ABSTRACT

Cilia are key regulators of animal development and depend on intraflagellar transport (IFT) proteins for their formation and function, yet the roles of individual IFT proteins remain unclear. We examined the Ift56<sup>hop</sup> mouse mutant and reveal novel insight into the function of IFT56, a poorly understood IFTB protein. Ift56<sup>hop</sup> mice have normal cilia distribution but display defective cilia structure, including abnormal positioning and number of ciliary microtubule doublets. We show that Ift56<sup>hop</sup> cilia are unable to accumulate Gli proteins efficiently, resulting in developmental patterning defects in Shh signaling-dependent tissues such as the limb and neural tube. Strikingly, core IFT proteins are unable to accumulate normally within Ift56<sup>hop</sup> cilia, including IFT88, IFT81 and IFT27, which are crucial for key processes such as tubulin transport and Shh signaling. IFT56 is required specifically for the IFTB complex, as IFTA components and proteins that rely on IFT function are unaffected in Ift56<sup>hop</sup> cilia. These studies define a distinct and novel role for IFT56 in IFTB complex integrity that is crucial for cilia structure and function and, ultimately, animal development.

KEY WORDS: IFT56, TTC26, Cilia, Intraflagellar transport, IFTB, Hedgehog signaling, Microtubule structure

INTRODUCTION

In vertebrates, cilia are essential for many developmental processes, including left-right specification, organogenesis, skeleton formation and neural patterning (Fliegauf et al., 2007; Oh and Katansis, 2012; Sharma et al., 2008). As a consequence, defects in cilia formation or function underlie a broad range of human diseases, with varying degrees of severity, collectively referred to as ciliopathies (Badano et al., 2006; Hildebrandt et al., 2011; Lancaster and Gleeson, 2009; Nigg and Raff, 2009; Sharma et al., 2008; Waters and Beales, 2011). Cilia are near-ubiquitous organelles that extend into the extracellular space and sense mechanical stimuli as well as several signaling molecules (Choksi et al., 2014; Goetz and Anderson, 2010). These include Hedgehog (Hh) ligands, which regulate the development of multiple organ systems, including the limb and neural tube (Litingtung and Chiang, 2000; Litingtung et al., 2002). Components of the Hh pathway, including the receptor Patched (Ptc1), the pathway mediator Smootherned (Smo), and Gli transcriptional mediators localize to the cilium, and cilia are required for normal Hh pathway activity (Haycraft et al., 2005; Rohatgi et al., 2007). Through a mechanism that is still unclear, the Gli proteins require cilia to form both their functional transcriptional activator (GliA) and repressor (GliR). Without proper cilia formation or function, both positive and negative (ligand-independent GliR) functions of Shh signaling are affected, leading to a range of Hh-related patterning defects in multiple tissues (Liu et al., 2005; May et al., 2005).

In addition to components of the Hh pathway, hundreds of other proteins transit through the cilium via intraflagellar transport (IFT) (Kozminski et al., 1993). This process is mediated by kinesin and dynein microtubule motor proteins, which direct anterograde and retrograde movement, respectively (Cole et al., 1998; Pedersen et al., 2008; Rosenbaum and Witman, 2002). Working with the motors are IFT particles, multiprotein complexes that transport proteins involved in cilia formation and function, including tubulin subunits and signaling pathway effectors (Kozminski et al., 1995; Pazour et al., 1998). The IFT particle itself can be biochemically grouped into IFTB and IFTA protein complexes that have distinct functions. Loss-of-function mutations in most IFTB genes lead to failure of cilia formation due to defects in importing key cilia-building components, whereas IFTA mutants form bulbous cilia with proteins accumulating at the distal tip (Blacque et al., 2008; Huangfu et al., 2003; Liem et al., 2012; Qin et al., 2011). This evidence has been interpreted to mean that anterograde trafficking is mediated by IFTB, whereas retrograde transport depends on IFTA function. However, the finding that a subset of IFTB mutants (e.g. Ift25<sup>/f7</sup>) retain cilia (Eguether et al., 2014; Keady et al., 2012) highlights the poorly understood complexity of individual IFT protein function.

