



Published in final edited form as:

Curr Top Dev Biol. 2008 ; 85: 225–260. doi:10.1016/S0070-2153(08)00809-0.

THE PRIMARY CILIUM: AT THE CROSSROADS OF MAMMALIAN HEDGEHOG SIGNALING

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Abstract

Cilia function as critical sensors of extracellular information, and ciliary dysfunction underlies diverse human disorders including situs inversus, polycystic kidney disease, retinal degeneration and Bardet-Biedl syndrome. Importantly, mammalian primary cilia have recently been shown to mediate transduction of Hedgehog (Hh) signals, which are involved in a variety of developmental processes. Mutations in several ciliary components disrupt the patterning of the neural tube and limb bud, tissues that rely on precisely coordinated gradients of Hh signal transduction. Numerous components of the Hh pathway, including Patched, Smoothed and the Gli transcription factors, are present within primary cilia, indicating that key steps of Hh signaling may occur within the cilium. Because dysregulated Hh signaling promotes the development of a variety of human tumors, cilia may also have roles in cancer. Together, these findings have shed light on one mechanism by which primary cilia transduce signals critical for both development and disease.

Hh signals pattern diverse developing tissues

Hh proteins are secreted lipoproteins that specify cell patterning in many metazoa and in various developmental contexts (Lum and Beachy 2004; Huangfu and Anderson 2006; Wang et al. 2007b). Investigations into Hh signal transduction have proved to be a rich source of insight into fundamental mechanisms by which cells coordinate their functions during development and homeostasis, and have also revealed that dysregulated Hh signaling underlies a variety of human congenital diseases and cancer (Ruiz i Altaba et al. 2002; McMahon et al. 2003; Hooper and Scott 2005). Indeed, studies on Hh signaling have continued to yield unanticipated surprises, one of which, the involvement of the primary cilium in vertebrate Hh signaling, is the focus of this review.

Hh was initially identified by Nüsslein-Volhard and Wieschaus in a systematic screen for larval segmentation defects in *Drosophila melanogaster* (Nüsslein-Volhard and Wieschaus 1980). Whereas flies possess a single Hh gene, mammals have three, the best studied of which encodes Sonic hedgehog (Shh), an important regulator of both embryonic development and post-natal homeostasis (Echelard et al. 1993; Chiang et al. 1996). The other two members of this family, Indian hedgehog and Desert hedgehog, participate in bone development and spermatogenesis, respectively (Bitgood et al. 1996; Vortkamp et al. 1996; St-Jacques et al. 1999).

All Hh proteins are initially synthesized as inactive precursors that possess an amino-terminal signaling domain and a carboxy-terminal intein-like domain which is later removed by autocatalytic cleavage (Perler 1998). In the choanoflagellate *Monosiga brevicollis*, separate proteins containing domains homologous to the Hh signaling domain and the intein-like

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domain have recently been identified, suggesting that Hh proteins evolved through shuffling of domains found in our unicellular antecedents (Snell et al. 2006; King et al. 2008). However, some organisms, most notably *Caenorhabditis elegans*, lack proteins with homology to the Hh amino-terminal signaling domain, suggesting that the Hh pathway has subsequently diverged in some metazoan phyla (Bürglin 1996).

In addition to its role in segment polarity, *Drosophila* Hh regulates the development of a variety of other tissues, including the wing, eye, muscle and gut (McMahon et al. 2003; Hooper and Scott 2005). Similarly, vertebrate Shh is important for proper morphogenesis of organs such as the skin, eye, lung, muscle and pancreas (McMahon et al. 2003). Perhaps the best understood roles of Shh are in the patterning of the neural tube and limb bud.

In the developing mouse embryo, *Shh* is first expressed at embryonic day (E) 7.5 in the ventral node (Echelard et al. 1993). By E9.5, *Shh* is expressed by cells in the ventral forebrain, notochord, floor plate of the neural tube, and posterior limb buds. In the mouse neural tube, Shh is critical for specifying neuronal cell fates in a concentration- and time-dependent manner (Martí et al. 1995; Roelink et al. 1995; Ericson et al. 1997; Dessaud et al. 2007). Shh is initially produced by cells in the notochord and is essential for the specification of cells in the overlying neural tube as floor plate (or, in zebrafish, as lateral floor plate) (Martí et al. 1995; Roelink et al. 1995; Chiang et al. 1996; Schauerte et al. 1998). Once specified, the floor plate also expresses Shh, becoming a second source of axial midline Shh that patterns the remainder of the ventral neural tube.

