immunofluorescence (IF) in transverse cryosections. An advantage to this method is that it is relatively simple and double or triple labeling can reveal the spatial relationships of multiple distinct cell types simultaneously in the same tissue. These markers can be assayed in the E10.5 neural tube but, in cases where certain mutants arrest earlier, they can also be studied at E9.5. It is often convenient to counterstain these sections with a nuclear stain such as DAPI to delineate the neural tube. Here we show the expression of markers for proliferating progenitor cells of the ventricular zone, but because these progenitors give rise to distinct types of postmitotic neurons, the expression of neuronal markers, such as Isl1 (motor neurons), Chx10 (V2 interneurons), En1 (V1 interneurons), and Evx1 (V0 interneurons), may also be examined at stages when neurogenesis is well underway (E10.5 or later).

The floor plate markers FoxA2 (previously called Hnf3 β) and Shh are induced at the ventral pole of the neural tube by the highest levels of Shh signaling (Marti *et al.*, 1995; Roelink *et al.*, 1995). These markers show the highest sensitivity to diminished Shh signaling and neither marker is expressed in the neural tissues of *Kif3a*^{−/−} or *Ifi88*^{fxo/−} mutants (Fig. 4C, E, H, J). Shh is also expressed in the notochord, lying ventral to the neural tube, and its initial expression here does not rely on Shh pathway activity (Epstein *et al.*, 1999). In IFT mutants with hyperactive Shh signaling, such as *Ifi122*^{sopb}, the domains of Shh and FoxA2 expand dorsally beyond the ventral midline (Fig. 4B and G). These effects can vary with respect to the rostral-caudal level of the neural tube. For example, inappropriate ventralization of cell fates is limited to the lumbar regions of the spinal neural tube in *Ifi122*^{sopb} mutants.

The expression of homeobox and bHLH transcription factors such as Nkx2.2, Nkx6.1, Olig2, Dbx1, Irx3, and Pax6 is controlled by distinct levels of Shh signaling and thus provide sensitive readouts for this pathway in neural tissues (Dessaud *et al.*, 2008). Nkx6.1 (Fig. 4P, red) is normally expressed in the ventral third of the neural tube in response to high and intermediate levels of Shh signaling in progenitors giving rise to floor plate, V3 and V2 interneurons, and motor neurons (Briscoe *et al.*, 2000), whereas Nkx2.2 (Fig. 4K, red) is normally induced only by high levels in a progenitor domain adjacent to the floor plate that gives rise to V3 interneurons (Briscoe *et al.*, 1999). The motor neuron progenitor marker Olig2 (Fig. 4K, green) is induced by intermediate levels of signaling and is therefore expressed in a narrow band of cells just dorsal to the Nkx2.2+ domain (Takebayashi *et al.*, 2002). Pax6 (Fig. 4P, green) is upregulated by weak Shh signaling in progenitors giving rise to V0, V1, and V2 interneurons, but its expression is downregulated or fully repressed in cells experiencing higher levels of Shh signaling (Ericson *et al.*, 1997). Msx1 and Msx2 (Msx1/2) are expressed in the most dorsal neural progenitors (e.g., Fig. 4U) in response to BMP signaling (Liem *et al.*, 2000). Although the Msx1/2 expression domain in the neural tube is not affected by loss of Shh signaling, increased Shh signaling downregulates Msx expression (Cho *et al.*, 2008; Liem *et al.*, 2000).

In *Ifi88*^{fxo/−} mutants, the level of Shh pathway activation is partially reduced. This level is sufficient to induce Olig2 and Nkx6.1 expression. However, Nkx2.2 expression is lost and the ventral repression of Pax6 expression is incomplete. *Kif3a*^{−/−} mutants, analyzed at E9.5,

show a further reduction in the level of Shh signaling. This is manifested by the loss of Olig2 expression, restriction of Nkx6.1 expression to a just few cells in the ventral midline, and complete derepression of Pax6 throughout the ventral neural tube. As the ventral border of Msx1/2 expression is not normally set by Shh signaling, the Msx1/2 domain remains unchanged in *Ift88^{fxo/-}* and *Kif3a^{-/-}* mutants.

These expression patterns are quite different in *Ift122^{sopb}* mutants. Rather than being repressed or ventrally restricted, the Nkx2.2+ and Nkx6.1+ domains expand toward the dorsal pole, similar to the Shh+ and FoxA2+ domains. The Olig2+ domain dorso-laterally shifts to regions, while ventral border of the Pax6+ domain is restricted to the dorsal third of the neural tube. The increase in Shh signaling in such mutants also disrupts specification of dorsal cell fates, as shown by the absence of Msx1/2 expression in the dorsal neural tube.

The expression patterns of some genes are not easily monitored by immunofluorescent methods due to lack of availability of suitable commercially available antibodies. In such cases, section *in situ* hybridization provides a viable alternative. As an example, *in situ* hybridization analysis of neural identity genes, such as *Irx3* and *Dbx1*, and Shh signaling pathway components is shown in Figs. 5 and 6.

Irx3 and *Dbx1* (Fig. 5) are normally repressed in the ventral neural tube by intermediate to high levels of Shh signaling (Briscoe *et al.*, 2000; Pierani *et al.*, 1999). Hence, they are frequently expressed in ventrally expanded domains in *Kif3a^{-/-}* mutants showing strongly attenuated Shh signaling, while they are expressed in dorsally shifted/restricted domains in *Ift122^{sopb}* mutants. The expression patterns of *Dbx1* and *Irx3* are largely unchanged in *Ift88^{fxo/-}* mutants because they retain a higher level of Shh pathway activity in comparison to *Kif3a^{-/-}* mutants.

Transcriptional targets of the Shh pathway are not restricted to effector genes, such as the homeobox transcription factors that specify cell fate, but also include components of the signaling pathway itself. The expression of such targets is shown in Fig. 6. As described above, Shh from the notochord induces its own expression in the ventral midline of the neural tube (Fig. 6A and D). In addition, expression of the genes encoding the Shh receptor *Ptch1* and the transcription factor *Gli1* are induced by Shh signaling in the ventral neural tube, although they are not expressed in floor plate cells experiencing the highest levels of Shh signaling (Fig. 6F, I, K, N). In contrast, expression of *Gli3* is restricted dorsally in the neural tube due to repression by Shh signaling (Fig. 6P). Because of the lack of suitable antibodies, monitoring *Ptch1*, *Gli1*, and *Gli3* expression is best performed through *in situ* hybridization.