The IFTB complex comprises 16 proteins, and recent studies have begun to dissect the biochemical and structural interactions within the complex (Katoh et al., 2016; Taschner et al., 2014, 2016). IFT56 (also known as TTC26, Dfy-13 and PIFTC3) is a highly conserved IFTB protein that localizes to flagella/cilia of organisms ranging from the single-cell alga <i>Chlamydomonas reinhardtii</i> to vertebrates (Blacque et al., 2005; Follit et al., 2009; Franklin and Ullu, 2010; Ishikawa et al., 2014; Zhang et al., 2012). In <i>C. elegans</i> and <i>Chlamydomonas</i>, disruption of IFT56 function reduces cilia/flagella length, and zebrafish Ift56 morphants display reduced cilium number (Blacque et al., 2005; Ishikawa et al., 2014). In <i>Chlamydomonas</i>, IFT56 associates with motility factors, including outer dynein arms, and loss of IFT56 results in a decrease in these proteins leading to impaired motility (Ishikawa et al., 2014). The classic mouse mutant hop-sterile (Johnson and Hunt, 1971) was recently reported to harbor a nonsense mutation within Ift56 (Swiderski et al., 2014). In contrast to IFT56 mutants in
non-mammalian systems, as well as most other IFTB mouse mutants, *Ift56* mutants are viable and display normal cilia numbers and morphology. This provides a unique opportunity to study IFT function within intact cilia and suggests distinct roles for IFT56 as compared with other IFTB proteins.

*Ift56* mutants are characterized by preaxial polydactyly, gait abnormalities and male sterility (Johnson and Hunt, 1971). In this study we uncover three key functions of IFT56 in mammalian primary cilia that explain the developmental defects and shed new light on the roles of IFT56. First, IFT56 is required for the accumulation of Gli2 and Gli3 at the distal tips of cilia. Reduced ciliary Gli2/3 in *Ift56* mutants would account for the Shh-dependent limb and neural tube patterning defects observed in *Ift56* mutants. Second, although IFT56 is not required to form primary cilia, it is essential for normal microtubule architecture within the ciliary axoneme. *Ift56* mouse mutants show a reduced number and abnormal arrangement of ciliary microtubule doublets. Finally, IFT56 is crucial for the normal levels and distribution of IFTB proteins, but not IFTA, within cilia. These studies reveal previously undescribed roles of IFT56 as an essential core component of the IFTB complex that is required for the integrity of both IFTB and the cilium structure itself. These results also challenge the current model of IFTB-dependent IFTA transport and demonstrate that IFTA components can undergo the IFT process independently of a complete IFTB particle.

**RESULTS**

**The hop-sterile phenotype is caused by a nonsense mutation in *Ift56***

The phenotype of *hop-sterile* homozygotes was suggestive of an underlying defect in cilia formation or function. We used a capture and sequencing approach to test whether the *hop* phenotype was caused by a mutation in a known cilia gene (Johnson and Hunt, 1971). Through exome capture, we identified a cytosine-to-adenosine transversion located at mouse Chr6:38,362,071 in mutants (see Materials and Methods). This mutation fell within the coding region of the intraflagellar transport 56 (*Ift56*) gene (also known as *Ttc26*) (c.1290C>A) and was predicted to result in a premature truncation (Y430X) of the protein (Fig. S1). We confirmed complete linkage between the *hop-sterile* phenotype and the *Ift56* mutation, which has been further corroborated by Bánfi and colleagues (Swiderski et al., 2014).

**Ciliary IFT56 is required for induction of Shh targets in the neural tube***

*Ift56* mutants were initially described with an abnormal gait (Johnson and Hunt, 1971), which is consistent with a patterning defect in the developing neural tube. As most IFTB mutants lose cilia or form short cilia throughout the embryo including the neural tube, we first tested whether *Ift56* mutants showed a similar defect. However, we found that *Ift56* mutants are able to form grossly normal cilia both in the neural tube and in culture, at a similar density as controls (Fig. 1A, Fig. S2). Consistent with Swiderski et al. (2014), we observed increased cilia length in *Ift56* mutants (Fig. S3). However, *Ift56* mutant cilia lack IFT56 protein (Fig. 1B), suggesting potential defects in neural tube cilia function.

