Primary Cilia and Mammalian Hedgehog Signaling

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It has been a decade since it was discovered that primary cilia have an essential role in Hedgehog (Hh) signaling in mammals. This discovery came from screens in the mouse that identified a set of genes that are required for both normal Hh signaling and for the formation of primary cilia. Since then, dozens of mouse mutations have been identified that disrupt cilia in a variety of ways and have complex effects on Hedgehog signaling. Here, we summarize the genetic and developmental studies used to deduce how Hedgehog signal transduction is linked to cilia and the complex effects that perturbation of cilia structure can have on Hh signaling. We conclude by describing the current status of our understanding of the cell-type-specific regulation of ciliogenesis and how that determines the ability of cells to respond to Hedgehog ligands.

Hedgehog (Hh) is one of a handful of signaling pathways that is used repeatedly for intercellular communication in development. Hh is critical for the development of nearly every organ in mammals, as well as in homeostasis and regeneration, and Hh signaling is disrupted in several types of cancer. Unlike other core developmental signaling pathways, vertebrate Hh signaling is completely dependent on a highly specialized organelle, the primary cilium.

The *Hh* gene was discovered in *Drosophila* based on the striking phenotype of fly larvae that lack Hh—the mutants do not develop the segmented anterior-to-posterior body plan and have ectopic denticles resembling a Hedgehog (Nüsslein-Volhard and Wieschaus 1980). Mutations in other genes with related phenotypes defined a signaling pathway in which Hh is the

ligand that acts through the membrane receptor Patched (PTCH1) and the seven transmembrane spanning protein Smoothened (SMO) to control the activity of a transcription factor Cubitus interruptus (Ci) (Forbes et al. 1993; Ingham 1998). In the mid-1990s, embryological experiments in chicks and targeted mutations in the mouse homologs of the *Drosophila* Hh pathway genes showed that the Hh signaling pathway is also critical for the development of many tissues and organs in vertebrates. Both the core players and their pathway relationships are conserved (Fig. 1) (Ingham and McMahon 2001). Because of this evolutionary conservation, it was completely unexpected when phenotype-based genetic screens in the mouse identified a set of proteins required for the formation of primary cilia that were also required

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Figure 1. The backbone of the Hedgehog (Hh) signal transduction pathway. The core of the Hh signaling pathway is conserved between *Drosophila* and vertebrates. In the absence of ligand, the Hh receptor Patched (PTCH1) keeps the pathway off by inhibiting the activity of the seven transmembrane-domain protein Smoothed (SMO). When SMO is inactive, the GLI/Ci (glioblastoma/Cubitus interruptus) transcription factors are proteolytically processed to make a transcriptional repressor that binds to Hh target genes and blocks their transcription. Binding of Hh to PTCH1 inhibits its activity, relieving the repression of SMO, which promotes conversion of full-length GLI/Ci into a transcriptional activator. In vertebrates, cilia are required for the production of both GLI-repressor and GLI-activator.

for mammalian (but not *Drosophila*) Hh signaling. The relationship between primary cilia and mammalian Hh signaling has turned out to be fascinating, complex, and directly relevant for specific human diseases, the ciliopathies.

Here, we describe the nature of primary cilia Hh-dependent patterning during mammalian development, with a primary focus on proteins that have been shown to affect Hh signaling in vivo in genetic studies. The combination of genetics, developmental biology, and cell biology has provided overwhelming evidence that mammalian Hh signal transduction absolutely depends on the primary cilium, defined the features of the cilium that are important for signaling, and characterized how different changes in cilium structure have distinct effects on Hh signaling. From these studies, the view emerges that Hh signaling and the primary cilium have coevolved, concentrating critical signaling components in the very small volume at the tip of the cilium to allow efficient responses to low levels of ligand.

HEDGEHOG SIGNALING IN MAMMALIAN DEVELOPMENT

There are three mammalian Hh proteins, Sonic (SHH), Indian (IHH), and Desert (DHH). SHH and IHH have important, and sometimes overlapping, functions in many tissues. SHH has particularly striking roles in specification of cell types in the nervous system and in patterning of the limbs, whereas IHH has critical roles in skeletal development. Many studies have connected both SHH and IHH signaling to the primary cilium (see below). DHH appears to be restricted to the gonads and may also depend on primary cilia for its activity (Nygaard et al. 2015).

Drosophila Hedgehog has important roles in the patterning of adult organs, such as the eye, wing, and leg (Burke and Basler 1997). In these contexts, Hh acts as a short-range inducer of cell fate, inducing target gene expression in a range of 2–3 cell diameters away from the source of the signal. *Drosophila* Hh can activate

expression of secreted proteins, such as Dpp (Bmp4) that can move further in tissues; as a result, *Drosophila* Hh has a profound role in the patterning of entire organs.

In contrast to the short-range signaling of *Drosophila*, the vertebrate Hh proteins can act over a field of cells that are many cell diameters away from the source of the protein. In neural patterning, SHH is first expressed in the notochord, which lies below the neural tube, and acts directly on cells in the ventral half of the neural tube to specify neural progenitors (Fig. 2) (Briscoe and Ericson 2001), and it appears that cells at least 20 cell diameters away from the source respond to Shh. In the limb, Shh is expressed in a classical organizer, the zone of polarizing activity (ZPA), and influences the pattern across most of the developing limb, again at a distance from its source (Ahn and Joyner 2004; Harfe et al. 2004). Indian hedgehog also acts at a distance to control proliferation and differentiation of chondrocytes (Long et al. 2001; Mak et al. 2008). Thus, one significant difference between Hh signaling in vertebrates and *Drosophila* appears to be that Hh ligands act at greater distances from their source in vertebrates; we hypothesize that long-range signaling requires a sensitized signal transduction system dependent on the primary cilium.