Consistent with the effects described above, expression of *Gli1* and *Ptch1* in the neural tubes of *Ift88^{fxo/-}* mutants is weaker or is ventrally restricted when compared to wild type, and is reduced to basal levels in *Kif3a^{-/-}* mutants. In contrast, the expression domains of *Gli1* and *Ptch1* are either shifted or expanded dorsally in *Ift122^{sopb}* mutants due to hyperactivation of the Shh pathway. The level of Shh pathway activity in *Ift88^{fxo/-}* and *Kif3a^{-/-}* mutants is not reduced enough to appreciably affect *Gli3* expression ventrally, but the increase in pathway activity is sufficient to downregulate *Gli3* in the dorsal domains of *Ift122^{sopb}* mutant neural tubes. Thus, the expression of these Shh targets can provide an independent measure of the effects of IFT mutations on the activity of the Shh pathway in the neural tube.

C. Limb Patterning

As in the neural tube, signaling by Shh controls the expression of a large number of target genes in the developing mouse limb buds and its role in limb patterning and growth has been extensively studied (McGlinn and Tabin, 2006). These target genes can be positively or

negatively regulated by Shh signaling and the individual target genes have different response thresholds. Shh is expressed in a small group of mesenchymal cells in the posterior region of the limb, and it signals to cells throughout much of the limb bud along the anterior–posterior axis. In contrast to the neural tube, much of the patterning is established by the activity of the Gli3 repressor, whose formation is inhibited by Shh signaling (te Welscher *et al.*, 2002; Vokes *et al.*, 2008; Wang *et al.*, 2000). Gene expression in limb buds is easily assayed by whole-mount *in situ* hybridization. Because patterning of the limbs by Shh is not easily seen at earlier stages (before E10.5), this type of analysis is suitable for those mutants surviving until E10.5 or later (e.g., *Ift88^{fxo/-}* and *Ift122^{sopb}* mutants), as opposed to those arresting around E9.5 (e.g., *Kif3a^{-/-}* mutants).

Figure 7 shows examples of forelimb buds of *Ift88^{fxo/-}* and *Ift122^{sopb}* mutants at E10.5 analyzed by *in situ* hybridization. At this stage, the size and the shape of *Ift88^{fxo/-}* limb buds are normal, although at later stages the mutant limb buds exhibit preaxial polydactyly (extra digits on the anterior side of the limbs). In contrast, *Ift122^{sopb}* mutant forelimb buds are shorter and broader than in wild-type, although the overall size of *Ift122^{sopb}* mutants is normal at this stage. The altered gene expression patterns in IFT mutant forelimbs described below are also observed in the hindlimb buds (not shown).

The *Hoxd* genes are expressed in the limb bud mesenchyme in nested patterns (Robert and Lallemand, 2006). The Gli3 repressor form, Gli3Rep, whose production is differentially inhibited along the anterior–posterior axis by Shh signaling, controls these expression patterns. Thus, loss of Shh leads to overproduction of Gli3Rep and strong downregulation of *Hoxd* genes such as *Hoxd11* and *Hoxd13*, whereas loss of Gli3Rep results in their ectopic expression in anterior regions (te Welscher *et al.*, 2002; Zakany *et al.*, 2004). *Gremlin1* (*Grem1*) is also induced by Shh signaling in the mesenchyme, but its expression is excluded from the most posterior regions where *Shh* is expressed (Nissim *et al.*, 2006). *Grem1* expression is regulated by both GliAct and Gli3Rep (Vokes *et al.*, 2008). As a result of *Grem1* upregulation by Shh, *Fgf4* expression is upregulated in the apical ectodermal ridge (AER) overlying the mesenchyme through inhibition of Bmp signaling (Zuniga *et al.*, 1999).

Anterograde IFT is required not only for activation of GliAct forms but also for appropriate generation of Gli3Rep. Thus, although the most prominent phenotypic features observed in *Ift88^{fxo/-}* mutant neural tubes are explained by *attenuated* Shh pathway activity, many of the altered gene expression patterns observed in the limbs are explained by ectopic pathway activity. This can be seen by the anterior expansion of the *Hoxd11*, *Hoxd13*, *Grem1*, and *Fgf4* expression domains in *Ift88^{fxo/-}* mutants (Fig. 7B, E, H, K). Despite their abnormal shapes, *Ift122^{sopb}* mutant limb buds exhibit similar anterior expansion and/or shifting of these expression domains (Fig. 7C, F, I, L).

The expression of Shh pathway components in the limb buds also serves as an informative readout of the pathway. Figure 8 shows expression of such targets in the limb buds. *Shh* expression is initiated in the posterior limb bud independently of Shh pathway activity but is maintained by positive feedback via ectodermal FGFs (Niswander *et al.*, 1994). In *Ift88^{fxo/-}* mutants, *Shh* expression is limited to the posterior domain, whereas in *Ift122^{sopb}* mutant limb buds, it is slightly expanded. *Gli1* and *Ptch1* are induced by Shh signaling in the limbs as they are in the neural tube. These genes are induced broadly in the posterior regions of normal limb buds (Fig. 8D, and G). In contrast to *Hoxd11* and *Hoxd13*, the expression domains of *Ptch1* and *Gli1* appear to be set primarily by GliAct forms, rather than by Gli3Rep (te Welscher *et al.*, 2002; Vokes *et al.*, 2008). As a result, both genes are strongly downregulated and posteriorly restricted in *Ift88^{fxo/-}* mutant limb buds (Fig. 8E and H). In *Ift122^{sopb}* mutants, GliAct (Gli2Act, in particular) appears to be constitutively active. Thus, both *Gli1* and *Ptch1* are ectopically expressed in anterior regions in these mutants (Fig. 8F

and I). *Gli3* expression is normally inhibited by strong Shh signaling and is therefore repressed in the most posterior regions of the limb buds. *Gli3* expression in *Ift88^{fxo/-}* mutants expands slightly toward the posterior side in comparison to wild type, while it is more anteriorly restricted in *Ift122^{sopb}* limb buds (Fig. 8K and L). Thus, constitutive activity of the GliAct forms in *Ift122^{sopb}* mutant limb buds results in a general expansion of posterior cell fates at the expense of anterior fates. By contrast, the combined effects of diminished formation of the Gli3Rep form and reduced activity of GliAct forms in *Ift88^{fxo/-}* mutants result in ectopic expansion of some Shh targets (e.g., *Hoxd* genes and *Grem1*), as well as downregulation/posterior restriction of others (e.g., *Ptch1* and *Gli1*).