In the developing neural tube, Shh forms a ventral gradient of signaling that is transduced primarily through the function of Gli2 activator, with the most ventral cell types requiring the highest level of Shh signaling (Chiang et al., 1996; Ericson et al., 1997; Marti et al., 1995). Cilia function is required in the neural tube to receive the Shh signal (Huangfu et al., 2003). In the rostral neural tube (cervical and forelimb level) of *Ift56* embryos, the most ventral cells expressed FoxA2, a high-level target of Shh, similar to controls (Fig. 1C). V3 interneuron progenitors, marked by Nkx2.2, are specified by slightly lower levels of Shh and were largely found within their normal domains in *Ift56* mutants, dorsal to the floorplate (Fig. 1D). However, Nkx2.2 was abnormally expressed in

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**Fig. 1. *Ift56* neural tube cilia show reduced sensitivity to Shh signaling.** (A,B) *Ift56* neural tube cilia form a similar number of cilia as controls but lack IFT56 protein. Neural tube sections were stained for Arl13b (A, green) to mark ciliary axonemes or IFT56 (B, cyan) and γ-tubulin to mark basal bodies (A, red). (C-J) E10.5 neural tube sections were stained with DAPI (blue) and for markers of neuronal progenitors (green), FoxA2 (C,G) is expressed normally in the rostral (forelimb region) floor plate in *Ift56* neural tubes but dramatically reduced caudally (hindlimb region, bracket). A subset of V3 interneuron progenitors (Nkx2.2*) are misplaced and found at the *Ift56* midline rostrally (D,D′, arrowheads) and almost completely absent caudally (H, bracket). Motor neuron progenitors (Olig2*) are unaffected in rostral *Ift56* sections (E) but are specified throughout the entire ventral caudal neural tube (I, bracket). Pax6 appears unaffected throughout the *Ift56* neural tube (F,J). n=3 biological replicates each from control and mutant, with three sections per replicate analyzed. Scale bars: 2 μm in A,B; 100 μm in C-J.
a subset of ventral cells in Ift56<sup>hop</sup> mutants, which were mixed with floor plate cells (FoxA2<sup>−/−</sup>) at the ventral midline (Fig. 1D', arrowheads). Motor neuron progenitors, specified at intermediate levels of Shh signaling, appeared normal in the Ift56<sup>hop</sup> neural tube (Fig. 1E). The ventral boundary of Pax6<sup>+</sup> neuronal progenitors is limited by Shh signaling, and this restriction was also maintained in Ift56<sup>hop</sup> mutants (Fig. 1F). These data indicate a mild effect on rostral neural tube patterning in Ift56<sup>hop</sup> mutants.

Strikingly, we observed differential, but consistent, patterning effects of the Ift56<sup>hop</sup> mutation between the rostral and caudal neural tube. Ventral neuronal specification defects were more severe in the caudal neural tube (hindlimb level) of Ift56<sup>hop</sup> embryos compared with rostral regions, with the exception of Pax6, which appeared relatively normal (Fig. 1J). FoxA2<sup>−/−</sup> floor plate cells were almost completely absent (Fig. 1G; data not shown) and few V3 interneuron progenitors were specified in caudal regions of Ift56<sup>hop</sup> mutants (Fig. 1H). Although motor neuron markers maintained their dorsal border they were additionally expressed throughout the entire ventral half of the Ift56<sup>hop</sup> caudal neural tube (Fig. 1I), indicating reduced ventral Shh signaling in the mutants.

**IFT56 is essential for GliA and GliR function**

Preaxial polydactyly was initially described in Ift56<sup>hop</sup> mutants as an extra digit on the anterior side of the limbs (Johnson and Hunt, 1971). When we examined the developing limbs in more detail, we observed variable expressivity of this trait in Ift56<sup>hop</sup> mutants, ranging from a thickening of digit 1 to a complete ectopic digit, including duplicated metacarpal and metatarsal elements. In the mutant forelimbs, the ectopic digit always displayed digit 1 identity (i.e. two phalanges; Fig. 2A). However, ectopic hindlimb digits occasionally adopted a more posterior identity (i.e. three phalanges; 25%, n=5/20 hindlimbs; Fig. 2B; data not shown).

Shh signaling regulates digit number and identity through the function of GliA in the posterior, and a ligand-independent GliR function in the anterior (Bowers et al., 2012; Chiang et al., 2001; Lettice, 2003; Park et al., 2008; te Welscher et al., 2002; Wang et al., 2007). An extra digit on the anterior side of the limbs (Johnson and Hunt, 1971). Ectopic Shh expression in the anterior limb has been reported in most IFTB mutants, although the Ift56<sup>hop</sup> phenotype is milder. Unlike most IFTB mutants, Ift56<sup>hop</sup> animals form normal numbers of cilia, which might account for the milder phenotype, but also suggests that abnormal Gli activity is not due to cilia loss but rather defective cilia function. Thus, we examined mouse embryonic fibroblast (MEF) cilia to better understand how the Shh pathway activation, is likely to underlie preaxial polydactyly in Ift56<sup>hop</sup> limbs.