INTRAFLAGELLAR TRANSPORT IS REQUIRED FOR SHH SIGNAL TRANSDUCTION IN THE MOUSE EMBRYO

In the developing mouse embryo, perhaps the best-studied function of Shh is to direct patterning of neural progenitors, in which molecular markers make it possible to distinguish six different cell types that are specified by different levels of Shh activity (Briscoe and Ericson 2001; Dessaud et al. 2008). SHH made by the notochord induces formation of the floor plate and then specifies five additional neural cell types—V3 interneuron progenitors are specified adjacent to the floor plate by prolonged high levels of Shh signaling; motor neurons are determined by lower levels of Shh activity; and more dorsal interneurons are specified by yet lower levels of Shh. The final activity of the pathway depends

on both ligand concentration and duration of exposure to ligand, and final cell fate depends on a regulatory network set into motion by SHH (Dessaud et al. 2007; Balaskas et al. 2012). In the absence of SHH or its positive effector SMO, ventral neural cell types fail to be specified, whereas loss of the negative regulator PTCH1 leads to the formation of ventral cell types by all neural progenitors (Fig. 2).

The seminal discovery that linked the primary cilium to Hedgehog signaling in the mouse came from the characterization of chemically induced mutations, such as the *wimple* mutation that lacked Shh-dependent ventral neural cell types and affected genes that encoded intraflagellar transport (IFT) proteins (Huangfu et al. 2003). IFT is the process by which the cilium is assembled and maintained. It is powered by two conserved, dedicated microtubule motors—anterograde transport from the base to the tip depends on a plus-end-directed heterotrimeric kinesin-2 complex, KIF3a, KIF3b, and KIF associated protein 3 (KAP3) (Cole 1999) and retrograde transport from the tip to the base requires the minus-end-directed cytoplasmic dynein2 motor made up of a heavy chain, an intermediate chain, light intermediate chain, and several light chains (Hou and Witman 2015). These motors are coupled to the cargos by large electron-dense IFT trains, made up of two protein complexes, IFT-A and IFT-B (Pigino et al. 2009; Behal and Cole 2013).

Map-based cloning showed that the *wimple* mutation disrupted the gene encoding IFT172, an IFT-B complex protein, resulting in a complete lack of primary cilia. The same screen identified a partial loss-of-function allele of another IFTB complex protein, IFT88, which partially disrupted cilia assembly and showed a milder disruption of neural patterning. Targeted null alleles of *Ifi88* and *Kif3a* also caused the complete absence of cilia accompanied by a loss of Shh-dependent ventral neural cell types. Although *wimple* mutant embryos lacked ventral neural cell types, the mutants still expressed SHH, suggesting that without a cilium these mutants are unable to respond to Shh signaling (Huangfu et al. 2003). It was subsequently discovered that null mutations in other IFT-B

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Figure 2. Mutations in cilia genes alter Hedgehog (Hh)-dependent neural patterning. Schematics of the spatial distribution of neural cell types in the developing lumbar neural tube in mutants with abnormal cilia. Dorsal up, ventral down; the notochord is the small oval below the neural tube. SHH ligand released from the notochord and floorplate patterns the dorsoventral axis of the neural tube. The highest levels of Shh signaling specify floorplate cells (pink). Dorsal to the floorplate are V3 neural progenitors, which also require high levels of Shh signaling (magenta). Motor neurons (green) require Shh but are specified at lower concentrations of ligand. V2, V1, and V0 interneurons (orange) require even lower levels of Shh activity. Dorsal progenitors (yellow) are specified by default, and high levels of Shh signaling inhibit the specification of dorsal progenitors. *Smo* mutant embryos fail to specify ventral neural subtypes and all neural progenitors follow a dorsal fate. In *Patched1* mutant embryos, all neural progenitors follow a ventral fate and express markers of the floorplate and V3 neural progenitors. Mouse embryos that lack a core intraflagellar transport (IFT-B) protein or kinesin-II lack primary cilia and therefore are unable to respond to Hh signaling; these mutants lack floorplate, V3 interneurons, and most motor neurons, V0-V2 interneurons and dorsal cell types extend ventrally, but V0-V2 interneurons are specified. *Dync2h1* mutants, which lack the heavy chain of the dynein retrograde IFT motor and strong loss of IFT-A mutant embryos, have very short, bulged primary cilia and cannot transduce Hh signals efficiently; these mutants lack floor plate V3 interneurons and some motor neurons. In combination with Shh, *Smo*, or *Patched1*, double mutant embryos resemble single dynein or strong IFT-A mutants indicating that like IFT-B these components of the cilium assembly machinery are required downstream from SMO and PTCH1. Surprisingly, IFT-A mutant embryos that have a milder effect on cilia morphology (e.g., with cilia of near-normal length and bulged tips) have the opposite effect on neural patterning, with expanded ventral neural cell types. Cilia in *Arl13b*^{hnn/hnn} mutant embryos have structural defects in the microtubule axoneme, and the mutants fail to specify both the most ventral and most dorsal neural cell types. Double mutants that lack both a cilia component and *Smo* or *Patched1* have phenotypes similar to the cilia single mutants, indicating that the cilia machinery is required downstream from *Ptch1* and *Smo*.

genes (IFT52, IFT54, and IFT57) also block cilia formation and Shh-dependent specification of ventral neural cell types (Table 1), providing strong evidence that the primary cilium is required for a cell to receive Shh signals.

Double-mutant analysis defined the step in the Hh signaling pathway that requires

IFT. PTCH1, the Hh receptor, is a negative regulator of Hh signaling. *Ptch1*^{-/-} mutant embryos die at E9.0 and express markers of ventral neural cell types (floor plate and V3 interneuron progenitors) throughout the neural plate, even far from the source of SHH (Fig. 2). In contrast, double mutants that

Table 1. Subtle changes in cilia structure can decrease or increase Hh pathway activity, suggesting that the cilium provides more than just a permissive context for Hh signaling