III. Methods

A. Dissection/Fixation of Embryos and Cryosectioning

To analyze mouse embryonic phenotypes, crosses of heterozygous mice are set up and vaginal plugs are checked each morning to establish the day of conception (noon of the day when a plug is found is considered to be E0.5). After a suitable period of gestation, pregnant female mice are sacrificed by regulated CO₂ inhalation, and the abdominal cavity is opened so that the uterine horns (containing individual decidua) may be removed into a dish with chilled 1× PBS containing 0.4% bovine serum albumin (PBS/BSA). Decidua are dissected from the uterus with watchmaker forceps in PBS/BSA. Individual decidua are then dissected to remove the embryos and their surrounding yolk sacs. The latter are removed and saved individually for genotyping by PCR. The thin amniotic membrane surrounding the embryo is also removed, after which embryos are fixed in 4% paraformaldehyde.

For *in situ* hybridization (ISH), embryos are fixed overnight (o/n) at 4°C. For whole-mount ISH, embryos are washed 3× [5 min each at room temperature (r.t.)] in DEPC-treated PBSw and dehydrated through a methanol series (25, 50, and 75% MeOH in DEPC-PBSw, 5 min each), washed twice (5 min each) in 100% MeOH, and stored in MeOH at -20°C until subjected to ISH. For section ISH, embryos are washed 3× (5 min each) in DEPC-treated PBS (DEPC-PBS), immersed o/n in 30% sucrose (prepared in DEPC-PBS) at 4°C, and then embedded in Tissue-Tek O.C.T. mounting medium in plastic molds. Embryos are positioned upright in the O.C.T. for transverse sections through the spinal neural tube and the molds are transferred to dry ice to freeze. Blocks prepared this way may be stored at -80°C for at least 2 years.

For IF, embryos are fixed in 4% paraformaldehyde for 2 h at 4°C, washed twice in PBS (5 min each) and then immersed o/n in 30% sucrose solution in PBS at 4°C. The following day, embryos are embedded in O.C.T. blocks as described above, and stored at -80°C.

Ten- to twelve-micrometer-thick sections of embryos for section ISH or IF are prepared using a cryostat (e.g., Leica CM3000). Sections should be collected on positively charged microscopy slides (e.g., Superfrost Plus, Fisher Scientific, Pittsburgh, PA, USA), labeled, and stored in slide boxes. Slides prepared this way may be stored for at least 1 month before analysis.

B. Immunofluorescence Analysis

1. Slides are thawed and air dried at r.t. (20 min) and immersed in wash buffer with goat serum (see *Materials*) for 10 min to block and wash away O.C.T. For staining with primary antibodies raised in goats (e.g., α -Olig2), wash buffer with horse serum should be used throughout.
2. Primary antibodies (see *Materials*) are diluted in wash buffer, preparing 150 μ l per slide. Multiple antibodies may be used together if they are raised in different

species. Slides are placed in humidified flat chambers, antibody solution is added to cover sections, and chambers are incubated o/n at 4°C.

3. Slides are washed 3× (20 min each) at r.t. (with agitation).
4. Fluorescently-labeled (e.g., Cy2- or Cy3-conjugated) secondary antibodies (see *Materials*) are diluted in wash buffer, which includes DAPI (at 200 ng/ml). The secondary antibody/DAPI solution is added (150 µl/slide) to slides in flat, humidified chambers, which are covered in foil, and the slides are incubated for 1 h at r.t.
5. Slides are washed as in step 3.
6. Slides are mounted with cover slips in fluorescence mounting medium (FMM) and stored at 4°C until imaging.
7. Imaging of neural patterning markers is performed with a conventional compound fluorescent microscope (e.g., Nikon E800) using 10 or 20× objectives with appropriate excitation/emission filters for DAPI, Cy2, and Cy3 (355-, 492-, and 550-nm peak excitation, respectively).

C. Immunostaining of Primary Cilia

Primary cilia may be stained in embryo sections using the protocol described above (see *Materials* for description of antibodies). Acetylated alpha tubulin is a commonly used marker for cilia. However, it is difficult to use it for staining cilia of the neural progenitors, as these cilia stain weakly with this marker, while there is intense staining throughout the cell body. For this reason, we have chosen to image primary cilia on cells of the lateral plate mesoderm (Fig. 2). Due to their small size (~1 µm long), primary cilia are best imaged by confocal microscopy using 60 or 100× (1.4 NA) objectives. Here, we have used a Leica TCS SP5 microscope and Velocity software (Improvision, Waltham MA, USA) and we have shown 5-µm-thick Z-stack projections.

D. ISH: Probe Preparation

Digoxigenin-labeled riboprobes are synthesized from plasmid templates (see *Materials*) using a digoxigenin-UTP labeling mix (Roche, Branford, CT, USA) following the protocol provided by the manufacturer. Briefly, plasmids are linearized at the 5' ends of the inserts (for generating antisense probes) and 20 µl *in vitro* transcription reactions are set up using SP6, T7, or T3 RNA polymerases and DIG labeling mix with 1 µl RNasin for 2 h at 37°C. One microliter of RNase-free DNase is added to the reactions (to digest template DNA), and the reaction is incubated for an additional 15 min at 37°C. Two microliters of the reaction is combined with loading buffer, heated at 95°C for 5 min, and run with a DNA ladder on an agarose gel to check synthesis. Probes are precipitated by the addition of 1 µl glycogen (20 mg/ml), 2 µl 0.2 M EDTA, 2.5 µl 4M LiCl, and 75 µl ethanol, followed by incubation at -20°C for 2 h and centrifugation for 15 min. The pellets are washed with 75% ethanol, air dried, and the probes are resuspended in DEPC-treated dH₂O. The probe concentration is determined with a spectrophotometer.

E. Section ISH Protocol

We follow a modified version of previously developed protocols for section ISH (Schaeren-Wiemers and Gerfin-Moser, 1993) as follows: Unless otherwise stated, all procedures are performed at r.t.