**Ciliary accumulation of Gli proteins is reduced in Ift56<sup>hop</sup> tissues**

Our analyses of Ift56<sup>hop</sup> mutant neural tubes and limbs indicate that IFT56 is crucial for both GliA and GliR function, similar to other IFTB mutants, although the Ift56<sup>hop</sup> phenotype is milder. Unlike most IFTB mutants, Ift56<sup>hop</sup> animals form normal numbers of cilia, which might account for the milder phenotype, but also suggests that abnormal Gli activity is not due to cilia loss but rather defective cilia function. Thus, we examined mouse embryonic fibroblast (MEF) cilia to better understand how the Ift56<sup>hop</sup> mutation affects the Shh pathway. Smo accumulates in cilia in the presence of the Hh ligand or a Smo agonist (SAG), and we discovered that the
distribution and intensity of ciliary Smo were unaffected in Ift56<sup>hop</sup> cilia when the Hh pathway is activated (Fig. 3A,B). Gli proteins normally accumulate at the distal tips of cilia in response to Hh pathway activation. Strikingly, Gli2 and Gli3 ciliary tip accumulation was significantly reduced in Ift56<sup>hop</sup> cilia, and a large percentage of mutant cilia showed no detectable Gli2/3 localization (Fig. 3C-F). To test the physiological relevance of this finding, we also examined Gli2 in Ift56<sup>hop</sup> cilia, both in the caudal (hindlimb region) neural tube epithelium and in limb mesenchyme. Throughout the posterior limb, few cilia showed strong accumulation of Gli2 in Ift56<sup>hop</sup> mutants, and most exhibited low or no levels of Gli2 within cilia (Fig. 3G-I). We similarly observed fainter Gli2 in the neural tube cilia of Ift56<sup>hop</sup> mutants overall (Fig. S5). These data show that although cilia form in Ift56<sup>hop</sup> mutants, IFT56 is required for the proper accumulation of ciliary Gli proteins during development.

**IFT56 is required for integrity of the IFTB complex in cilia**

Movement of cargo such as Gli proteins and ciliary subunits into and out of the cilium requires the function of IFT complexes (Follit et al., 2009). IFT56 is a known component of the IFTB complex, but its role in primary cilia was unclear. We began by examining IFT protein localization to better understand the effect of IFT complex integrity and function when IFT56 is disrupted. Surprisingly, we discovered that, in Ift56<sup>hop</sup> MEFs, although the amount of IFT88 along the length of the ciliary axoneme was not significantly altered, IFT88 accumulated at the proximal base of cilia in Ift56<sup>hop</sup> MEFs (Fig. 4A,B). As IFT88 is crucial for cilia formation, this result was unexpected and prompted us to further examine whether additional IFT complex components require IFT56 for their ciliary localization.

Recent studies have begun to dissect out distinct roles of IFTB proteins within the complex. For example, in contrast to IFT88, IFT81 and IFT74 serve as a ‘tubulin module’ that binds and transports tubulin within cilia (Bhogaraju et al., 2013). IFT25 (HSPB11) and IFT74 serve as a ‘tubulin module’ that binds and transports tubulin within cilia (Eguether et al., 2014; Keady et al., 2012; Liew et al., 2014). Similar to IFT88, we observed pooling of IFT81 at the Ift56<sup>hop</sup> ciliary base. Additionally, there was a significant reduction of IFT81 protein in the axoneme in Ift56<sup>hop</sup> cells (Fig. 4C,D). Unlike IFT88 and IFT81, IFT27 did not pool at the base of mutant cilia, but was dramatically decreased in Ift56<sup>hop</sup> axonemes (Fig. 4E,F). To test whether this was physiologically relevant, we examined limb mesenchyme cilia in vivo and confirmed a strong and significant reduction of both IFT81 and IFT27 in Ift56<sup>hop</sup> mutants (Fig. 4G-L). Similar to our MEF studies, IFT27 was significantly reduced in limb Ift56<sup>hop</sup> mesenchyme cilia (Fig. 4J-L). However, IFT81 showed a stronger phenotype in vivo compared with MEFs and was significantly reduced throughout the entire Ift56<sup>hop</sup> ciliary axoneme (Fig. 4G-I). This could suggest different requirements for IFT56 within cilia of distinct tissues.