Shh produced by the notochord, the floor plate and possibly the gut moves away from these sources, establishing a dorsoventral gradient in the neural tube that specifies the fate of five additional neuronal subtypes (Figure 1A) (Ericson et al. 1997). The ventral-most cells in the neural tube—those of the floor plate and the V3 interneurons—are normally exposed to and specified by the highest concentrations of Shh, whereas more dorsal cells are exposed to successively lower concentrations of Shh. Thus, *Shh* mutants display a loss of the floor plate and almost all ventral neural subtypes, with a concomitant expansion of more dorsal neural subtypes into the ventral neural tube (Figure 1B) (Chiang et al. 1996). Conversely, ectopic placement of Shh sources is sufficient to specify ventral neural cell types in the lateral neural tube (Roelink et al. 1994).

In addition to concentration of Shh, duration of Hh signaling is also an important factor in determining neural cell fates. Neural explants can be induced to become progressively more ventral neural subtypes either by exposing these cells to increasing concentrations of Shh or by lengthening the time of exposure (Dessaud et al. 2007). Similarly, ventral neural cells that eventually become V3 interneurons initially express a marker of the more dorsal motor neuron fate, suggesting that ventral cell fates are sequentially determined *in vivo* by the duration of Hh pathway activity (Dessaud et al. 2007). Together, these results provide evidence that both concentration and duration of Hh signaling generate the diversity of ventral neural tube precursors.

A similar scenario occurs in the limb buds, where Shh specifies digit identity and number in a concentration- and time-dependent manner. During embryonic development, three organizing centers are crucial for establishing the axes of the nascent limb bud: the apical ectodermal ridge, which is necessary for the outgrowth of the limb and patterning of the proximal-distal axis; the surface ectoderm, which regulates dorsal-ventral patterning; and the zone of polarizing activity (ZPA), which specifies digit number and identity. The ZPA is located in the posterior mesenchyme of the limb bud and secretes Shh, which forms a gradient as it moves anteriorly (Figure 1A) (Riddle et al. 1993). Posterior cells within the ZPA are exposed to high concentrations of Shh for the longest period of time and are specified to form digit five (the

little finger in the human hand), whereas anterior cells in the limb bud are exposed to little or no Shh, and are specified into digit one (thumb) (Ahn and Joyner 2004; Harfe et al. 2004; Scherz et al. 2007). The rest of the digits are specified by intermediate levels and/or durations of Shh signaling. In *Shh* mutant embryos, only a single digit similar to digit one is formed (Figure 1B) (Chiang et al. 1996).

Although vertebrate Hh signaling impinges on many aspects of development, the importance of this pathway later in life appears more limited. During adulthood, the Hh pathway maintains normal tissue homeostasis and plays regulatory roles for stem cells found, for instance, in the central nervous system and hair follicle (Lai et al. 2003; Levy et al. 2005; Han et al. 2008). Improper activation of the Hh pathway may lead to cancer, and in two tumor types in particular—basal cell carcinoma and medulloblastoma—dysregulated Hh signaling appears to play a causative role.

Hh signal transduction: activating an activator and repressing a repressor

Hh initiates signaling by binding to its receptor, the twelve-transmembrane protein Patched (Ptch). Unlike receptors in many signaling pathways, in the absence of Hh, Ptch tonically inhibits the downstream pathway by repressing the function of Smoothed (Smo), a seven-transmembrane protein that acts as the central positive mediator of Hh signaling (McMahon et al. 2003; Hooper and Scott 2005). Vertebrates have two homologs of Ptch. Consistent with its function as a negative regulator of the Hh pathway, *Ptch1* mutant embryos display increased and ectopic Hh pathway activity, leading to an expansion of ventral neural cell types and polydactyly (Goodrich et al. 1997; Milenkovic et al. 1999; Motoyama et al. 2003).