Mouse gene	Function in cilia	Mouse embryo Hh pathway null phenotype (neural and limb)	Human phenotype
<i>Kif3a</i>	Anterograde IFT motor	Loss of SHH-dependent ventral neural cell types; polydactyly; midgestation lethality (Huangfu et al. 2003)	
<i>Dync2h1</i>	Retrograde IFT motor	Partial loss of ventral neural cell types; polydactyly; midgestation lethality (Huangfu and Anderson 2005; May et al. 2005)	Short rib dysplasia, with or without polydactyly (SRP) (Dagoneau et al. 2009; Merrill et al. 2009)
<i>Dync2li1 (D2lic)</i>	Dynein light intermediate chain		SRP (Taylor et al. 2015), Jeune syndrome (JATD) (Kessler et al. 2015)
<i>Wdr34</i>	Dynein light chain		SRP (Huber et al. 2013)
<i>Tctex1d2</i>	Light chain, IFT dynein		Jeune syndrome
<i>Ift25 (Hspb11)</i>	IFT-B	Perinatal lethality; weak loss of ventral neural cell types; partially penetrant polydactyly (Keady et al. 2012)	
<i>Ift27</i>	IFT-B	Perinatal lethality; weak loss of ventral neural cell types; partially penetrant polydactyly (Eguether et al. 2014)	
<i>Ift38 (Cluap1)</i>	IFT-B	Midgestation lethality; abnormal neural patterning (Botilde et al. 2013)	
<i>Ift52</i>	IFT-B	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Liu et al. 2005)	
<i>Ift54 (Traf3ip1)</i>	IFT-B	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Berbari et al. 2011)	Senior-Løken syndrome (Bizet et al. 2015)
<i>Ift57 (Hippi)</i>	IFT-B	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Houde et al. 2006)	
<i>Ift80 (WDR56)</i>	IFT-B	Hypomorphic allele only: short long bones; polydactyly; late gestation lethality (Rix et al. 2011)	SRP, JATD
<i>Ift88 (Polaris; Tg737)</i>	IFT-B	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Huangfu et al. 2003)	
<i>Ift172</i>	IFT-B	Loss of SHH-dependent ventral neural cell types; midgestation lethality (Huangfu et al. 2003)	Jeune (Halbritter et al. 2013)
<i>Ttc26 (hop)</i>	IFT-B	Partial loss of function allele only; abnormal neural patterning (Swiderski et al. 2014)	
<i>Ift121 (Wdr35)</i>	IFT-A	Polydactyly (Mill et al. 2011)	SRP (Mill et al. 2011); Ellis-van Creveld (EVC) syndrome (Caparrós-Martín et al. 2015)

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Table 1. *Continued*

Mouse gene	Function in cilia	Mouse embryo Hh pathway null phenotype (neural and limb)	Human phenotype
<i>Ift122</i>	IFT-A	Expansion of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Qin et al. 2011)	Sensenbrenner syndrome (Walczak-Sztulpa et al. 2010)
<i>Ift139</i> (<i>Ttc21b</i> / <i>Thm1</i> / <i>alien</i>)	IFT-A	Expansion of SHH-dependent ventral neural cell types; late gestation lethality; polydactyly (Tran et al. 2008)	NPHP12, JATD/SRP (Davis et al. 2011)
<i>Ift140</i>	IFT-A	Polydactyly, neural tube closure defects, (Miller et al. 2013)	SRTD9 (Perrault et al. 2012)
<i>Ift144</i> (<i>Wdr19</i>)	IFT-A	Weak allele: GOF neural; polydactyly. Strong allele: LOF (Liem et al. 2012) (Wicking)	Various: Senior-Løken, Sensenbrenner, Jeune, NPHP, RP (Bredrup et al. 2011; Fehrenbach et al. 2014)
<i>Tulp3</i>	IFT-A-associated	Expansion of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Norman et al. 2009; Patterson et al. 2009)	
<i>Gpr161</i>	GPCR	Expansion of SHH-dependent ventral neural cell types; midgestation lethality (Mukhopadhyay et al. 2013)	
<i>C2cd3</i> (<i>chick</i> <i>talpid2</i>)	Distal appendage protein	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Hoover et al. 2008; Ye et al. 2014)	Skeletal dysplasia (Cortés et al. 2016)
<i>Ofd1</i>	Centriole and centriolar satellites	Loss of SHH-dependent ventral neural cell types; midgestation lethality (Ferrante et al. 2006)	Oral-facial-digital syndrome (Ferrante et al. 2001)
<i>Ttbk2</i>	Kinase required for cilia initiation; associates with distal appendages	Loss of SHH-dependent ventral neural cell types; midgestation lethality (Goetz et al. 2012)	Spinocerebellar ataxia type 11 (Houlden et al. 2007)
<i>Talpid3</i>	Associated with distal appendages and PCM	Loss of SHH-dependent ventral neural cell types; midgestation lethality; polydactyly (Bangs et al. 2011)	Ciliopathy spectrum (Alby et al. 2015); Joubert syndrome (Stephen et al. 2015)
<i>Inpp5e</i>	Cilia membrane composition	Bielas et al. 2009; Jacoby et al. 2009	Joubert syndrome (JBTS1) (Bielas et al. 2009); MORM syndrome (Jacoby et al. 2009)
<i>Rab23</i>	Not clear	GOF neural; polydactyly (Eggenchwiler et al. 2001)	Carpenter syndrome (Jenkins et al. 2007)
<i>Kif7</i>	Kinesin-4 protein; controls dynamics of axonemal microtubules	Mild expansion of SHH-dependent ventral neural cell types; polydactyly, defective diaphragm (Cheung et al. 2009; Endoh-Yamagami et al. 2009; Liem et al. 2009; Coles and Ackerman 2013; He et al. 2014)	Joubert syndrome; acrocallosal syndrome

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Table 1. *Continued*

Mouse gene	Function in cilia	Mouse embryo Hh pathway null phenotype (neural and limb)	Human phenotype
<i>Tectonic</i> (<i>Tctn1</i>)	Transition zone protein	Abnormal patterning of SHH-dependent ventral neural cell types (Reiter and Skarnes 2006)	Joubert (Garcia-Gonzalo et al. 2011)
<i>Mks1</i>	Transition zone protein	Abnormal patterning of SHH-dependent ventral neural cell types (Weatherbee et al. 2009)	Meckel syndrome (Kyttälä et al. 2006)
<i>Fuzzy</i>	CPLANE complex	Loss of SHH-dependent ventral neural cell types; late gestation lethality; polydactyly (Heydeck et al. 2009)	
<i>Inturned</i>	CPLANE complex	Loss of SHH-dependent ventral neural cell types; late gestation lethality; polydactyly (Zeng et al. 2010)	
<i>Jbts17</i>	CPLANE complex	Skeletal and heart defects; no change in neural patterning detected (Damerla et al. 2015)	Joubert syndrome (Srour et al. 2012)
<i>Rsg1</i>	Small GTPase, CPLANE-associated	Loss of SHH-dependent ventral neural cell types; late gestation lethality; polydactyly (S Agbu and KV Anderson, in prep.)	
<i>Evc1/Evc2</i>	Ciliary membrane protein	EVC-like (Ruiz-Perez et al. 2007; Caparrós-Martin et al. 2013)	Ellis–van Creveld syndrome (Ruiz-Perez et al. 2003)
<i>Arl13b</i>	Ciliary membrane GTPase	Loss of both the most dorsal and most ventral neural cell types (Caspary et al. 2007)	Joubert syndrome (Cantagrel et al. 2008)
<i>Sas-4</i> (<i>p53</i>) (<i>Cpap/Cenpj</i>)	Centriole duplication	Loss of SHH-dependent ventral neural cell types; midgestation lethality (Bazzi and Anderson 2014)	Microcephaly (Bond et al. 2005)
<i>DZip1/Iguana</i>	Cilia base	Loss of SHH-dependent ventral neural cell types; midgestation lethality (Wang et al. 2013)	