Together, these data indicate a key role for IFT56 in the ciliary localization of other IFTB components. In particular, the strong reduction in IFT27 could explain the Shh-related cilia defects that lead to subsequent developmental patterning abnormalities in Ift56<sup>hop</sup> mutants.

**The Ift56<sup>hop</sup> mutation disrupts ciliary microtubule architecture**

One crucial function of IFT proteins is transporting components required to build the cilium, and at least three IFT proteins appear to be involved in ciliary tubulin transport (Bhogaraju et al., 2013;
Taschner et al., 2016), including IFT81, which we observed pooling at the base of Ift56hop cilia. We used transmission electron microscopy (TEM) to examine Ift56hop neural tube cilia for potential defects in the structure of the cilium. Consistent with normal cilia numbers in Ift56hop mutants, basal bodies appeared to dock normally at the apical side of cells lining the neural tube (Fig. 5A,B). Sections through the basal bodies further showed the typical 9+0 ring of microtubule triplets in both mutants and controls (Fig. 5C,D). However, whereas the axoneme of control primary cilia contain a 9+0 ring of microtubule doublets (Fig. 5E), Ift56hop cilia frequently displayed disorganized structures, including abnormal positioning and number (e.g. 8+0, 7+0) of the microtubule doublets (Fig. 5F-H). These misalignments and tubule losses were never observed in control cilia. These data demonstrate that IFT56 is essential for proper microtubule organization within the ciliary axoneme, which may be linked to defects in IFT81 localization (Fig. 4C).

**DISCUSSION**

The IFT complex consists of 16 IFTB proteins and 6 IFTA proteins, which together are crucial for the formation, maintenance and function of cilia. Ciliary cargo transport is carried out by the combined actions of IFTB and IFTA, which together form the IFT particles. To test whether the IFTB defects observed in Ift56hop cilia (Fig. 4) in turn affect ciliary transport of the IFTA complex, we analyzed the localization of two core IFTA proteins, IFT140 and IFT122. We were surprised to find that both IFT140 and IFT122 protein levels and localization were normal in Ift56hop cilia (Fig. 6A-D).

To verify that IFTA components were functioning normally in Ift56hop cilia, we examined several proteins that depend upon IFTA for ciliary localization (Liem et al., 2012; Qin et al., 2011). Adenylate cyclase III (AC3, also known as ADCY3) is involved in determining cilia length (Ou et al., 2009; Wang et al., 2011) and was present at normal levels in Ift56hop cilia (Fig. 6E). Polycystin 2 (PC2, also known as PKD2) is a calcium channel required for mechanosensation (Cai et al., 1999) and Arl13b is a small GTPase required for normal ciliary ultrastructure and Shh signaling (Caspary et al., 2007); both of these proteins localized normally in Ift56hop cilia (Fig. 6F,G). These results demonstrate that IFT56 is dispensable for IFTA localization and function but instead is specifically required for IFTB complex integrity and ciliary trafficking.

**IFT56 is not required for IFTA localization or function**

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signaling properties of cilia. Recent studies uncovered key biochemical and structural details of protein-protein interactions within the IFTB subcomplex (Katoh et al., 2016; Taschner et al., 2014, 2016), yet the functional roles of individual IFTB components within cilia are still being elucidated. Defining the specific requirements of IFTB proteins in cilia has been particularly difficult as most IFTB mutants lack cilia altogether, precluding efforts to study their roles in protein trafficking and cilia maintenance. Using the Ift56<sup>hop</sup> mutant, which exhibits ciliopathic phenotypes but retains its cilia, we uncovered several distinct roles for IFT56 in the integrity of IFTB and trafficking of Shh signaling proteins, as well as in the microtubule organization of mammalian primary cilia.