1. Slides prepared for section ISH are thawed and air dried for 20 min.

2. Fixation: slides are placed in a rack and fixed in 4% paraformaldehyde for 10 min, then washed 3× (5 min each) in DEPC-PBS.
3. Acetylation: 0.63 ml acetic anhydride is added to acetylation buffer. The slides are immediately immersed in the solution and incubated for 10 min, followed by a quick rinse in DEPC-treated PBS.
4. Permeabilization: slides are incubated in 1% Triton X-100 (in DEPC-PBS) for 30 min, then washed 3× (5 min each) with DEPC-PBS.
5. Prehybridization: hybridization buffer A is added to cover sections (0.2–0.3 ml/slide) in trays prepared with paper towels soaked in DEPC-treated 5× SSC (for humidification). The trays are covered and incubated at r.t. o/n.
6. Hybridization: the riboprobes are diluted at 200–400 ng/ml in hybridization buffer A, heated to 80°C for 5 min, and then placed on ice. A sense riboprobe is included to control for nonspecific hybridization signals. Probe solution (0.1 ml/slide) is added and coverslips carefully applied to avoid air bubbles. It is best to use a new box of coverslips for each experiment to avoid RNase contamination. Slides are placed in trays prepared with paper towels soaked in 50% formamide/5× SSC solution (for humidification). Trays are carefully taped shut with electrical tape to make them air tight and then placed o/n in an oven set to 72°C.
7. Washes and blocking: Slides are removed from trays and individually immersed in 5× SSC (preheated to 72°C) to allow coverslips to gently slide off (avoid applying pressure). Slides are moved to a rack in preheated 0.2× SSC and incubated for 1 h at 72°C. Slides are then transferred to 0.2× SSC at r.t. for 5 min, followed by incubation in B1 solution (5 min at r.t.).
8. Antibody labeling and developing: Slides are moved to humidified trays, B2a solution (1 ml/slide) is added, and slides are blocked for 1 h at r.t. Blocking solution is removed and replaced with B2b solution (0.5 ml/slide) and slides are incubated o/n at 4°C. Slides are washed 3× (5 min each) in B1 solution, followed by one wash (5 min) in B3 solution. B3 solution is removed by blotting, 90 µl B4 solution is added per slide, and coverslips are carefully applied. Slides are moved to humidified trays, covered, and incubated for 6–24 h.
9. Upon inspection, the staining is stopped by immersion in 1× TE (10 mM Tris pH8.0, 1 mM EDTA). Slides are then mounted with coverslips in 80% glycerol and imaged using a dissecting microscope/digital camera (e.g., Leica MZ16 fitted with Optonics digital camera).

F. Whole-Mount ISH Protocol

We follow a modified version of previously developed protocols for whole-mount ISH developed by Domingos Henrique and David Ish-Horowitz as follows: Unless otherwise stated, all procedures are performed at r.t.

1. Embryos are transferred in MeOH to mesh-bottomed “baskets” (see *Materials*) fitting in wells of 12-well culture plates. Solutions are changed by moving the baskets from one well to the next.
2. Embryos are rehydrated through a series of 5-min washes in 75, 50, and 25% MeOH (in PBSw) and then washed 3× (5 min) in PBSw. Embryos are then incubated in freshly diluted 10 µg/ml proteinase K in PBSw for 12 min, washed 2× (5 min each) in PBSw containing 2 mg/ml glycine, washed 3× (5 min each) in PBSw, fixed for 15 min in 4% paraformaldehyde/0.2% glutaraldehyde prepared in PBSw, and washed 3× (5 min each) in PBSw.

3. Embryos are washed 5 min in PBSw/hybridization buffer B (1:1 mix), 5 min in 100% hybridization buffer B, then transferred to 3 ml fresh 100% hybridization buffer B. Plates are taped shut with electrical tape and incubated at 70°C for at least 3 h (prehybridization).
4. 200 ng of riboprobe is added to 0.1 ml hybridization buffer B, heated at 95°C for 5 min, then cooled on ice. The riboprobe solution is then added to prehybridized embryos, the plates are taped shut, and the embryos are hybridized o/n at 70°C.
5. Baskets are transferred to prewarmed (70°C) fresh hybridization buffer B (without probe) and incubated 5 min at 70°C; 1.6 ml hybridization buffer B + 0.75 ml 2X SSC (pH 4.5) is then added to the wells and incubated for 5 min at 70°C. 0.75 ml more 2× SSC (pH 4.5) is added and then incubated again (5 min at 70°C); 0.75 ml more 2× SSC (pH 4.5) is then added, followed by another incubation (5 min at 70°C).
6. Baskets are transferred to 2× SSC (pH7.0)/0.1% CHAPS and washed twice for 30 min each at 70°C. Embryos are then equilibrated for 5 min at r.t. in maleic acid buffer (MAB), washed 2× (30 min each) in MAB at 70°C, washed 2 in PBS at r.t., and then washed 1× (5 min) in PBSw.
7. Embryos are incubated in blocking solution for 2 h at 4°C with agitation on a nutator. Blocking solution is then replaced with AP-conjugated α -digoxigenin antibody (Roche) diluted 1:2000 in blocking solution and incubated o/n at 4°C on a nutator.
8. Embryos are rinsed once and then washed 5× (45 min each) in PBSw + 0.1% BSA at r.t. Embryos are then washed 2× (30 min each) in PBSw, then 2× in B3 solution for 10 min.
9. Embryos are incubated in B4 solution in the dark until signal is sufficiently strong (4–18 h). Staining is stopped by washing the embryos 3× (5 min each) in PBSw, followed by stepwise dehydration through a MeOH series (25, 50, 75, and 100%, made in PBSw, 5 min each).
10. Embryos are imaged using a dissecting microscope/digital camera (e.g., Leica MZ16 fitted with Optronics digital camera). Dehydrated embryos may be stored at –20°C for at least 1 month.

IV. Materials

A. Dissection/Fixation/Embedding

Dissection buffer: 1× PBS 0.4% BSA. Chill to 4°C.

Fixative to: 4% Paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA, Cat. #6148) made in 1× PBS (heat to 60°C to dissolve). Store in 10-ml aliquots at –20°C. Thaw to room temperature just before use.

Embedding: Tissue-Tek O.C.T. mounting medium (Sakura Finetek, Torrance, CA, USA, Cat. #4583), plastic embedding molds (Peel-A-Way, Polysciences, Warrington, PA, USA, Cat. #18986).