References noted in table are for specific mouse and human proteins and phenotypes. This is not a complete list of references, and includes only the earliest mouse references, and is not a complete list of phenotypes.

IFT, intraflagellar transport; GPCR, G-protein-coupled receptor; PCM, pericentriolar material; CPLANE, ciliogenesis and planar polarity effector; SHH, Sonic Hedgehog; GOF, gain of function; LOF, loss of function; MORM, mental retardation, truncal obesity, retinal dystrophy, and micropenis.

lack both *Ptch1* and *Ift172* lack all ventral neural cell types, like the *Ift172* single mutants (Fig. 2) (Huangfu et al. 2003). Thus, the ectopic activation of the Hh pathway caused by loss of *Ptch1* depends on IFT172, arguing that IFT is required at a step in the signal transduction pathway downstream from PTCH1. Similar double mutant analysis showed that IFT172, IFT88, and KIF3A act downstream from both PTCH1 and SMO and upstream of the GLI proteins, at the heart of the signal transduction cascade (Fig. 1).

CENTRIOLE AND BASAL BODY PROTEINS ARE ALSO REQUIRED FOR THE RESPONSE TO Hh

In addition to IFT proteins, other proteins that build cilia are also required for the ability of cells to respond to Hh family ligands (Table 1). The doublet microtubules of the cilium are templated by the triplet microtubules of the mother centriole. Embryos that lack centrioles because of the lack of proteins required for centriole duplication, such as SAS4 (CPAP/CENPJ),

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can go through normal segregation at mitosis but fail to assemble cilia. These embryos can survive to midgestation (if a cell-cycle checkpoint is blocked by removal of p53) but Shh-dependent ventral neural cell types are not specified (Bazzi and Anderson 2014). The distal appendages of the mother centriole are required for docking on the plasma membrane (Tanos et al. 2013). The distal appendage protein C2cd3 is required for the centriole to dock on the ciliary vesicle (Ye et al. 2014) and *C2cd3* null mutant embryos lack cilia and lack Hh-dependent ventral neural cell types (Hoover et al. 2008). *Talpid3* and *OFD1* are both centrosome-associated proteins required for ciliogenesis and for the response to SHH in the neural tube (Ferrante et al. 2006; Bangs et al. 2011). *Tau tubulin kinase 2* (*TTBK2*) is required for cilia initiation and mutants lack all cilia and Hh-dependent ventral neural cell types (Goetz et al. 2012). Embryos that lack *CP110*, a protein that localizes to the distal ends of mother centrioles, also have fewer cilia and mild defects in Hh signaling (Yadav et al. 2016).

The CPLANE (ciliogenesis and planar polarity effector) protein complex is located at the basal body and includes downstream mediators of planar cell polarity in *Drosophila*, *Fuzzy*, *Inturned*, and *Fritz* (*Wdpcp*), and *Jbts17*, a gene responsible for Joubert syndrome in humans (Damerla et al. 2015; Toriyama et al. 2016). The CPLANE complex is required for efficient ciliogenesis as it functions to recruit IFT-A proteins to the cilium *RSG1*, a small GTPase that appears to be a peripheral member of the CPLANE complex. *Rsg1* mutant mice have fewer cilia of normal morphology, suggesting *Rsg1* has a specific role in cilia initiation (S Agbu and KVAnderson, in prep.). These mutants survive longer than IFT-B mutants, yet still display Hh signaling defects. Thus, diverse classes of proteins are required to build cilia and ensure efficient ciliogenesis. Loss of any type of protein required for normal cilia formation in the mouse prevents or attenuates cells from responding to Shh. Thus, it is the primary cilium, rather than another activity of the IFT machinery, which is required for mammalian cells to respond to Hh ligands.

CILIA ARE REQUIRED FOR RESPONSES TO Hh FAMILY LIGANDS IN ALL VERTEBRATE TISSUES

Conditional genetic deletion of *Ift88* and/or *Kif3a* has been used to test the role of cilia in Hh signaling in other mouse tissues. In every context examined, removal of cilia blocks the response to both SHH and IHH (Table 2). The defects observed include shortened bones of the limb, craniofacial defects, and loss of neural stem and progenitor cells.

Cilia are also required for Hh signaling in other vertebrates. As in the mouse, absence of cilia in the chick blocks the ability of cells to receive Hh signals (Yin et al. 2009). In zebrafish, loss of cilia leads to milder defects in Hh-dependent patterning, in part because of the maternal contribution of cilia proteins or mRNAs to the zebrafish embryo, which masks the phenotype in homozygous embryos, and in part because of the different roles of the zebrafish GLI proteins in Hh responses (Huang and Schier 2009; Tay et al. 2010; Ben et al. 2011).

In humans, it is not possible to directly examine the requirement of cilia for Hh signaling during development, as mutations that completely disrupt IFT genes would presumably result in lethality in the first half of gestation, as in mice, and would not be detected. However, many mutations in human cilia genes do not completely ablate cilia, allowing survival to the end of gestation with comparatively mild Hh pathway defects (Table 1). For example, mutations in the cytoplasmic dynein-2 complex cause short ribs (narrow chest)—short-rib dysplasia, with or without polydactyly (SRP) or Jeune asphyxiating thoracic dystrophy (JATD) (Dagoneau et al. 2009; Merrill et al. 2009).

