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The IFT-A complex regulates Shh signaling through cilia structure and membrane protein trafficking

Karel F. Liem Jr., Alyson Ashe, Mu He, Peter Satir, Jennifer Moran, David Beier, Carol Wicking, and Kathryn V. Anderson

Two intraflagellar transport (IFT) complexes, IFT-A and IFT-B, build and maintain primary cilia and are required for activity of the Sonic hedgehog (Shh) pathway. A weak allele of the IFT-A gene, Ift144, caused subtle defects in cilia structure and ectopic activation of the Shh pathway. In contrast, strong loss of IFT-A, caused by either absence of Ift144 or mutations in two IFT-A genes, blocked normal ciliogenesis and decreased Shh signaling. In strong IFT-A mutants, the Shh pathway proteins Gli2, Sufu, and Kif7 localized correctly to cilia tips, suggesting that these pathway components were trafficked by IFT-B. In contrast, the membrane proteins Arl13b, ACIII, and Smo failed to localize to primary cilia in the absence of IFT-A. We propose that the increased Shh activity seen in partial loss-of-function IFT-A mutants may be a result of decreased ciliary ACIII and that the loss of Shh activity in the absence of IFT-A is a result of severe disruptions of cilia structure and membrane protein trafficking.

Introduction

The Shh signaling pathway is critical for development of nearly every organ system in vertebrate embryos and has important roles in tumorigenesis (Ingham et al., 2011). Recent studies have shown that Hedgehog (Hh) signaling in vertebrates, unlike in Drosophila melanogaster, depends on the primary cilium, a microtubule-based organelle that projects from the surface of most interphase cells (Goetz and Anderson, 2010). Two evolutionarily conserved intraflagellar transport (IFT) protein complexes, IFT-A and IFT-B, are required to build and maintain cilia structure (Cole, 2003; Pedersen et al., 2006). IFT-B proteins and Kinesin-II are required for anterograde trafficking from the base to the tip of the cilium, whereas the IFT-A complex is believed to work with the cytoplasmic dynein-2 motor to control retrograde ciliary trafficking (Pedersen and Rosenbaum, 2008).

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to pathway activation, including the Shh receptor Patched, the membrane protein Smoothened, the Kinesin-4 family member Kif7, the negative regulator Sufu, and the Gli transcription factors (Goetz and Anderson, 2010).

The roles of IFT-A proteins in ciliogenesis appear to be more complex than those of IFT-B proteins. Biochemical studies have identified six evolutionarily conserved proteins in the IFT-A complex in both Chlamydomonas reinhardtii and mammalian cells, IFT122, IFT144, IFT139 (Tc21b), IFT140, IFT121 (WDR35), IFT43, and IFT144 (WDR19) (Mukhopadhyay et al., 2010; Behal et al., 2012). A role for IFT-A in retrograde trafficking in cilia and flagella was first defined in the alga Chlamydomonas, where temperature-sensitive mutations in two Chlamydomonas IFT-A genes, fla15 (IFT144) and fla17 (IFT139), cause bulged flagella of normal length, associated with decreased rates of retrograde IFT (Iomini et al., 2001, 2009). Null mutations in mouse Ift139a/Tc21b and Ift122 cause similar phenotypes: null mutant cilia are of approximately normal length but have bulges at the tips of the axoneme, consistent with a defect in retrograde trafficking (Tran et al., 2008; Cortellino et al., 2009; Qin et al., 2011), and slower rates of retrograde trafficking were measured after knockdown of Ift139a (Tran et al., 2008). In contrast to the relatively mild ciliary defects of Ift139a and Ift122 mutants, null mutations in mouse Ift121/Wdr35, another IFT-A gene, lead to the formation of very short cilia (Mill et al., 2011). Caenorhabditis elegans null mutations in the IFT-A gene encoding IFT140 (che-11) allow formation of normal-length cilia that fail to function normally (Qin et al., 2001), whereas df-2 mutants, which lack WDR19/IFT144, make very short cilia, similar to those seen in IFT-B mutants (Efimenko et al., 2006). Thus, some mutants reveal a role for IFT-A proteins in retrograde ciliary trafficking, and others suggest that IFT-A has a role in building cilia. Despite the differences among the mouse and worm phenotypes, mutations in all six human IFT-A genes—Ift139a/TTC21b, Ift121/WDR35, IFT43, Ift122, IFT144/WDR19, and IFT140—have recently been shown to cause a set of related human genetic syndromes, including short-rib polydactyly, Jeune asphyxiating thoracic dystrophy, Sensenbrenner syndrome/cranioectodermal dysplasia, and Mainzer-Saldino syndrome (Gilissen et al., 2010; Arts et al., 2011; Bredrup et al., 2011; Davis et al., 2011; Mill et al., 2011; Perrault et al., 2012).

Paralleling the different effects on cilia structure, IFT-A proteins appear to have different roles in mouse Shh signaling than the IFT-B proteins. Whereas IFT-B–null mutants fail to respond to Shh, embryos that lack Ift139a/Tc21b and Ift122 show ectopic, ligand-independent activation of the Shh pathway in the neural tube. The basis of the inappropriate activation of the Shh pathway in the IFT-A mutants is not clear.