B. Immunofluorescence

Wash buffer: 1× PBS + 0.1% Triton X-100 and 1% heat-inactivated goat serum (HINGS) or horse serum (HIHS). Heat inactivate serum at 55°C for 25 min.

DAPI: Make 500× stock of 4',6-diamidino-2-phenylindole (Sigma Aldrich, Cat. #D9542, 100 µg/ml in dH₂O). Store as aliquots at −20°C.

FMM: 90 ml glycerol, 10 ml 10× PBS, 5 g *n*-propyl gallate (Sigma Aldrich, Cat. #P3130). Store at 4°C.

Primary antibodies: Mouse monoclonal antibodies against Shh (clone 5E1), Nkx2.2 (74.5A5), FoxA2 (4C7), Nkx6.1 (F55A10), and Msx1/2 (4G1) are available from the Developmental Studies Hybridoma Bank (DSHB), University of Iowa. Tissue culture supernatants are typically used at a 1:10 dilution. Rabbit anti-Pax6 and goat anti-Olig2 polyclonal antibodies are from Covance (Berkeley, CA, USA, Cat. #PRB-278P, use at 1:1000) and R&D Systems (Minneapolis, MN, USA, Cat. #AF2418, use at 1:200), respectively. Other useful antibodies for neural patterning analysis include α-Pax7 (DSHB, Pax7), α-Dbx2 (Abcam, Cambridge, MA, USA, ab25554), α-MNR2/HB9 (DSHB, 81.5C10), α-Isl1/2 (DSHB, 39.4D5), α-Lhx3 (DSHB, 67.4E12), α-En1 (DSHB, 4G11), and α-Evxl (DSHB, 99.1-3A2). Primary cilia are stained with mouse antiacetylated alpha tubulin monoclonal antibodies from Sigma (Cat. #T6793), rabbit α-IFT88 polyclonal antiserum (generous gift from Dr Gregory Pazour, used at 1:1000), and rabbit α-Arl13b polyclonal antiserum (generous gift from Dr Tamara Caspary, used at 1:3000).

Secondary antibodies: Donkey Cy3-labeled antimouse IgG (Cat. #715-165-151), Donkey Cy2-labeled antirabbit IgG (Cat. #711-225-152), donkey Cy2-labeled antigoat IgG (Cat. #705-225-147) from Jackson ImmunoResearch (West Grove, PA, USA).

C. Riboprobes

Reagents for riboprobes synthesis: T3, T7, or SP6 polymerases (Roche), 10× DIG labeling mix (Roche, Cat. #11277073910) and RNasin (Promega, Madison, WI, USA, Cat. #N251A), Rnase-free DNase (Roche, Cat. #776785).

Probe templates: Plasmids used for synthesis are *Shh* (Echelard *et al.*, 1993), *Gli1* (Hui *et al.*, 1994), *Ptch1* (Goodrich *et al.*, 1996), *Gli3* (Bulgakov *et al.*, 2004), *Dbx1* (Takahashi and Osumi, 2002), *Irx3* (Bosse *et al.*, 1997), *Hoxd11* (Dolle *et al.*, 1989), *Hoxd13* (Dolle *et al.*, 1989), *Fgf4* (Hebert *et al.*, 1990), and *Grem1* (Pearce *et al.*, 1999).

D. Solutions/Reagents for ISH (whole mount and sections)

Baskets for whole mount ISH: 15 mm Netwell Insert, Corning, Lowell, MA, USA, Cat. #3478

DEPC-treated solutions: add 1 ml diethyl pyrocarbonate (Sigma Aldrich, Cat. #D5758) per liter solution, stir o/n at 37°C, autoclave to inactivate DEPC.

PBSw: DEPC-treated 1× PBS + 0.1% Tween-20.

Hybridization buffer A (sections): 50% formamide, 5× SSC, 5× Denhardt's solution (Invitrogen, Carlsbad, CA, USA), 0.25 mg/ml Bakers yeast RNA (Sigma Aldrich), 0.5 mg/ml boiled/sonicated Salmon Sperm DNA (Sigma Aldrich). All stock solutions made in DEPC-treated dH₂O.

Hybridization buffer B (whole mount): Sock solutions should be made in DEPC-treated dH₂O. For 50 ml, combine 25 ml formamide, 12.5 ml 20× SSC (pH 7.0), 0.5 g Boehringer Blocking Reagent (Roche, Cat. #109676). Heat at 65°C for 1 h with stirring to dissolve. Add 6 ml DEPC-dH₂O, 5 ml 10 mg/ml Bakers yeast RNA (Sigma Aldrich), 100 µl 50 mg/ml

heparin (Sigma Aldrich), 250 μ l 20% Tween-20, 500 μ l 10% CHAPS, 500 μ l 0.5 M EDTA. Filter solution and store at -20°C .

Acetylation buffer: 246 ml DEPC-treated dH_2O , 3.3 ml triethanolamine (Sigma Aldrich), 0.44 ml concentrated HCL. Add 0.625 ml acetic anhydride (Sigma Aldrich) and mix immediately before use.

MAB: 100 mM maleic acid, 150 mM NaCl, pH 7.5.

Blocking Solution: Dissolve Boehringer Blocking Reagent to 1% in PBSw with 10% HINGS by heating to 65°C and vortexing repeatedly. Store at -20°C .

B1 solution: 0.1 M Tris-HCl pH 7.5, 0.15 M NaCl.

B2a solution: B1 solution + 10% HINGS.

B2b solution: B1 solution: + 1% HINGS, and alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche, Cat. #11093274910) diluted 1:1000.

B3 solution: 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM MgCl_2 .

B4 solution: B3 solution + 5 μ l/ml Nitro blue tetrazolium chloride (NBT, Roche, Cat. #11383213001), 3.75 μ l/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Roche, Cat. #11383221001).

V. Summary

Developmental geneticists studying mammalian Hh signaling and cell biologists studying IFT and ciliogenesis have been unexpectedly brought together by recent findings. As a result, both groups are expanding their research efforts to explore the fascinating connection between the two systems. Here, we have attempted to provide a framework for analyzing Shh signaling in mouse tissues for those cell biologists less familiar with the topic. Due to the complexities of the Shh signaling pathway, the patterning phenotypes of mutants defective for IFT components are complex and distinct from one another, as illustrated by the examples shown here. However, this very complexity provides clues for understanding how Shh signal transduction components interact with one another in the context of the highly organized structure of the primary cilium.