Further evidence that cilia are important for Hh signaling in humans comes from their impact on two human tumor types, medulloblastoma and basal cell carcinoma, which are both caused by gain of Hh signaling. In mouse medulloblastoma or basal cell carcinoma triggered by activated SMO, conditional removal of cilia blocks tumor formation, consistent with the requirement for cilia at a step downstream from SMO. The same tumors can also be caused by

Table 2. In every context examined, removal of cilia blocks the response to both SHH and IHH

Tissue	Conditional allele	Cre	Phenotype
Cranial neural crest	<i>Kif3a</i>	<i>Wnt1</i>	Craniofacial defects (Liu et al. 2014)
Endochondral bone (Ihh-dependent)	<i>Ift88</i>	<i>Prx1</i>	Short bones (Haycraft et al. 2007)
Postnatal cartilage	<i>Kif3a</i>	<i>Col2a1-Cre</i>	Craniofacial defects (Koyama et al. 2007)
Neural stem cells	<i>Kif3a</i>	<i>hGFAP</i>	Loss of hippocampal stem cells (Han et al. 2008)
Hippocampal stem cells	<i>Ift20</i>	<i>mGFAP</i>	Reduction hippocampal amplifying progenitors (Amador-Arjona et al. 2011)
Postnatal B1 SVZ neural stem cells	<i>Kif3a</i>	<i>Adeno-Cre (injected)</i>	Decreased proliferation of neural stem cells (Tong et al. 2014)
Cerebellar granule cell precursors	<i>Kif3a</i>	<i>hGFAP</i>	Loss of cerebellar granule cell progenitors (Chizhikov et al. 2007; Spassky et al. 2008)
Basal cell carcinoma	<i>Kif3a; Ift88</i>	<i>Keratin14</i>	Removal of cilia inhibited tumors induced by activated Smoothened. Removal of cilia accelerated tumors induced by activated Gli2 (Wong et al. 2009)
Medulloblastoma	<i>Kif3a</i>	<i>hGFAP</i>	Removal of cilia inhibited tumors induced by activated Smoothened. Removal of cilia accelerated tumors induced by activated Gli2 (Han et al. 2009)

SHH, Sonic Hedgehog; IHH, Indian Hedgehog; SVZ, subventricular zone.

activation of GLI2; in these cases, removal of cilia makes the tumors more aggressive because GLI3 repressor is no longer generated resulting in a greater net GLI activation.

TRAFFICKING OF PATHWAY PROTEINS WITHIN THE CILIUM IS REQUIRED FOR Hh SIGNALING

Immunolocalization experiments showed that all the proteins required for transduction of Hh signals are enriched in primary cilia and change their distribution in response to ligand (Fig. 3). The first demonstration of cilia localization came for the membrane protein SMO, which accumulates in the cilium within an hour following stimulation of the Hh pathway (Corbit et al. 2005; Rohatgi et al. 2007). As total SMO protein levels are unaltered, accumulation of SMO in the cilium is a consequence of translocation of a ready-made pool of SMO and not from newly synthesized protein (Rohatgi et al. 2007). A common hydrophobic and basic residue motif following the seventh transmembrane domain at the carboxyl terminus of SMO is required for localization of SMO to the cilium in the presence of SHH (Händel

et al. 1999; Brailov et al. 2000; Dwyer et al. 2001; Corbit et al. 2005; Aanstad et al. 2009). Single-molecule imaging indicates that once in the cilium SMO moves by diffusion within the membrane, rather than by IFT (Milenkovic et al. 2015). Localization of SMO to the cilium is, however, not sufficient for signaling, as SMO accumulates in primary cilia that lack Dync2h1, the heavy chain of the retrograde dynein IFT motor, but *Dync2h1* mutants lack ventral neural cell types (Huangfu and Anderson 2005; May et al. 2005).

Even more remarkable, all three transcription factors of the GLI family, the mediators of Hh-regulated transcription, are enriched at cilia tips in the absence of Hh ligand and become further enriched in response to pathway activation (Haycraft et al. 2005). Only full-length GLI proteins localize to cilia, whereas the proteolytically processed repressor forms that lack the carboxy-terminal half of the protein do not (Wen et al. 2010; Santos and Reiter 2014). Deletion analysis identified a 330-amino-acid (out of the 1544 in the full-length protein) central region of GLI2 is required for cilia targeting. SUFU, a key negative regulator of vertebrate Hh signaling, binds to GLI preventing its acti-

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Figure 3. Hedgehog (Hh) signal transduction in the primary cilium. The primary cilium has several spatially distinct regions that promote normal Hh signal transduction. Entry into the cilium is gated at the transition zone; the EVC zone facilitates pathway activation in some cell types; the cilia tip compartment, defined by KIF7, is the site of enrichment and activation of the Gli1/SUFU complex; and proteolytic processing to make Gli1 repressors that may occur at the base where protein kinase A (PKA) is localized and leads to formation of Gli3R. (*Legend continues on following page.*)

vation and is also highly enriched at cilia tips (Haycraft et al. 2005).

PTCH1 has 12 transmembrane domains and the region carboxy-terminal to the last transmembrane domain is required for cilia localization and signaling (Kim et al. 2015). PTCH1 is localized to cilia in the absence of ligand and binding of SHH to PTCH1 triggers its removal from the cilium, although removal of PTCH1 from the cilium is not required for Hh pathway activation. During the same interval after exposure to ligand when PTCH moves out of the cilium, SMO translocates into the cilium (Rohatgi et al. 2007).

CILIA ARE REQUIRED FOR BOTH KEEPING THE PATHWAY OFF AND TURNING THE PATHWAY ON

In mammals, the GLI family of transcription factors, GLI1, GLI2, and GLI3 implement the transcriptional responses to Hh family ligands (Bai et al. 2004). In the absence of ligand, proteolytically processed forms of GLI proteins repress expression of Hh target genes (primarily GLI3, with a minor role for GLI2). In the presence of ligand, processing of the repressor forms is blocked and, instead, full-length GLI proteins are converted (by an unknown mechanism) to GLI activators, principally mediated by GLI2. The formation of both GLI repressor and activator depend on cilia (Huangfu and Anderson 2005; Liu et al. 2005; May et al. 2005).