Here, we carry out studies that resolve the apparently disparate phenotypes caused by loss of different mouse IFT-A proteins. We first compare two different ethynitrosourea (ENU)-induced mutations that disrupt mouse IFT144, a core component of the mammalian IFT-A complex (Mukhopadhyay et al., 2010). We find that a partial loss-of-function Ift144 allele causes ectopic activation of the Shh pathway in the neural tube, similar to the phenotypes seen in mouse Ift122 and Ift139a mutants. In contrast, mutant embryos homozygous for a strong allele of Ift144 appear to have the opposite phenotype: neural cell types that require a high level Shh activity are not specified. We find that the structure of the weak Ift144 mutant cilia is relatively normal, whereas cilia of the null mutant are short and have highly disrupted axonemes. Analysis of double mutants carrying mutations in two different IFT-A genes confirms that strong loss of IFT-A disrupts ciliogenesis and blocks high-level activity of the Shh pathway. The data indicate that IFT-B is sufficient for trafficking of key proteins required for Shh signaling (Gli2, Sufu, and Kif7) into cilia. In contrast, we find that IFT-A is required for localization of a set of membrane-associated proteins to cilia, and we suggest how the loss of these membrane proteins may account for the Shh pathway phenotypes of both weak and strong IFT-A mutants.

**Results**

**Identification of two alleles of mouse Ift144/Wdr19**

We identified a recessive mouse mutant, diamondhead (dmhd), in an ENU mutagenesis screen based on altered expression of a transgenic motor neuron reporter gene (Liem et al., 2009). The HB9-GFP transgene is expressed throughout the spinal cord in motor neurons, a cell type that is specified by intermediate levels of Shh pathway activity (Fig. 1A). In the dmhd mutant, HB9-GFP was not expressed at rostral levels of spinal cord but was strongly expressed in caudal regions. Homozygous dmhd mutant embryos also had a set of defects in external morphology including exencephaly, with an open diamond-shaped forebrain, and left–right randomization of heart looping. The dmhd mutant embryos arrested at midgestation, at approximately embryonic day 10.5 (E10.5).

We used meiotic recombination mapping to localize the dmhd mutation on chromosome 5 (see Materials and methods). Because disruption of primary cilia can cause defects in both left–right asymmetry and Shh signaling, we examined candidate genes in the interval that might affect the formation of primary cilia. We identified a splice site mutation that was predicted to lead to a truncated protein in the gene encoding Wdr19/IFT144 (Fig. 2A), the homolog of the Chlamydomonas IFT144 and C. elegans Dyf-2, which are required for normal formation of flagella and cilia (Efimenko et al., 2006; Iomini et al., 2009). We were unable to detect wild-type Ift144 transcript by PCR from mutant embryo cDNA and could not detect IFT144 protein with a polyclonal antibody to the N-terminal domain of the protein by immunofluorescence (Fig. 2B), suggesting that dmhd is a strong loss-of-function or null allele. Consistent with disruption of an IFT protein, cilia structure was strongly disrupted in the dmhd mutant, and only very short cilia were formed (Fig. 2B).

A second allele of Ift144, twinkletoes (twt), was identified in an independent screen for ENU-induced mutations (Ash et al., 2012). twt homozygotes survived to the end of gestation, when they showed polydactyly and craniofacial abnormalities. The twt mutation was associated with a missense change in the first tetratricopeptide repeat of IFT144, but the mutation did not affect the amount of protein made (Fig. S1B; Ash et al., 2012). The twt mutants showed increased HB9-GFP expression.
Figure 1. Mutations in Ift144 alter Shh-dependent neural patterning. (A) The motor neuron marker H89-GFP (green) is not expressed in the rostral neural tube of Ift144<sup>dmhd</sup> embryos (arrow) but is strongly expressed in the caudal spinal cord. WT, wild type. (B) Ift144<sup>int</sup> mutants showed increased H89-GFP expression compared with wild-type littermates. (C) The two Ift144 alleles cause contrasting changes in dorsal-ventral neural patterning, shown in transverse sections at three rostral-caudal levels of the E10.5 neural tube. (D) Expression of the Shh target gene Pch1-lacZ in E10.5 Ift144 mutant and littermate control embryos at lumbar levels. Ift144<sup>dmhd</sup> mutants lack strong expression Pch1-lacZ at the ventral midline at all levels. Pch1-lacZ is ectopically expressed in the mesoderm adjacent to the neural tube in Ift144<sup>int</sup> mutants (arrows). Ift144<sup>int</sup> mutants show dorsally expanded Pch1-lacZ expression. Bars: (A and B) 1 mm; (C and D) 200 μm.

throughout the spinal neural tube (Fig. 1 B). In contrast to the very abnormal dmhd cilia, twt mutant cilia appeared nearly normal in structure, although IFT-140, another IFT-A protein, was not detectable in the twt mutant cilia (Fig. 2 B). dmhd/twt compound heterozygotes died at E12–13 with exencephaly and polydactyly, confirming that the two mutations disrupted the same gene. Because twt homozygotes survived longer than dmhd/twt embryos and because of the stronger disruption in cilia structure in dmhd than in twt, we concluded that twt is a hypomorphic allele of the gene. Therefore, we refer to the dmhd mutation as Ift144<sup>dmhd</sup> and twt as Ift144<sup>int</sup>.