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References

- Blacque OE, Li C, Inglis PN, Esmail MA, Ou G, Mah AK, Baillie DL, Scholey JM, Leroux MR. The WD repeat-containing protein IFTA-1 is required for retrograde intraflagellar transport. *Mol. Biol. Cell* 2006;17:5053–5062. [PubMed: 17021254]
- Bosse A, Zulch A, Becker MB, Torres M, Gomez-Skarmeta JL, Modolell J, Gruss P. Identification of the vertebrate Iroquois homeobox gene family with overlapping expression during early development of the nervous system. *Mech. Dev* 1997;69:169–181. [PubMed: 9486539]

- Briscoe J, Pierani A, Jessell TM, Ericson J. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* 2000;101:435–445. [PubMed: 10830170]
- Briscoe J, Sussel L, Serup P, Hartigan-O'Connor D, Jessell TM, Rubenstein JL, Ericson J. Homeobox gene *Nkx2.2* and specification of neuronal identity by graded Sonic hedgehog signalling. *Nature* 1999;398:622–627. [PubMed: 10217145]
- Bulgakov OV, Eggenschwiler JT, Hong DH, Anderson KV, Li T. FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues. *Development* 2004;131:2149–2159. [PubMed: 15105374]
- Caspary T, Larkins CE, Anderson KV. The graded response to Sonic Hedgehog depends on cilia architecture. *Dev. Cell* 2007;12:767–778. [PubMed: 17488627]
- Cho A, Ko HW, Eggenschwiler JT. FKBP8 cell-autonomously controls neural tube patterning through a Gli2- and Kif3a-dependent mechanism. *Dev. Biol* 2008;321:27–39. [PubMed: 18590716]
- Cole DG, Diener DR, Himelblau AL, Beech PL, Fuster JC, Rosenbaum JL. Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons. *J. Cell Biol* 1998;141:993–1008. [PubMed: 9585417]
- Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate Smoothed functions at the primary cilium. *Nature* 2005;437:1018–1021. [PubMed: 16136078]
- Dessaud E, McMahon AP, Briscoe J. Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* 2008;135:2489–2503. [PubMed: 18621990]
- Dolle P, Izpisua-Belmonte JC, Falkenstein H, Renucci A, Duboule D. Coordinate expression of the murine *Hox-5* complex homoeobox-containing genes during limb pattern formation. *Nature* 1989;342:767–772. [PubMed: 2574828]
- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP. Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 1993;75:1417–3140. [PubMed: 7916661]
- Eggenschwiler JT, Anderson KV. Cilia and developmental signaling. *Annu. Rev. Cell Dev. Biol* 2007;23:345–373. [PubMed: 17506691]
- Epstein DJ, McMahon AP, Joyner AL. Regionalization of Sonic hedgehog transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms. *Development* 1999;126:281–292. [PubMed: 9847242]
- Ericson J, Rashbass P, Schedl A, Brenner-Morton S, Kawakami A, van Heyningen V, Jessell TM, Briscoe J. *Pax6* controls progenitor cell identity and neuronal fate in response to graded *Shh* signaling. *Cell* 1997;90:169–180. [PubMed: 9230312]
- Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP. Conservation of the hedgehog/patched signaling pathway from flies to mice: Induction of a mouse patched gene by Hedgehog. *Genes Dev* 1996;10:301–312. [PubMed: 8595881]
- Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK. Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet* 2005;1:e53. [PubMed: 16254602]
- Hebert JM, Basilico C, Goldfarb M, Haub O, Martin GR. Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol* 1990;138:454–463. [PubMed: 2318343]
- Hoover AN, Wynkoop A, Zeng H, Jia J, Niswander LA, Liu A. *C2cd3* is required for cilia formation and Hedgehog signaling in mouse. *Development* 2008;135:4049–4058. [PubMed: 19004860]
- Huangfu D, Anderson KV. Cilia and Hedgehog responsiveness in the mouse. *Proc. Natl. Acad. Sci. USA* 2005;102:11325–11330. [PubMed: 16061793]
- Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* 2003;426:83–87. [PubMed: 14603322]
- Hui CC, Slusarski D, Platt KA, Holmgren R, Joyner AL. Expression of three mouse homologs of the *Drosophila* segment polarity gene *cubitus interruptus*, *Gli*, *Gli-2*, and *Gli-3*, in ectoderm- and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Dev. Biol* 1994;162:402–413. [PubMed: 8150204]