Proteolytic processing of the GLI proteins depends on phosphorylation by cAMP-dependent PKA, a key evolutionarily conserved com-

ponent of the Hh signal transduction pathway (Wang et al. 2014). In contrast to *Drosophila*, mammalian PKA does not act only by promoting formation of GLI repressor, it is also required to prevent inappropriate activation of GLI2 (Tuson et al. 2011). In addition to the phosphorylation sites on GLI2 required for proteolytic processing, phosphorylation of two additional PKA sites of GLI2 and GLI3 prevents formation of the fully activated form of the transcription factors (Niewiadomski et al. 2014).

Both the catalytic and regulator subunits of PKA are highly enriched at the base of the cilium (Barzi et al. 2010; Tuson et al. 2011). Mutant mouse embryos that lack both genes that encode catalytic subunits (*PKA nulls*) show a very strong activation of the Hh pathway, in which all cells in the neural tube acquire the most ventral fates (Tuson et al. 2011). Compound mutants that lack both PKA catalytic subunits and also lack cilia have a neural phenotype similar to cilia mutants, showing that the action of PKA depends on events that take place in cilia. Although both catalytic and regulatory subunits of PKA are highly enriched at the base of the cilium, recent proximity ligation experiments provide biochemical evidence that regulatory subunits of PKA localize to the cilium (Mick et al. 2015), suggesting that sensing of cAMP by PKA may take place inside cilia.

PKA is activated by cAMP, and several adenylyl cyclases that produce cAMP, including AC3, AC5, and AC6, are enriched in cilia (Bishop et al. 2007; Mick et al. 2015; Vuolo et al. 2015). These, in turn, are regulated by G-protein-coupled receptors (GPCRs), including

Figure 3. (Continued) In the absence of Hh signal, PTCH1 and Gpr161, both negative regulators of the pathway, are present in the cilia membrane. Gpr161 trafficking into the cilium depends on TULP3 and intraflagellar transport (IFT)-A; GPR161 appears to activate G α s, which activates adenylyl cyclase, which increases the levels of cAMP, thereby activating PKA. Activated PKA phosphorylates sites on GLI3 that promote partial proteolysis by β TrCP/Cul1 and the proteasome, generating Gli3 repressor (Gli3R), which moves to the nucleus and represses expression of Hh target genes. GLI2 and GLI3 are trafficked to the tip of the cilium in the absence of ligand in a complex with SuFu, and processing of GLI3 depends on it having transited the cilium. Binding of Hh to PTCH1 triggers its removal from the cilium, allowing Smoothened (SMO) to translocate into the cilium where it can activate downstream signaling. Gpr161 also exits the cilium after exposure to ligand. Binding of EvC to SMO near the base of the cilium promotes SMO activity. In the presence of ligand, the GLI/SUFU complex accumulates to high levels at the tip of the cilium, where dissociation of the complex allows formation of the activator form of GLI2.

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Gpr161, which activates ACIII and thereby increases cAMP levels (Mukhopadhyay et al. 2013). Like PTCH1, Gpr161 is present in the cilia membrane in the absence of ligand and moves out of the cilium in response to ligand. This movement is regulated by TULP3, a member of the vertebrate tubby-like family of proteins, which links GPCRs, including Gpr161, to IFT-A, which transports them into the cilium (Nishina et al. 1998; Mukhopadhyay et al. 2010).

Small G proteins, including G α s, mediate the actions of GPCRs. In mouse embryos that lack G α s (Gnas), the Hh pathway is ectopically activated with a phenotype similar to that of PKA or *Ptch1* mutant embryos, indicating that G α s negatively regulates the Hh pathway (REGARD et al. 2013). Thus, the Hh pathway is kept off in the absence of ligand by Gpr161, which is localized to the cilium by TULP3 and activated by G α s. Gpr161 subsequently activates adenylyl cyclase, leading to increased cAMP and increased PKA activity, which promotes GLI repressor formation and prevents GLI activation (Fig. 3) (Mukhopadhyay and Rohatgi 2014).

CHANGES IN AXONEMAL STRUCTURE OR CILIA MEMBRANE COMPOSITION CAN INCREASE OR DECREASE Hh PATHWAY ACTIVITY

The requirement for primary cilia for Hh signaling and the localization of pathway proteins to the cilium would be consistent with the cilium acting as a scaffold for Hh signal transduction or a site for concentration of Hh pathway proteins. However, subtle changes in cilia structure can decrease or increase Hh pathway activity, suggesting that the cilium provides more than just a permissive context for Hh signaling. (References to specific mouse and human proteins and phenotypes are in Table 1.)

Embryos carrying mutations in the gene that encodes the heavy chain of the retrograde motor cytoplasmic dynein 2 (*Dync2h1*) assemble cilia; however, the axoneme is bulged because of accumulation of IFT complexes and other proteins trapped in the cilium as a consequence of defective retrograde transport. Like IFT-B or *Kif3a* mutant embryos, *Dync2h1* mu-

nants lack floor plate and V3 interneuron progenitors indicating these cells cannot respond to high levels of Shh signaling. However, unlike IFT-B or *Kif3a* mutants, motor neurons are specified in the caudal spinal cord of *Dync2h1* mutants in which they are intermingled with V2 interneurons (Fig. 2) (Huangfu and Anderson 2005; May et al. 2005). Thus, low levels of Hh signaling can still be transduced in the bulged *Dync2h1* cilia.

Although null mutations in most IFT-B components block cilia formation, abnormal cilia form in most mutants that lack one of the six proteins of the IFT-A complex. Mutations that strongly disrupt the mouse IFT-A proteins IFT122 or IFT139 cause bulged cilia, similar to mutants that lack Dynein2. However, unlike mutants that disrupt Dynein2, the IFT-A mutants show a gain-of-activity of the Hh pathway in the neural tube, with expanded domains of Shh-dependent ventral neuron cell types (e.g., motor neurons) (Fig. 2) (Tran et al. 2008; Qin et al. 2011). This gain-of-function for Hh pathway activity is independent of ligand, suggesting that IFT-A plays a specific role in keeping the pathway off in the absence of ligand. A similar phenotype is seen in embryos that lack TULP3 (Mukhopadhyay et al. 2010).