IFTB is compartmentalized into a stable core known as IFTB-1 [IFT88, 81, 74, 70 (TTC30B), 52, 46, 27, 25, 22] and a second subcomplex known as IFTB-2 [IFT172, 80, 57, 54 (TRAF3IP1), 38...
Current study, we demonstrate that IFT56 is also required for proteins have varied functions within the IFT particle in different throughout the axoneme. It is likely that IFT56 and/or other IFT where in limb mesenchyme cilia the same IFT proteins are reduced ciliary IFT56 can lead to pooling of specific IFTB components (IFT88, 81) at the proximal basal of embryonic fibroblast cilia, whereas in limb mesenchyme cilia the same IFT proteins are reduced throughout the axoneme. It is likely that IFT56 and/or other IFT proteins have varied functions within the IFT particle in different tissue systems. Overall, our data suggest that IFT56 is essential in maintaining IFTB complex integrity in mammalian primary cilia, and that loss of ciliary IFT56 may affect a subset of the complex (IFT27) more strongly than others (IFT88, 81), depending on tissue type. Despite the abnormal localization of IFTB proteins, the IFTB complex retains enough function in Ift56hop mutants to form cilia, although the cilia that do form cannot transduce Shh signaling effectively. We observed defective Gli2 accumulation at Ift56hop cilia tips both in vitro and in vivo, and others have reported decreased responsiveness of a Gli reporter gene during Shh induction (Swiderski et al., 2014), indicating that the reduction in Gli2 accumulation affects downstream Shh signaling. This defect in Gli2 accumulation is likely to be due to the strong reduction in ciliary IFT27. Recent evidence suggests that IFT27/25 promotes transport of ciliary Smo and Ptc1 through a BBSome-dependent mechanism (Eguether et al., 2014; Keady et al., 2012; Liew et al., 2014). However, there is little evidence of whether or how Gli might be transported by IFT proteins. IFT mutants lack or have short cilia, further complicating experiments to examine how Gli proteins function in cilia without efficient protein transport. Here, we find that loss of ciliary IFT56 and improper localization of a subset of IFTB components, including IFT27, directly affects Gli accumulation at cilia tips independently of Smo transport. This suggests that individual IFTB proteins affect cilia-dependent Shh signaling through different mechanisms, whereby IFT27/25 is important for Smo transport, and IFT56 is crucial for Gli accumulation. Whether the loss of other IFT proteins from cilia affects the localization of signaling function similarly remains to be determined.

One surprise from our study is that core IFTA proteins (IFT122, 140) localized normally in Ift56hop mutant cilia despite defects in ciliary IFTB. Ciliary membrane and associated proteins, including Smo, Arl13b, AC3 and PC2, which have been shown to depend on ciliary IFTA (Liem et al., 2012), also localize normally, further suggesting that IFTA is functioning in the absence of IFT56 and the complete IFTB particle. This is unexpected, as IFTA proteins have long been thought to enter cilia together with IFTB proteins through the function of kinesin 2 (Blacque et al., 2008; Hsiao et al., 2012). In mice, most IFTB and kinesin 2 mutants lose cilia (Berbari et al., 2011; Botilde et al., 2013; Houde et al., 2006; Huangfu et al., 2003; Lee et al., 2015; Marszalek et al., 1999), suggesting that IFTB and kinesin 2 are crucial for the anterograde transport of cilia proteins and ciliogenesis. IFTA and dynein mouse mutants have short, bulbous cilia that accumulate IFT proteins (Cortellino et al., 2009; May et al., 2005; Mill et al., 2011; Qin et al., 2011; Tran et al., 2008), implicating a role in retrograde transport and the recycling of ciliary proteins. However, distinct exceptions, including IFT56, 27, 25 (IFTB) mutants (Eguether et al., 2014; Keady et al., 2012; Swiderski et al., 2014), provide evidence suggesting that IFTB and IFTA may not be restricted to anterograde and retrograde trafficking, respectively. Our data suggest that IFTA can enter cilia and function properly despite the loss and reduction of several IFTB proteins, and does not depend on the complete IFTB particle. Instead, individual IFTB proteins have differential roles within the complex, some of which might be crucial for IFTA transport and others for ciliary cargo. Rather than attributing specific transport roles to each complex, it is more likely that IFTA and IFTB act as one unit that travels throughout the cilium by interacting either directly or indirectly with kinesin and dynein motors, and that individual IFT proteins have distinct and specific functions in ciliary protein transport and subsequent vertebrate development.

In addition to defects in signaling, Ift56hop primary cilia also exhibited microtubule doublet loss and misalignment, similar to abnormalities originally described in Ift56hop sperm tail flagella (Johnson and Hunt, 1971). This suggests that there might be global cilia structural defects in Ift56hop mutants. We also observed a strong reduction of IFT81 in Ift56hop cilia, one of the IFTB proteins with tubulin-binding capabilities (Bhogaraju et al., 2013; Taschner et al., 2016). The abnormal IFT81 localization to Ift56hop cilia might explain the microtubule defects. However, it is unclear at this point whether the primary defect is the inability of microtubules to build or maintain proper cilia structure during ciliogenesis and thus disrupt IFTB localization, or if loss of IFT56 affects IFTB complex integrity and potentially other cargo to disrupt cilium ultrastructure. Interestingly, we did not observe microtubule defects at the basal body level (microtubule triplets), suggesting that the cilium begins to form normally and is unable to continue at the axonemal level, or is unable to be maintained properly.