Weak and strong alleles of Ift144 have opposing effects on specification of Shh-dependent ventral neural cell types

To define how IFT144 affects Shh signaling, we examined neural tube patterning in the mutants. Neural patterning in Ift144<sup>dmhd</sup> embryos was unlike that of other previously characterized IFT mutants. Null mutations in two other IFT-A genes, Ift122 and Ift139a, cause a ventralization of the neural tube, in which the most ventral cell types that require the highest level of Shh (the floor plate and V3 interneuron progenitors) are expanded as a result of elevated ectopic activity of the Shh pathway (Tran et al., 2008; Qin et al., 2011). In contrast, Ift144<sup>dmhd</sup> mutants lacked the cell types that require high levels of Shh activity, the FoxA2+ floor plate and Nkx2.2+ V3 progenitors, throughout the spinal cord (Figs. 1 C and S1 A). In anterior (cervical) regions, Ift144<sup>dmhd</sup> embryo mutants lacked all Shh-dependent cell types, but motor neurons, which require intermediate levels of Shh activity, were present in the posterior (lumbar) neural tube, where they expanded to more dorsal positions than seen in wild type (Figs. 1 C and S1 A).

Whereas the ventral neural cell types that require high levels of Shh activity were absent in Ift144<sup>dmhd</sup> at all rostrocaudal positions, Ift144<sup>int</sup> showed an opposing phenotype: the floor plate, V3 progenitor, and motor neuron domains were dorsally expanded in the caudal neural tube (Fig. 1 C). This ectopic specification of Shh-dependent ventral neural cell types in the Ift144<sup>int</sup> neural tube was similar to that caused by null mutations in two IFT-A genes, Ift139a<sup>dm</sup> and Ift139a<sup>ph</sup> (Tran et al., 2008; Cortellino et al., 2009; Qin et al., 2011).

To test whether the changes in neural patterning were a result of changes in activity of the Shh pathway, we examined expression of Pch1, a direct transcriptional target of the Shh pathway, using a Pch1-lacZ reporter (Goodrich et al., 1997). As predicted by the loss of ventral neural cell types, Pch1-lacZ was reduced in the Ift144<sup>dmhd</sup> neural tube anterior to the forelimbs (Fig. S1, C and D). In the caudal neural tube, Pch1-lacZ was expressed ectopically in dorsal regions, although the strong Pch1-lacZ expression at the ventral midline was absent (Figs. 1 D and S1 D). Pch1-lacZ was ectopically
whether cilia were required for the specification of motor neurons in Ift144<sup>dmhd</sup> embryos, we generated double mutants that lacked both IFT144 and the IFT-B complex protein IFT172. Embryos homozygous for the Ift172<sup>mut</sup> mutation lack cilia and fail to specify Shh-dependent cell types in the neural tube, including motor neurons (Huangfu et al., 2003). The Ift144<sup>dmhd</sup> Ift172<sup>mut</sup> double mutants showed the same loss of ventral neural cell types seen in Ift172<sup>mut</sup> single mutants (Fig. S3, A–D). Thus, the changes in the activity of the Shh pathway in Ift144<sup>dmhd</sup> embryos must be a result of changes in cilia structure or ciliary trafficking caused by loss of this IFT-A protein.

**Weak and strong alleles of Ift144 have distinct effects on cilia structure**

Each of the neural progenitor cells that respond to Shh has a single primary cilium that projects from its apical surface into the central lumen of the neural tube. When cells of the neural tube were examined en face by scanning electron microscopy, wild-type cilia on E10.5 neural progenitors were ~1 µm in length, with shorter cilia emanating from ciliary pockets (Fig. 3 A), consistent with the appearance of primary cilia in other cell types (Sorokin, 1962; Molla-Herman et al., 2010; Rohatgi and Snell, 2010). In contrast, the primary cilia on Ift144<sup>dmhd</sup> neural epithelial cells were short and bulbous (Fig. 3 B and Table S1). The neural cilia in Ift144<sup>ext</sup> were approximately normal in length (Fig. 3 C and Table S1), whereas Ift144<sup>dmhd/ext</sup> compound heterozygote neural cilia were shorter and wider than wild type (Fig. 3 D and Table S1). The vast majority of Ift144<sup>ext</sup> and Ift144<sup>dmhd/ext</sup> cilia lacked the bulges near the tips that have been described in other IFT-A mutants.