The effects of mutations in IFT-A genes on Hh signaling are complex. A weak allele of *Ift144* (WDR19) shows bulged cilia and ventralization of the neural tube similar to that seen in IFT122 or 139 (Table 1). However, a stronger allele that produces only very short bloated cilia with a highly disrupted axoneme causes a loss of Hh signaling in the neural tube. Double mutants that carry both the weak allele of *Ift144* and an allele of *Ift122* show the short bulged cilia and loss-of-function Hh neural phenotype, suggesting that some IFT-A proteins have overlapping functions and that a complete lack of IFT-A complex activity leads to the formation of very short cilia and a loss of Hh activity (Liem et al. 2012). Human mutations in IFT-A genes cause a spectrum of phenotypes, ranging in severity from retinitis pigmentosa in the adult to SRP associated with perinatal lethality (Table 1).

Even small changes in IFT function can have subtle effects on Hh signaling activity. IFT25

and IFT27 form an IFT-B subcomplex. In contrast to other IFT-B proteins, mutants that lack either of these proteins make primary cilia that have apparently normal structure. The mouse mutants survive to birth, when they show mild Hh pathway phenotypes and defects in trafficking of GLI2, SMO, and PTCH1. IFT27 appears to be important for loading of retrograde IFT cargo in trypanosomes (Huet et al. 2014), and given the evolutionary conservation of IFT proteins, it is likely that the Hh defects in mouse *Ift25* and *Ift27* mutants are the result of relatively subtle changes in global IFT. This indicates that Hh signaling is sensitive to even small changes in IFT function.

ARL13B is an ARF-family GTPase that is a component of the ciliary membrane that is required for normal cilia structure—in the absence of ARL13B, cilia are short and the B-tubules of the axoneme are frequently open. The neural tube of *Arl13b* mouse mutants lacks both the most ventral and the most dorsal cell types, indicating that when ciliary structure is disrupted in this way it is not possible to achieve the highest level of Hh signaling or to prevent ectopic low-level activation of the pathway.

It has been supposed that cilia length affects Hh signaling; however, it appears not to be the case. Cilia on *Rfx3* mutant cells are about half the normal length (Bonnafe et al. 2004) and embryos that overexpress ARL13B have cilia that are 50% longer than wild-type (Bangs et al. 2015), but both genotypes are viable and apparently have a normal pattern of Hh-dependent neural cell types.

The ciliary membrane has a distinct lipid composition; the inositol phosphate PI(4)P is enriched in the entire ciliary membrane and PI(4,5)P₂ is enriched at the base of the cilium. Inpp5e, a ciliary phosphoinositide 5-phosphatase, is required to maintain this distribution and is one of the genes mutated in Joubert syndrome (Chávez et al. 2015; Garcia-Gonzalo et al. 2015). In *Inpp5e* mutant mice, ciliary PI(4,5)P₂ levels are elevated, causing disrupted Hh signaling, apparently a result of inappropriate accumulation of TULP3, which binds to these phosphoinositides. *Inpp5e* mutant mice survive to birth when they show polydactyly, suggesting

that they have a significant, but relatively mild disruption of Hh signal transduction.

KIF7 ORGANIZES THE CILIA TIP COMPARTMENT

One rationale for why Hh signaling requires the primary cilium is that it concentrates components of the Hh signal transduction cascade in a small volume, promoting their interactions by mass action. It has been calculated that concentrating the entire pool of a protein into the cilium would increase its concentration by two to three orders of magnitude (Nachury 2014). However, certain Hh components, GLI, SuFu, and KIF7, are strongly enriched at the cilia tip. Full-length Gli proteins form complexes with SuFu, and data indicate that separation of SuFu and GLI proteins is critical for GLI activation (Humke et al. 2010); it is likely that this separation occurs or is regulated by events that happen at the tip of the cilium. KIF7 also binds directly to both GLI2 and GLI3 (Cheung et al. 2009; Endoh-Yamagami et al. 2009).

The tip compartment of the primary cilium is organized, at least in part, by KIF7, an evolutionarily conserved core component of the Hh pathway (He et al. 2014). KIF7 and its *Drosophila* homolog Cos2 have both positive and negative roles in Hh signal transduction. KIF7, a member of the Kinesin-4 family, binds microtubule plus-ends at the tip of the cilium to organize microtubule architecture at the tip (He et al. 2014). In the absence of KIF7, axonemal microtubules have variable lengths, and GLI and SuFu are localized in multiple puncta along the ciliary axoneme that appear to correspond to ectopic cilia tip compartments. The mislocalization of the GLI/SUFU complex causes inappropriate, ligand-independent GLI activation and the mild ectopic activation of the pathway seen in *Kif7*^{-/-} mutant embryos.

CELL-TYPE-SPECIFIC DIFFERENCES IN CILIA COMPOSITION CAN MODULATE Hh SIGNALING

Most proteins that have a role in Hh signaling in the cilium act in all cell types. Two exceptions

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are EVC and EVC2, transmembrane proteins responsible for Ellis–van Creveld syndrome, a distinctive ciliopathy associated with short limbs, a narrow chest (short ribs), and polydactyly (Ruiz-Perez et al. 2003). Mouse *Evc* is highly expressed in differentiating chondrocytes and all the cartilaginous components of the skeleton. *Evc* mutant mice survive to birth, and some can survive to adulthood; like the affected people, the mice have short bones, short ribs, and abnormal teeth, although the response to Shh in the neural tube appears to be nearly normal (Caparrós-Martín et al. 2013). Specific partial loss-of-function mutations that disrupt the IFT-A protein IFT121/WDR35 also cause EVC syndrome, and both EVC proteins and SMO fail to localize to *Ift121/Wdr35* mutant MEF cilia (Caparrós-Martín et al. 2015). As other IFT-A proteins are also required for the recruitment of transmembrane proteins, including SMO, to cilia (Liem et al. 2012), it appears IFT-A has a general role in recruiting membrane proteins, including the EVC proteins, to the cilium, and chondrocytes appear to be particularly sensitive to the loss of IFT-A.