Despite reduced Shh signaling functions, cilia structure abnormalities and key IFTB localization defects, the Ift56hop phenotype is fairly mild compared with that of most IFTB mutants, which display severe defects including neonatal lethality, left-right defects and cyst formation (Berbari et al., 2011; Botilde et al., 2013; Eguether et al., 2014; Huangfu et al., 2003; Keady et al., 2012). Intriguingly, studies in Chlamydomonas show that although IFT56 mutants lose the distal tips of flagella and flagellar motility, mutant flagella did not display changes in IFT particle rates, suggesting that IFT56 is not essential for IFT complex movement within cilia (Ishikawa et al., 2014). This differs from most other IFTB mutants, which lack the IFT process altogether and thus lose cilia completely. Instead, these prior studies along with our current data suggest that IFT56 has a specific role within IFTB that promotes complex integrity and potentially other cargo to disrupt cilia ultrastructure.

Recently, independent of our studies, Bánfi and colleagues isolated the Ift56hop mutation via standard meiotic recombination mapping (Swiderski et al., 2014). Similar to our findings, they found that the Ift56hop phenotype results from reduced Shh signaling; however, Bánfi and colleagues reported no significant changes in Gli2 and Gli3 accumulation in Ift56hop cilia or changes in Gli protein levels. This contrasts with our studies, which found reduced Gli2/3 accumulation following pathway activation (SAG treatment) in MEFs. One possible explanation for this discrepancy is differences in experimental conditions between the studies, including amounts and duration of Hh pathway activation. Our in vivo studies showing that Gli2 is reduced in Ift56hop mutant cilia in both the limb and neural tube argue that our cell culture parameters more accurately reflect the situation in the embryo. Our data further suggest that the defects in ciliary accumulation of Gli
proteins and reduction in Shh signaling are likely to be secondary effects of abnormal IFTB trafficking and cilia function.

Having been long overlooked, cilia research has recently emerged as essential both for understanding developmental pathways in many animals as well as in deciphering human disease. An increasing number of human diseases (e.g. nephronophthisis, Jeune’s asphyxiating thoracic dystrophy, COACH) and syndromes (e.g. Joubert, Bardet-Biedl, oral-facial-digital type 1, Meckel) have been linked to abnormal development, morphology or function of the cilium (Badano et al., 2006; Lancaster and Gleeson, 2009; Nigg and Raff, 2009; Sharma et al., 2008; Waters and Beales, 2011). Given the breadth and severity of cilia-associated defects, elucidating the genetics of ciliopathies is essential to our understanding of how cilia impact development and human health. Our identification of IFT56 as a key regulator of IFTB ciliary localization and function further sheds light on the mechanisms of the IFT process and its importance in cilia function.

MATERIALS AND METHODS

Mouse strains

Mouse experiments were performed in accordance with Yale Institutional Animal Care and Use Committee guidelines. The hop-sterile (hop) strain was obtained from The Jackson Laboratory. All analyses were performed on the BALB/cByJ background, with the exception of skeletal preparations, which were analyzed on an F1 C57BL/6J-BALB/cByJ intercross background. Mice of both sexes were used and adult animals were maintained until ∼1-1.5 years of age. Ages of animals used in experiments ranged from E10.5 to E18.5.

Exome sequencing of hop

Genomic DNA from a hop homozygote and BALB/cByJ control was obtained from the Jackson Laboratory DNA Resource. Exomes were captured at the Yale Center for Genome Analysis using the NimbleGen SeqCap EZ Mouse Exome, following NimbleGen protocols. Captured pools were sequenced (75 bp, paired-end) on an Illumina HiSeq 2000 using previously described methods (Choi et al., 2009). We obtained ∼91 million (BALB/cByJ) to ∼113 million (hop) high-quality reads.