To characterize the aspects of ciliogenesis that require IFT144, we analyzed cilia and basal bodies in neuroepithelial cells by transmission EM (TEM). Cross-sections showed that wild-type basal bodies had the typical triplet microtubule structure, and cilia had the characteristic 9 + 0 doublet microtubule structure between the membrane proteins Patched and Smoothened and the Gli transcription factors. Because the Ift144<sup>dmhd</sup> phenotype was different than that of other IFT mutants, we used double-mutant analysis to identify the step in the Shh pathway affected by the mutation. Analysis of neural pattern in double mutants showed that Ift144<sup>dmhd</sup> acted genetically downstream of the two membrane proteins in the pathway, Patched and Smoothened (Fig. S2, A and B), similar to other IFT-B and IFT-A genes (Huangfu et al., 2003; Huangfu and Anderson, 2005; Qin et al., 2011). Double-mutant analysis showed that the neural patterning phenotype of Ift144<sup>dmhd</sup> was the result of changes in the activity of the Gli2 and Gli3 transcription factors (Fig. S2, C–L), as in other IFT mutants.

**The expansion of the motor neuron domain in Ift144<sup>dmhd</sup> depends on the presence of cilia**

IFT144 could, in principle, exert its effect on Shh signaling through either a ciliary or nonciliary role of the protein. To test
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Figure 4. Ultrastructure of wild-type and mutant neural tube cilia. (A–F) Longitudinal sections (left) and cross-sections of cilia (top right) and basal bodies (bb; bottom right) from the neural tube in wild type (WT) and mutants. (A) Wild-type cilia have a 9 + 0 doublet microtubule organization in the axoneme and triplet microtubules in the basal body. (B and C) The microtubules in Ift144mutant (B) and Ift144dmhd/twt (C) mutant cilia appear normal, whereas the cilia are slightly wider than wild type. (D) Ift144dmhd mutant cilia lack axonemal microtubules and contain vesicle-like structures and have a normal-appearing basal body. (E) Longitudinal sections show that neural tube Dyn2hlmutant cilia are swollen and filled with arrays of electron-dense particles that resemble trains of IFT particles. A few singlet microtubules can be seen in cross-sections of Dyn2hlmutant cilia (arrow). (F) The cilia of Ift144dmhd/Dyn2hlmutant double mutants are less swollen than the Dyn2hlmutant single mutants and do not accumulate IFT trains but show a more severe phenotype than Ift144dmhd cilia. Cilia are from E9.5 (wild type and Ift144dmhd) and E10.5 (Ift144mutant, Ift144dmhd/twt, Dyn2hlmutant, and Ift144dmhd/Dyn2hlmutant) embryos. Bars, 200 nm.

IFT-A and cytoplasmic dynein-2 have distinct roles in cilia structure

Because previous studies have shown that both IFT-A proteins and cytoplasmic dynein-2 are required for normal retrograde ciliary trafficking, we compared the structure of Ift144 mutant cilia with that of mutants homozygous for a strong allele of the gene encoding the heavy chain of the retrograde dynein motor Dyn2hlmutant (Liem et al., 2009; Ocbina et al., 2011). TEM sections showed that Dyn2hlmutant cilia were filled with electron-dense particles between the axoneme and the ciliary membrane (Fig. 4 E), similar to the accumulation of particles in Chlamydomonas flagella that lack cytoplasmic dynein-2 (Pazour et al., 1999). In cross-sections, it was apparent that Dyn2hlmutant cilia lacked most axonemal microtubules, and most of the remaining microtubules were singlet rather than doublets. The particles in the Dyn2hlmutant cilia appeared to be highly organized and were regularly spaced at ~40-nm intervals, very different than the unstructured content of the Ift144dmhd cilia.

As both IFT-A and cytoplasmic dynein-2 are important for retrograde ciliary trafficking, we analyzed the ultrastructure of cilia of Ift144mutant/Dyn2hlmutant double mutants. The double-mutant cilia lacked the regular repeating electron-dense particles and were less swollen than Dyn2hlmutant single mutants, and, in the best cases, a reasonably normal-appearing axoneme was restored (Fig. 4 F). Thus, the accumulation of trains of IFT particles in Dyn2hlmutant cilia was relieved by the Ift144mutant mutation. The partial rescue of cilia structure was paralleled by a partial rescue of Shh signaling, as Ift144mutant/Dyn2hlmutant double mutants specified the most ventral neural cell type, the floor plate, which was never seen in Dyn2hlmutant single mutants (Fig. S3, E–J).

IFT-A is required for recruitment of specific cilia components, but not IFT88, to cilia

The TEM analysis showed that Ift144dmhd cells have short, abnormal cilia. However, standard molecular markers for cilia were not detectable in the mutant cilia. Arl13b, a palmitoylated membrane-associated protein of the ARL family of small GTPases (Casparry et al., 2007; Cevik et al., 2010; Larkins et al., 2011), is localized to all cilia in the wild-type embryos and mouse embryo fibroblasts (MEFs). Arl13b was present in primary cilia of Ift144mutant and Ift122dmhd embryos and

Ift122 or Ift139 (Tran et al., 2008; Qin et al., 2011). To test whether this represented a unique property of Ift144 in the IFT-A complex, we analyzed double mutants that were homozygous for both the weak allele Ift144mutant and the null allele Ift122mutant. Whereas neural cilia in both Ift144mutant and Ift122mutant embryos were of nearly normal length (Fig. 3, C and E), the Ift144mutant/Ift122mutant double-mutant cilia were less than half the length of wild type and more bulbous than either single mutant (Fig. 3 F and Table S1). The Ift144mutant/Ift122mutant double-mutant cilia were similar in size and shape to those present in Ift144dmhd embryos.