The Tectonic complex localizes to the transition zones and appears to have complex tissue-specific functions in ciliogenesis (Table 1) (see Vaisse et al. 2016). Mutant fibroblasts assemble cilia but cells in the mutant embryonic node and mesenchymal cells adjacent to the neural tube fail to form cilia. As mutants show both defects in neural patterning and polydactyly, the tissue specificity of the Tectonic complex merits further study.

LINEAGE DETERMINES WHICH CELLS HAVE PRIMARY CILIA IN THE MOUSE EMBRYO

In cell culture, primary cilia are present in the G₀ phase of the cell cycle and can be induced by serum removal (Plotnikova et al. 2009). In contrast, many proliferating cells in the mouse embryo and adult organs in vivo have a primary cilium. Scattered reports indicate that some specific cell types in the whole animal, such as acinar cells of the adult pancreas (Aughstien 2001), lack primary cilia. Transgenic mice that

carry fluorescent markers for basal bodies and cilia have been used to systematically identify ciliated and unciliated cells (Bangs et al. 2015). No cilia are present on cells of the preimplantation embryo; cilia first appear on cells of the epiblast lineage (the embryo proper) shortly after cavitation (E6.0) and all cells derived from the epiblast (ectoderm, mesoderm, and definitive endoderm) are ciliated in the midgestation embryo. In contrast, cells of extraembryonic lineages, the visceral endoderm and trophoctoderm, have centrioles but lack primary cilia. Cells of both the trophoctoderm lineage and from the embryonic mesoderm contribute to the placenta and cells of the visceral endoderm and mesoderm contribute to the yolk sac. These lineages lack cilia until at least E14.5 (Bangs et al. 2015).

Stem cell lines that correspond to the different embryonic lineages (Ralston and Rossant 2005) recapitulate the cilia status of the lineages in the embryo—all nondividing epiblast stem cells (EpiSCs) (which recapitulate the status of the E6.5 epiblast) are ciliated, whereas extraembryonic endoderm stem cells (XEN cells) and trophoctoderm stem cells (TS cells) lack cilia (Bangs et al. 2015). For all three stem cell types, the presence or absence of primary cilia on embryo-derived stem cells is independent of the presence of serum.

The absence of primary cilia on cells of extraembryonic origin implies that these cells cannot respond to Hh ligands. The first time that Hh signaling is active in the mouse embryo is at the beginning of gastrulation, when Ihh expressed in the extraembryonic visceral endoderm signals to the adjacent epiblast derived extraembryonic mesoderm, which is ciliated. This is necessary to promote blood island formation (Fig. 4) (Farrington et al. 1997; Dyer et al. 2001). In the placenta, the only cells that respond to Hh signaling are embryo-derived ciliated cells that surround the fetal blood vessels (Fig. 4) (Jiang and Herman 2006). It is interesting to speculate that normal development of the placenta and yolk sac depends on preventing extraembryonic lineages from responding to Hh signals and this is achieved by blocking ciliogenesis in these cells.

Figure 4. Lineage-dependent cilia formation in the early mouse embryo. (A) Three lineages defined in the preimplantation embryo persist in the postimplantation embryo (E8.0 embryo shown). Two extraembryonic lineages, the trophectoderm (blue) and visceral endoderm (red) contribute to the placenta and yolk sac respectively; these two lineages lack primary cilia. Nearly all nonmitotic cells of the third lineage, the epiblast (black), are ciliated; this lineage gives rise to almost all cells of the embryo proper, as well as to mesoderm-derived components of the placenta and yolk sac. (B) Paracrine Hh signaling in the yolk sac. *Upper* drawing represents the two layers of the E8.0 yolk sac (lassoed in panel A). The unciliated visceral endoderm layer produces IHH, but does not activate Hh target genes. The adjacent ciliated extraembryonic mesoderm responds to the IHH produced by the visceral endoderm and produces BMP4, which leads to formation of the blood islands (*lower* panel). (Image based on data in Baron 2001.) (C) A blood vessel in the E14.5 yolk sac from an embryo expressing a *Centrin2-GFP*, a marker for the basal body, and *ARL13b-mCherry*, a marker of the cilia membrane. The lineage relationships are preserved from earlier development—the visceral endoderm (cell layer at the top of the image) has *GFP*⁺ centrioles, but lacks cilia (arrowhead), whereas the extraembryonic mesoderm that surrounds the vessel filled with round blood cells is ciliated (arrow). (Image from Bangs et al. 2015; modified, with permission, from the authors.)

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These cases of Hh ligand made by extraembryonic epithelia signaling to adjacent Hh-responsive ciliated cells reflect one of the common modes of Hh signaling in other organs. Paracrine signaling from ligand-producing cells to adjacent Hh responsive cells has been seen in a number of organs, including the digestive tract and the prostate (Yu et al. 2009; Mao et al. 2010). Thus, it will be interesting to see whether tissue-specific absence of primary cilia enforces paracrine, rather than autocrine Hh signaling.

CONCLUDING REMARKS

Vertebrate Hedgehog signal transduction, which is essential for the development and maintenance of most organs, takes place in the primary cilium. The responses to Hh ligands are exquisitely sensitive to alterations in cilia structure—disruptions of many cilia components reduce the response to Hh ligands, but disruption of specific cilia proteins can also cause inappropriate activity of the pathway, and some proteins can be altered in different ways that enhance or interfere with Hh signal transduction. Dozens, and probably hundreds, of proteins are required to build the primary cilium; because of the many essential roles of Hh signaling, mutations in cilia genes collectively have a broad impact on human health.

The central effectors of Hh signaling, GLI proteins and the negative regulator SUFU, are regulated in the tiny compartment at the tip of the primary cilium. A single core conserved component of the Hh pathway, the kinesin-family protein KIF7, has an essential role in organizing the cilia tip. The dual roles of KIF7 in Hh signal transduction and primary cilia structure argue for an ancient intertwining of Hh signal transduction with the structure of the primary cilium.

The distribution of primary cilia determines the ability of tissues to respond to Hh ligands, but the tissue distribution of primary cilia and the mechanisms that regulate cilia assembly and disassembly in vivo are poorly understood. A deeper understanding of the mechanisms that control cilia formation in vivo is likely to pro-

vide the foundation for therapeutic interventions in both ciliopathies and cancer.

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