Illumina reads were first trimmed based on their quality scores to remove low-quality regions using the program Btrim (Kong, 2011). A cutoff of 20 for average quality scores within a moving window of 5 bp was used. Minimum acceptable read length was 25 bp. Other parameters of Btrim were set to defaults. The preprocessed reads were then aligned to the mouse genome reference sequence (mm9) using the BWA mapping program (Li et al., 2009). The mapping results were converted into SAMtools pileup format using SAMtools programs (Li et al., 2009). PCR duplicates were removed using the mdup command from SAMtools, resulting in ∼84× (BALB/cByJ) or ∼100× (hop) coverage across the exome. >97% of all bases included in the exome showed at least 8× coverage and >90% of the bases showed at least 20× coverage.

Mutation analysis

Single-nucleotide variations (SNVs) were called using SAMtools pileup command. Further filtering was performed using in-house scripts to exclude those SNV calls that had fewer than three reads or an SNP score less than 20.

Annotation was added based on the UCSC RefSeq gene model (Prutt et al., 2009). To identify homozygous mutations the filters were set to exclude any mutations with a major allele frequency <90%. We scanned the genome using a 100 kb sliding window to identify regions with homozygous single-nucleotide polymorphisms (SNPs). This provided unbiased mapping data and resulted in a list of potential causative mutations. The list of SNPs was then filtered for known SNPs (NCBI dbSNP build 128) and repeat elements.

Skeletal staining and expression analysis

Skelets were prepared and stained with Alcian Blue and Alizarin Red using standard methods (Nagy et al., 2003). In situ hybridizations and immunofluorescence analyses (primary antibodies are detailed in the supplementary Materials and Methods) were performed using standard methods (Nagy et al., 2003). RT-PCR is described in the supplementary Materials and Methods.

Transmission electron microscopy

E11.5 embryos were dissected in ice-cold 4% paraformaldehyde (PFA). A transverse cut was made between the forelimbs and hindlimbs, and the caudal portion of each sample was fixed with 2% PFA and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at room temperature, followed by an additional hour at 4°C. Embryos were washed three times in sodium cacodylate buffer at 4°C, and then processed for TEM according to standard procedures by the Yale Center for Cellular and Molecular Imaging.

Cell culture and immunostaining

Mouse embryonic fibroblasts (MEFs) were derived using standard methods (Nagy et al., 2003) and maintained in a mix of DMEM (1×hi-glucose, Gibco) plus 10% fetal bovine serum (FBS; Atlanta Biologicals). For cilia staining, cells were plated on coverslips, grown to confluence, and starved for 48-72 h in DMEM containing 0.5% FBS. Cells on coverslips were fixed with 2% PFA for 20 min at room temperature followed by 100% methanol for 5 min at room temperature, and then blocked with 1% goat serum and 0.1% Triton X-100 in PBS for 1 h at room temperature. Primary antibody (supplementary Materials and Methods) in blocking solution was added and incubated overnight at 4°C. Secondary antibody in blocking serum was added for 2 h at room temperature. Samples were mounted on slides (Denville) using Vectashield (Vector Laboratories). For cells subjected to SAG treatment, 0.1 mM SAG was added for 8 h after 48 h of cell starvation. All experiments involving MEFs were replicated on at least three animals of the same genotype.

Imaging and image analysis

Skeletal stainings were imaged on a Zeiss StReO Discovery microscope; neural tube and MEF cilia stainings (AC3, Arl13b, PC2, stable cell lines) were imaged on a Zeiss Axiowert using Zeiss AxioVision Product Suite. Gli2, Gli3, IFT81, IFT88, IFT27, IFT140, IFT122 (MEFs and tissue) samples were imaged with a Leica SP5 inverted confocal microscope using Leica Application Software. At least 20 cilia in three or more sections were imaged per slide, and biological and technical replicates were performed at least three times each. Image analysis was performed in ImageJ (NIH). To compare the intensity of Gli and IFT protein markers in MEF cilia, integrated density values were generated for individual axonemes by selecting the entire cilium, as marked by acetylated α-tubulin, and normalized to cilia length. The same analyses were performed for cilia in vivo, but Arl13b was used to mark the cilium. Tip and base intensity measurements were performed similarly, but with a fixed rectangular selection at the tip and base of individual cilia across multiple samples. Two-tailed Student’s t-tests were performed in Excel to compare two groups and determine significance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors performed some of the experiments. In addition, Y.K. analyzed the exome data; D.X. and S.D.W. prepared and edited the manuscript.

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disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. Dev. Biol. 287, 378-389.