Whereas Ift144mutant and Ift122mutant single mutants showed expansion of Shh-dependent ventral neural cell types, neural patterning in Ift144mutant/Ift122mutant double mutants was similar to that seen in Ift144dmhd embryos. Like Ift144dmhd, the Ift144mutant/Ift122mutant double mutants lacked all ventral cell types in spinal cord regions anterior to the forelimbs and lacked floor plate and most Nkx2.2+ V3 progenitors cells at more posterior positions (Figs. 5 [A and B] and S1 A). Also like Ift144dmhd, motor neurons in the double mutant spanned the midline and were expanded dorsally in the caudal neural tube (Fig. 5 B). We conclude that the Ift144mutant/Ift122mutant double-mutant and Ift144dmhd single-mutant phenotypes in cilia formation and neural patterning are the result of stronger disruption of the IFT-A complex than seen in Ift122-null single mutants.

IFT-A and cytoplasmic dynein-2 have distinct roles in cilia structure

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IFT-A is required for recruitment of specific cilia components, but not IFT88, to cilia

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ACIII localization in wild type but appeared to be present in mesenchymal cells of the weak IFT-A mutants, and little or no ACIII was detected in strong IFT-A mutants (Fig. S5 and Table S2).

However, it was possible to visualize Itf144<sup>dmd</sup> cilia by immunofluorescent staining, as the mutant cilia showed strong expression of the IFT-B protein IFT88. IFT88 was localized to neural cilia that point into the lumen of the neural tube in all genotypes analyzed: wild type, Itf144<sup>dmd</sup>, Itf144<sup>dmnt</sup>, Itf122<sup>sopb</sup>, and Itf144<sup>dmnt</sup> Itf122<sup>sopb</sup> (Fig. 6 A). Thus, we infer that IFT-A is not required for targeting or entry of IFT88 into cilia. In wild-type MEFs, IFT88 was enriched at the transition zone at the base of the cilium and in a punctate pattern along the ciliary axoneme (Figs. 2 B and 6 [B–D]). In Itf144<sup>dmd</sup> and Itf144<sup>dmnt</sup> Itf122<sup>sopb</sup> MEF cilia, IFT88 extended distal to the basal bodies (marked by \(\gamma\)-tubulin) and often appeared to be continuous from the transition zone into the short mutant axoneme, in contrast to its more punctate localization in wild type, suggesting that this IFT-B protein accumulates in cilia in the absence of IFT-A.

**IFT-A is required for recruitment of Smo, but not Gli2, Sufu, and Kif7, to cilia**

To determine how the changes in cilia structure and composition in the mutants affected the activity of the Shh pathway, we analyzed the localization of Shh pathway proteins in cilia of MEFs derived from mutant embryos. Smo was not detected in the ciliary axonemes of any of the genotypes analyzed in the absence of Shh pathway activation. In wild-type, Itf144<sup>dmnt</sup> and Itf122<sup>sopb</sup> MEF cilia, Smo moved into cilia when the Shh pathway was activated with the small molecule Smoothened agonist (SAG; Fig. 7 A). In contrast, no Smo was detected in the cilia of Itf144<sup>dmd</sup> mutant or Itf144<sup>dmnt</sup> Itf122<sup>sopb</sup> double-mutant MEFs when the pathway was activated with SAG (Fig. 7 A).

In wild-type cells, Gli2 localizes to cilia tips in the absence of ligand, and its concentration at cilia tips increases in response to pathway activation (Fig. 7 B; Chen et al., 2009; Endoh-Yamagami et al., 2009). In the absence of pathway activation, the amount of Gli2 at cilia tips appeared to be elevated in all of the IFT-A mutants examined (Fig. 7 B).

To confirm that the localization of Shh pathway proteins in MEFs reflected their behavior in the embryo, we examined the distribution of the proteins in mesenchymal cells surrounding the neural tube in wild-type and mutant embryos. In cells near the notochord, a source of Shh, Smo was present along the ciliary axoneme of wild-type cells (Fig. S5). In mesenchymal cells in Itf144<sup>dmd</sup> embryos, no Smo was detected in the axoneme, as in MEFs. As in MEFs, Gli2 was present at the tips of wild-type and Itf144<sup>dmd</sup> mesenchymal cilia (Fig. S5). Sufu and Kif7, essential components of the Shh signaling pathway that localize to the tips of wild-type cilia, were also present at the tips of Itf144<sup>dmd</sup> mesenchymal cilia. Mesenchymal cells in Itf144<sup>dmnt</sup> Itf122<sup>sopb</sup> showed a similar pattern of Shh pathway protein localization as Itf144<sup>dmd</sup> MEFs: Gli2, Sufu, and Kif7 were present at the tips of double-mutant cilia, but no Smo was detected in the cilia (Fig. S5). Thus, despite the gross structural defects, Gli2, Sufu, and Kif7 traffic into Itf144<sup>dmd</sup> and Itf144<sup>dmnt</sup> Itf122<sup>sopb</sup> cilia, but Smo does not move into these cilia in response to pathway activation.
IFT-A and Shh signaling