- Jiang J, Hui CC. Hedgehog signaling in development and cancer. *Dev. Cell* 2008;15:801–812. [PubMed: 19081070]
- Liem KF Jr, Jessell TM, Briscoe J. Regulation of the neural patterning activity of sonic hedgehog by secreted BMP inhibitors expressed by notochord and somites. *Development* 2000;127:4855–4866. [PubMed: 11044400]
- Liu A, Wang B, Niswander LA. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. *Development* 2005;132:3103–3111. [PubMed: 15930098]
- Marszalek JR, Ruiz-Lozano P, Roberts E, Chien KR, Goldstein LS. Situs inversus and embryonic ciliary morphogenesis defects in mouse mutants lacking the KIF3A subunit of kinesin-II. *Proc. Natl. Acad. Sci. USA* 1999;96:5043–5048. [PubMed: 10220415]
- Marti E, Bumcrot DA, Takada R, McMahon AP. Requirement of 19K form of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* 1995;375:322–325. [PubMed: 7753196]
- May SR, Ashique AM, Karlen M, Wang B, Shen Y, Zarbalis K, Reiter J, Ericson J, Peterson AS. Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. *Dev. Biol* 2005;287:378–389. [PubMed: 16229832]
- McGlenn E, Tabin CJ. Mechanistic insight into how Shh patterns the vertebrate limb. *Curr. Opin. Genet. Dev* 2006;16:426–432. [PubMed: 16806898]
- Murcia NS, Richards WG, Yoder BK, Mucenski ML, Dunlap JR, Woychik RP. The Oak Ridge Polycystic Kidney (orpk) disease gene is required for left–right axis determination. *Development* 2000;127:2347–2355. [PubMed: 10804177]
- Nissim S, Hasso SM, Fallon JF, Tabin CJ. Regulation of Gremlin expression in the posterior limb bud. *Dev. Biol* 2006;299:12–21. [PubMed: 16989805]
- Niswander L, Jeffrey S, Martin GR, Tickle C. A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* 1994;371:609–612. [PubMed: 7935794]
- Norman RX, Ko HW, Huang V, Eun CM, Abler LL, Zhang Z, Sun X, Eggenschwiler JT. Tubby-like protein 3 (TULP3) regulates patterning in the mouse embryo through inhibition of Hedgehog signaling. *Hum. Mol. Genet* 2009;18:1740–1754. [PubMed: 19286674]
- Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. *Mol. Cell Biol* 2006;26:3365–3377. [PubMed: 16611981]
- Pearce JJ, Penny G, Rossant J. A mouse cerberus/Dan-related gene family. *Dev. Biol* 1999;209:98–110. [PubMed: 10208746]
- Pedersen LB, Rosenbaum JL. Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. *Curr. Top. Dev. Biol* 2008;85:23–61. [PubMed: 19147001]
- Pierani A, Brenner-Morton S, Chiang C, Jessell TM. A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* 1999;97:903–915. [PubMed: 10399918]
- Piperno G, Siuda E, Henderson S, Segil M, Vaananen H, Sassaroli M. Distinct mutants of retrograde intraflagellar transport (IFT) share similar morphological and molecular defects. *J. Cell Biol* 1998;143:1591–1601. [PubMed: 9852153]
- Robert B, Lallemand Y. Anteroposterior patterning in the limb and digit specification: contribution of mouse genetics. *Dev. Dyn* 2006;235:2337–2352. [PubMed: 16894622]
- Roelink H, Porter JA, Chiang C, Tanabe Y, Chang DT, Beachy PA, Jessell TM. Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* 1995;81:445–455. [PubMed: 7736596]
- Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. *Science* 2007;317:372–376. [PubMed: 17641202]
- Ruiz-Perez VL, Blair HJ, Rodriguez-Andres ME, Blanco MJ, Wilson A, Liu YN, Miles C, Peters H, Goodship JA. Evc is a positive mediator of Ihh-regulated bone growth that localises at the base of chondrocyte cilia. *Development* 2007;134:2903–2912. [PubMed: 17660199]
- Schaeren-Wiemers N, Gerfin-Moser A. A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: *In situ* hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 1993;100:431–440. [PubMed: 7512949]

- Takahashi M, Osumi N. Pax6 regulates specification of ventral neurone subtypes in the hindbrain by establishing progenitor domains. *Development* 2002;129:1327–1338. [PubMed: 11880342]
- Takebayashi H, Ohtsuki T, Uchida T, Kawamoto S, Okubo K, Ikenaka K, Takeichi M, Chisaka O, Nabeshima Y. Non-overlapping expression of Olig3 and Olig2 in the embryonic neural tube. *Mech. Dev* 2002;113:169–174. [PubMed: 11960707]
- Taulman PD, Haycraft CJ, Balkovetz DF, Yoder BK. Polaris, a protein involved in left-right axis patterning, localizes to basal bodies and cilia. *Mol. Biol. Cell* 2001;12:589–599. [PubMed: 11251073]
- te Welscher P, Zuniga A, Kuijper S, Drenth T, Goedemans HJ, Meijlink F, Zeller R. Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* 2002;298:827–830. [PubMed: 12215652]
- Tran PV, Haycraft CJ, Besschetnova TY, Turbe-Doan A, Stottmann RW, Herron BJ, Chesebro AL, Qiu H, Scherz PJ, Shah JV, Yoder BK, Beier DR. THM1 negatively modulates mouse sonic hedgehog signal transduction and affects retrograde intraflagellar transport in cilia. *Nat. Genet* 2008;40:403–410. [PubMed: 18327258]
- Vierkotten J, Dildrop R, Peters T, Wang B, Ruther U. Ftm is a novel basal body protein of cilia involved in Shh signalling. *Development* 2007;134:2569–2577. [PubMed: 17553904]
- Vokes SA, Ji H, Wong WH, McMahon AP. A genome-scale analysis of the cis-regulatory circuitry underlying sonic hedgehog-mediated patterning of the mammalian limb. *Genes Dev* 2008;22:2651–2663. [PubMed: 18832070]
- Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. *Cell* 2000;100:423–434. [PubMed: 10693759]
- Zakany J, Kmita M, Duboule D. A dual role for Hox genes in limb anterior-posterior asymmetry. *Science* 2004;304:1669–1672. [PubMed: 15192229]
- Zuniga A, Haramis AP, McMahon AP, Zeller R. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* 1999;401:598–602. [PubMed: 10524628]

Fig. 1.

The Shh signaling pathway. Hedgehog ligands, such as Sonic hedgehog (Shh), bind to and inhibit the Patched receptor on the cell surface. The binding prevents Patched from antagonizing a second transmembrane protein, Smoothened. Once activated, Smoothened regulates the Gli transcription factors through a mechanism that is poorly understood. Active Smoothened prevents the generation of the Gli transcriptional repressor forms (GliRep) and promotes the activity of the Gli activator forms (GliAct). The IFT machinery acts in several different ways. For example, kinesin II and the complex B IFT proteins are required for GliAct function and for the formation of GliRep, whereas complex A IFT proteins such as Ift122 are required to restrict GliAct function in the absence of ligands.

Fig. 2.

Ciliogenesis in mouse IFT mutants. (A–D) Primary cilia in lateral plate mesenchyme tissues in wild-type, *Ift122^{sopb}*, *Ift88^{fxo/-}*, and *Kif3a^{-/-}* embryos labeled with anti-Arl13b antibodies (green). Nuclei are counterstained with DAPI, blue. Cilia are generated at a reduced frequency in *Ift88^{fxo/-}* mutants and are undetectable in *Kif3a^{-/-}* mutants. (E–L) Higher magnification images of cilia double labeled with antibodies against acetylated α -tubulin (red) and Arl13b (green, E–H) or acetylated α -tubulin (red) and IFT88 (green, I–L). Note that Arl13b and IFT88 show accumulation at *Ift122^{sopb}* mutant cilia tips (white arrowheads). Some weak IFT88 staining can be seen at the base and within the axonemes of *Ift88^{fxo/-}* mutant cilia. No cilia are observed using these antibodies in *Kif3a^{-/-}* mutant samples. Scale bars are 10 μ m in A–D and 1 μ m in (E–L). (See Plate no. 13 in the Color Plate Section.)

Fig. 3.