Figure 6. A subset of cilia markers is not detected in IFT-A mutant cilia. (A) Neural tube cilia. Arl13b is expressed in the cilia that project into the lumen of the E10.5 neural tube in wild-type (wt), Ift144<sup>dmhd</sup>, and Ift122<sup>sopb</sup> embryos. Arl13b cannot be detected in Ift144<sup>dmhd</sup> or Ift144<sup>wt</sup> Ift122<sup>sopb</sup> double-mutant cilia. IFT88 localizes to cilia in all genotypes analyzed. ACIII is present in neural cilia in wild type but is present in fewer neural cilia in the IFT-A mutants. Bars: (top row) 200 µm; (bottom rows) 10 µm. (B–D) MEF cilia. The basal body is marked by γ-tubulin and the axoneme by IFT88. (B) Arl13b marks the ciliary axoneme in wild-type (35/35), Ift144<sup>dmhd</sup> (0/9) and greatly reduced or Ift144<sup>wt</sup> Ift122<sup>sopb</sup> (1/12) mutant cilia. IFT88 accumulates in the cilia of Ift144<sup>dmhd</sup> or Ift144<sup>wt</sup> Ift122<sup>sopb</sup> mutants. (C) Like Arl13b, acetylated α-tubulin (ac α-tub) is detected in the primary cilia of wild-type (12/12), Ift144<sup>dmhd</sup> (1/13) or Ift122<sup>sopb</sup> cilia (8/21) showed ACIII staining, and, in the ACIII<sup>+</sup> cilia, the intensity was 22 ± 10% of wild type. Bars, 2 µm.

Figure 7. Localization of Shh pathway proteins in IFT-A mutant MEF cilia. (A and B) γ-Tubulin marks the basal body; IFT88 marks the ciliary axoneme. (A) Smo is present in the cilia of wild-type (wt), Ift144<sup>dmhd</sup>, and Ift122<sup>sopb</sup> MEFs in cells where the Shh pathway is activated by SAG (>90% of cilia are Smo<sup>+</sup>; n > 10). Smo movement into the cilium in response to activation of the Shh pathway by SAG is abolished in Ift144<sup>dmhd</sup> (0/9 cilia were Smo<sup>+</sup>) and greatly reduced Ift144<sup>wt</sup> Ift122<sup>sopb</sup> (1/17). (B) Gli2 is two- to threefold enriched at wild-type MEF cilia tips in response to SAG (n > 14 for each condition). The level of Gli2 is slightly elevated at the tips of the Ift144<sup>dmhd</sup> and Ift122<sup>sopb</sup> mutant cilia in the absence of SAG and is further enriched in the presence SAG (n > 16 for each condition). The level of Gli2 is elevated at the tips of the Ift144<sup>dmhd</sup> or Ift144<sup>wt</sup> Ift122<sup>sopb</sup> mutant cilia in the absence of SAG and does not increase further in the presence SAG (n > 9 for each condition). Bar, 2 µm.
Discussion

**IFT144 is required for anterograde, as well as retrograde, ciliary trafficking**

Our results define an allelic series in the mouse *Ift144* gene, which shows that partial loss of IFT-A and complete loss of IFT-A have very different effects on cilia structure and on cilia-dependent Shh signaling. Previous experiments have shown that the IFT-A complex in both *Chlamydomonas* and mouse is important for the normal rate of retrograde ciliary trafficking (Iomini et al., 2001, 2009; Tran et al., 2008). Embryos homozygous for the partial loss-of-function mutation *Ift144<sup>dmhd</sup>* have normal-length neural cilia that show characteristics expected for a slowed rate of retrograde trafficking: the cilia are somewhat wider than wild-type cilia, and they accumulate IFT88 and Gli2. In contrast, embryos homozygous for the apparent null allele *Ift144<sup>dshl</sup>* have a strong defect in ciliogenesis. The loss of microtubules and the absence of Arl13b, ACIII, and Smo in *Ift144<sup>dshl</sup>* mutant cilia suggest that the mutation decreases anterograde trafficking in the cilium.

Additional evidence for a role of IFT144 in anterograde trafficking comes from the analysis of double mutants that lack the dynein motor. Although both IFT-A and cytoplasmic dynein-2 are important for retrograde trafficking in the cilium, our data highlight the different requirements for the two classes of proteins in cilia structure. In *Ift144<sup>dshl</sup>* cilia, a membrane compartment filled with electron-dense material extends distal to the basal body, but the normal microtubule structure of the axoneme is absent, and only a few microtubules are present in the cilium. This contrasts with the structure of *Dync2h1<sup>mmi</sup>* cilia, which are filled with a regular array of particles spaced at ~40-nm intervals. These structures are likely to be trains of IFT particles, based on comparison with the arrays of IFT trains with 40-nm periodicity observed by electron tomography in *Chlamydomonas* LC8 mutants, which lack a dynein light chain (Pigino et al., 2009). *Ift144<sup>wt</sup>* *Dync2h1<sup>mmi</sup>* double-mutant cilia do not accumulate the ordered IFT trains seen in *Dync2h1<sup>wt</sup>*. This is consistent with our previous results showing that reduction of either IFT-B or IFT-A partially rescued the cilia phenotype of *Dync2h1* mutants because of roles of both IFT-B and IFT-A in anterograde ciliary trafficking (Ochina et al., 2011). Thus, we conclude that IFT144 is required for normal anterograde trafficking.

The cilia phenotypes of the mouse *Ift144* allelic series parallel findings with *Chlamydomonas* IFT-A mutants. At the permissive temperature, temperature-sensitive mutations in two *Chlamydomonas* IFT-A genes, *fla15* (**IFT144** and *fla17* (**IFT139**), cause bulged flagella, associated with slowed rates of retrograde IFT (Iomini et al., 2001, 2009). However, at restrictive temperature, *fla15* and *fla17* mutant cells appear to be agflagellate, similar to the phenotype of null mutants in *Chlamydomonas* *Ift140* (another IFT-A gene; Cole, 2003). Thus, in both *Chlamydomonas* and the mouse, partial loss of IFT-A proteins leads to a defect in retrograde trafficking, and complete loss of IFT-A function leads to the formation of very short cilia. Studies on Dyf-2, the *C. elegans* homolog of IFT144, are also consistent with roles of this protein in both retrograde and anterograde trafficking (Efimenko et al., 2006).

**Overlapping functions of mouse IFT-A genes**

Our genetic analysis of mouse mutants provides a different perspective on the function of individual IFT-A proteins than seen in biochemical experiments. Those studies suggested that mammalian IFT144/Wdr19 and IFT122 were core components of the mammalian IFT-A complex, whereas IFT139/Tec21b and IFT121/Wdr35 were peripheral proteins that were not required for the formation of the core complex (Mukhopadhyay et al., 2010). In contrast, the genetic data indicate that loss of IFT144 or IFT121/Wdr35 (Mill et al., 2011) has a stronger effect on IFT-A function than does loss of IFT139a or IFT122.

Double mutants that are homozygous for both a null allele of *Ift122* and the weak allele of *Ift144* (*Ift144<sup>wt</sup> *Ift122<sup>opb</sup>*) have stronger defects in ciliogenesis than either single mutant and are indistinguishable from *Ift144<sup>dshl</sup>* mutants in both cilia structure and protein localization. Therefore, we conclude that IFT144 and IFT122 partially overlap in function, and, in the absence of IFT122, decreased activity of IFT144 is sufficient to block the activity of the IFT-A complex. This conclusion is consistent with data from *Chlamydomonas* that some IFT proteins can substitute for each other (Iomini et al., 2009) and reveals functional differences among the IFT-A proteins.

**Loss of Shh pathway activity in strong IFT-A mutants is associated with the disruption in cilia structure**

Strong disruption of IFT-A, as in *Ift144<sup>dshl</sup>* mutants, blocks the formation of a normal ciliary axoneme and the specification of ventral neural cell types that require high levels of Shh activity (Fig. 8 C). Because of the important roles of cilia in promoting Shh activity, a simple model is that the decreased activity of the Shh pathway in *Ift144<sup>dshl</sup>* is a result of the collapse of cilia structure in the absence of the IFT-A complex and that normal cilia structure is required for cilia-associated Shh pathway proteins to respond correctly to ligand.

Despite the loss of high-level responses to Shh, the *Ift144<sup>dshl</sup>* and *Ift144<sup>wt</sup>* *Ift122<sup>opb</sup>* embryos show ectopic activation of the pathway in the caudal neural tube, albeit at a lower level than in *Ift144<sup>wt</sup>*. As the ectopic motor neurons seen in *Ift144<sup>dshl</sup>* embryos are also present in *Ift144<sup>dshl</sup>* *Dync2h1<sup>mmi</sup>* double-mutant embryos (Fig. S3 F), where retrograde trafficking should be completely disrupted, the data argue that the activation of the Shh pathway seen in IFT-A mutants is not the result of defects in retrograde trafficking. Instead, the results suggest that the ligand-independent activation of the Shh pathway is the result of a defect in IFT-A–dependent anterograde trafficking.

**Cilia localization of soluble Shh pathway proteins does not depend on IFT-A**

Given the absence of most microtubules in the ciliary axoneme in the null allele *Ift144<sup>dshl</sup>* it is remarkable that the mutants can specify the ventral neural cell types that require intermediate levels of Shh activity and that this intermediate level of pathway activity depends on the presence of the *Ift144<sup>dshl</sup>* cilium. We observed that Gli2, Kif7, and Sufu, which are critical for activity of the Shh pathway, are all present at the tips of the small *Ift144<sup>dshl</sup>* and *Ift144<sup>wt</sup>* *Ift122<sup>opb</sup>* double-mutant cilia,
which suggests that the IFT-B complex is sufficient for trafficking Gli, Kiif7, and Sufu to cilia tips and therefore for the activation of midlevel Shh activity. A critical role for IFT-B in cilia localization of Gli2 is consistent with the reduction of Gli2 at cilia tips caused by a modest reduction in the level of the IFT-B protein IFT172 (Friedland-Little et al., 2011).