Summary of gene expression patterns in the neural tube. Shh is expressed in the notochord (nc) and induces its own expression in the floor plate (fp). A ventral-to-dorsal gradient of Shh protein controls the spatial expression patterns of downstream targets. Targets may be induced by Shh signaling (e.g., FoxA2, Nkx2.2 and Olig2), inhibited by Shh signaling (e.g., Irx3 and Msx1/2), or induced by weak Shh signaling, yet repressed by strong Shh signaling (e.g., Pax6 and Dbx1).

Fig. 4.

Analysis of neural patterning by immunohistochemistry. Neural patterning is analyzed in transverse sections through the neural tubes of E10.5 (A–C, F–H, K–M, P–R, and U–W) and E9.5 (D, E, I, J, N, O, S, T, X, Y) embryos. (A–E) Shh (red) staining normally marks the notochord (arrow in A) and neural tube floor plate, which also expresses FoxA2 (red, F–J). The floor plate is expanded in *Ift122^{sopb}* mutants and is missing in *Kif3a^{-/-}* and *Ift88^{fxo/-}* mutants. (K–O) Nkx2.2 (red) labels p3 cells, which are expanded in *Ift122^{sopb}* mutants and missing in *Kif3a^{-/-}* and *Ift88^{fxo/-}* mutants, and Olig2 (green) labels motor neuron progenitors, which are dorsally shifted in *Ift122^{sopb}* mutants, missing in *Kif3a^{-/-}* mutants, and ventrally expanded at the expense of p3 cells in *Ift88^{fxo/-}* mutants. (P–T) Nkx6.1 (red) is expressed in several classes of ventral progenitors, which are expanded in *Ift122^{sopb}* mutants, largely missing in *Kif3a^{-/-}* mutants, and variably lost in *Ift88^{fxo/-}* mutants. Pax6 (green) is expressed strongly in lateral neural progenitors, which are dorsally shifted in *Ift122^{sopb}* mutants, and ventrally expanded to varying extents in *Kif3a^{-/-}* and *Ift88^{fxo/-}* mutants. (U–Y) Msx1/2 staining marks dorsal progenitors and is largely absent in *Ift122^{sopb}* mutant neural tubes. All neural tube sections are shown dorsal side up and are counterstained with DAPI (blue) to highlight their contours. (See Plate no. 14 in the Color Plate Section.)

Fig. 5.

Neural patterning markers analyzed by section *in situ* hybridization. *In situ* hybridization for neural patterning markers is an alternative to the analysis by immunofluorescence. The expression of two homeobox transcription factors, *Irx3* (A–E) and *Dbx1* (F–J) at E10.5 and E9.5 is shown for the different genotypes. Both genes are repressed by intermediate-to-high levels of Shh signaling and show dorsally shifted or ventrally expanded patterns in *Ift122^{sopb}* and *Kif3a^{-/-}* mutants, respectively. Neural tube sections are shown dorsal side up. (See Plate no. 15 in the Color Plate Section.)

Fig. 6.

Expression of Shh signaling components in IFT mutants. Neural tube sections analyzed by *in situ* hybridization at E9.5 and E10.5. *Shh* expression (A–E) is expanded in the neural tube of *Ift122^{sopb}* mutants (B), and is limited to the notochord in *Ift88^{fxo/-}* (C) and *Kif3a^{-/-}* mutants (E). *Shh* induces the expression of *Gli1* (F–J) and *Ptch1* (K–O) in the ventral neural tube. The expression of these targets is dorsally expanded or shifted in *Ift122^{sopb}* mutants (G, L) due to hyperactivation of the Shh pathway, whereas they are downregulated/ventrally confined in *Ift88^{fxo/-}* mutants (H, M) and expressed at basal levels in *Kif3a^{-/-}* mutants (J, O). *Gli3* expression is limited to the dorsal neural tube due to repression by Shh signaling. The residual pathway activity in *Ift88^{fxo/-}* (R) and *Kif3a^{-/-}* (T) mutants is sufficient to restrict *Gli3* expression dorsally, while the increased activity in *Ift122^{sopb}* mutants downregulates *Gli3* in the dorsal neural tube (Q). Neural tube sections are shown dorsal side up. (See Plate no. 16 in the Color Plate Section.)

Fig. 7.

In situ hybridization analysis of IFT mutant limb buds. *Hoxd11* (A–C), *Hoxd13* (D–F), and *Grem1* (J–L) are induced to different extents in the posterior limb bud mesenchyme in response to Shh produced at the posterior pole. Shh induces these genes primarily by blocking the production of Gli3Rep. In *Ift88^{fxo/-}* mutants, Gli3Rep production is diminished, leading to expansion of these markers toward the anterior side (B, E, K, arrows). *Ift122^{sopb}* limb buds are small and abnormally shaped, yet show a similar anterior expansion of *Hoxd11*, *Hoxd13*, and *Grem1* expression (C, F, L, arrows). Gremlin1 upregulates *Fgf4* expression (G–I) in the limb bud AER tissue overlying the mesenchyme. As a result of expanded *Grem1* expression, *Fgf4* expression expands anteriorly in *Ift88^{fxo/-}* and *Ift122^{sopb}* mutants. Forelimb buds from E10.5 embryos are shown with anterior side up. (See Plate no. 17 in the Color Plate Section.)

Fig. 8.

Expression of Shh signaling components in IFT mutant limb buds. *Shh* (A–C) is expressed at the posterior pole of the limb bud mesenchyme in wild-type, *Ift88^{fxo/-}*, and *Ift122^{sopb}* mutants. *Gli1* and *Ptch1* are normally induced in broad posterior domains in response to Shh signaling (D, G). Their expression is thought to be driven primarily by GliAct activity. As a result of diminished GliAct function in *Ift88^{fxo/-}* mutants, both genes are downregulated and restricted posteriorly in *Ift88^{fxo/-}* mutants. In contrast, these genes show expanded domains of expression in response to increased activity of GliAct in *Ift122^{sopb}* mutants (F, I). *Gli3* is repressed by high levels of Shh signaling, which sets the posterior limit of its expression domain (J, arrowhead). The posterior limit of *Gli3* expression is slightly shifted posteriorly in *Ift88^{fxo/-}* mutants (K, arrowhead), whereas this limit is shifted anteriorly in *Ift122^{sopb}* mutants (L, arrowhead). Forelimb buds from E10.5 embryos are shown with anterior side up. (See Plate no. 18 in the Color Plate Section.)