IFT-A–dependent trafficking of membrane proteins into cilia may account for the neural patterning phenotypes of IFT-A mutants

Our analysis indicates that a variety of proteins and protein complexes, including IFT88, Gli2, Sufu, and Kiif7, can translocate into cilia that lack a functional IFT-A complex. In contrast, several membrane proteins, including Arl13b, ACIII, and Smo, are not detectable in cilia of either of the strong IFT-A mutants (\textit{Ift144\textsuperscript{dmhd}} single mutants or \textit{Ift144\textsuperscript{wt} Ift122\textsuperscript{mhd}} double mutants). A role for the IFT-A–associated protein Tulp3 in trafficking of specific G protein–coupled receptors into cilia was previously defined (Mukhopadhyay et al., 2010), and our findings suggest that IFT-A has a general role in the trafficking of membrane proteins into cilia. Recent work has shown that a membrane-associated protein complex at the transition zone that includes the protein Tectonic1 is important for entry of some membrane proteins (ACIII and Arl13b) but had less effect on others (Smo) into cilia (Garcia-Gonzalo et al., 2011). This raises the possibility that the Tectonic1 and IFT-A complexes act in concert to promote the transport of membrane proteins into cilia.

The membrane proteins affected by loss of IFT-A (Arl13b, Smo, and ACIII) all have documented or plausible roles in Shh signaling and are likely to contribute to the Shh phenotypes of the IFT-A mutants. Mouse mutants that lack Arl13b show strong defects in Shh signaling and neural patterning (Caspar et al., 2007), so the absence of detectable ciliary Arl13b in the strong IFT-A mutants (\textit{Ift144\textsuperscript{dmhd}} and \textit{Ift144\textsuperscript{wt} Ift122\textsuperscript{mhd}}) could be responsible for some aspects of the neural patterning phenotypes of these embryos. Indeed, cell types that require high levels of Shh activity (the floor plate and V3 interneurons) fail to be specified, and the motor neuron domain is expanded in the caudal neural tube of \textit{Arl13b} mutants, as in \textit{Ift144\textsuperscript{dmhd}} and \textit{Ift144\textsuperscript{wt} Ift122\textsuperscript{mhd}} embryos. However, there must be additional targets of IFT-A that contribute to the neural patterning defects in the mutant embryos because the \textit{Arl13b} and IFT-A neural patterning phenotypes are not identical. For example, neural patterning is normal in the rostral neural tube of \textit{Arl13b} mutants, but ventral neural cell types are not specified in this region of the neural tube in the strong IFT-A mutants; this could be explained if an unidentified membrane protein with an \textit{Arl13b}-like function depends on IFT-A for its cilia localization in the rostral neural tube. However, even in the caudal neural tube, where the \textit{Arl13b} and IFT-A phenotypes are similar, double mutants reveal differences between the genotypes. For example, motor neurons are specified in \textit{Arl13b Gli2} double mutants but not in \textit{Ift144\textsuperscript{dmhd} Gli2} double-mutant embryos. Therefore, we suggest that the IFT-A neural patterning phenotype could be the result of the absence of several different membrane proteins in the cillum, including Arl13b.

The one core component of the Shh pathway that does not traffic into cilia of the strong IFT-A mutants (\textit{Ift144\textsuperscript{dmhd}} and \textit{Ift144\textsuperscript{wt} Ift122\textsuperscript{mhd}}) is Smo, a positive regulator of the pathway. However, the absence of Smo in the cillum cannot account for the neural patterning phenotypes in \textit{Ift144\textsuperscript{dmhd}} embryos, as our double-mutant analysis shows that the \textit{Ift144\textsuperscript{dmhd}} phenotype is independent of the presence or absence of Smo. Nevertheless, our data provide the first evidence that IFT proteins play a role for Smo movement into cilia and contrast with models that suggest...
IFT-A and human genetic disease

Mutations in all six IFT-A genes—IFT139a/TTC21b, IFT121/WDR35, IFT43, IFT122, IFT144, and IFT40—cause human ciliopathies (Gilissen et al., 2010; Arts et al., 2011; Bredrup et al., 2011; Davis et al., 2011; Mill et al., 2011; Perrault et al., 2012), whereas disease-causing mutations have been identified in only a single IFT-B gene (Beales et al., 2007). The higher frequency of disease-causing IFT-A mutations is likely to reflect the overlap in function we have observed among IFT genes, so that even null mutations cause relatively mild phenotypes that are compatible with postnatal life. As the Ift144mut mutant recapitulates many of the skeletal and craniofacial anomalies seen in these diseases (Ashe et al., 2012), our findings suggest that the complex phenotypes seen in IFT-A syndromes are likely to be a result of the loss of specific membrane proteins in the mutant cilia.
EM
EM describes the electron microscopic examination of samples. In this study, 10.5 embryos were fixed with 2% PFA and 2.5% glutaraldehyde. For scanning electron microscopy, embryos were dissected to expose the lumen of the neural tube in 0.1 M sodium cacodylate buffer. For transmission electron microscopy, wild type and mutants. Table S2 shows cilia localization of Shh pathway proteins in Table S1 shows cilia dimensions in wild type

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