In *Drosophila*, Ptch normally retains Smo in cytoplasmic vesicles. Upon binding of Hh to Ptch, inhibition of Smo is relieved, leading to phosphorylation and plasma membrane accumulation of Smo (Denef et al. 2000; Jia et al. 2004; Zhang et al. 2004). Activated Smo at the cell surface subsequently transduces signals through Cubitus interruptus (Ci), a zinc finger transcription factor that serves as a pivotal switch for downstream pathway activity.

In an intriguing demonstration of biological parsimony, the *Drosophila* Hh signaling pathway uses Ci both to repress and activate the Hh transcriptional program. In the absence of Hh signals, Ci is processed into a smaller repressor form (CiR) that inhibits the expression of Hh target genes (Aza-Blanc et al. 1997). This processing is facilitated by a kinesin-related protein known as Costal-2 (Cos2), which complexes with Ci and helps exclude it from the nucleus (Robbins et al. 1997; Sisson et al. 1997; Wang and Holmgren 1999; Zhang et al. 2005; Farzan et al. 2008). This Ci-Cos2 complex is also bound by additional proteins in the absence of Hh signals, including unphosphorylated Smo, the serine/threonine kinase Fu, and Sufu (Lum et al. 2003). Importantly, Cos2 recruits several kinases, including Protein kinase A (PKA), Casein kinase I (CKI) and Glycogen synthase kinase 3 (GSK3), which phosphorylate full length Ci (Price and Kalderon 1999; Price and Kalderon 2002). The sequential action of these kinases creates phosphopeptide binding motifs that recruit the Supernumerary limbs (Slimb) subunit of the Skp1/Cullin1/F-box (SCF) E3 ubiquitin ligase complex, which ubiquitinates Ci to direct it for processing into CiR (Jiang and Struhl 1998; Smelkinson and Kalderon 2006). The proteolytic processing required for formation of CiR is mediated by the proteasome, which acts in a regulated manner to degrade only the carboxy-terminal portion of Ci containing the transcriptional activator domain (Chen et al. 1999; Wang and Price 2008). Consistent with their roles in mediating Ci processing, loss of Cos2 or any one of the kinases that phosphorylate Ci can lead to accumulation of full length Ci and increased activity of the Hh pathway (Wang and Holmgren 1999; Price and Kalderon 2002; Zhang et al. 2005).

In the presence of Hh, *Drosophila* Smo traffics to the membrane and induces phosphorylation and activation of Fu, leading to subsequent phosphorylation of Cos2 and Sufu (Lum et al.

2003; Ruel et al. 2003). Phosphorylation of Cos2 weakens its interactions with Ci and Smo, permitting dissociation of Ci from the complex (Liu et al. 2007; Ruel et al. 2007). Sufu, which suppresses the Hh pathway through its direct binding to Ci, is also inhibited by phosphorylation (Dussillol-Godar et al. 2006). Once freed of its interactions with Cos2 and Sufu, an activator form of Ci can enter the nucleus, where it induces expression of downstream target genes (Chen et al. 1999). In addition to its role in promoting CiR formation, PKA also appears to have a positive role in Hh pathway activation (Zhou et al. 2006). However, how PKA mediates these positive effects and whether additional posttranslational events activate Ci are currently unknown.

Vertebrates possess three homologs of Ci, called Gli transcription factors (Gli1-3). Like Ci, Gli2 and Gli3 possess an amino-terminal repressor domain and a carboxy-terminal activator domain flanking the central DNA-binding zinc fingers (Ruiz i Altaba 1999; Sasaki et al. 1999). Gli1, however, lacks the amino-terminal repressor domain and functions only as a transcriptional activator. Gli2 similarly functions mainly as an activator of Hh signaling, whereas Gli3 functions primarily as a repressor. Ultimately, it is the balance of the collective activator and repressor functions of these Gli transcription factors that determines the status of the Hh transcriptional program.

As with Ci, proteasome-mediated proteolysis regulates the abundance and transcriptional activity of the vertebrate Gli proteins. In the absence of Hh signals, Gli3 is phosphorylated by PKA, CKI and GSK3, generating a phosphopeptide motif bound by β -transducin repeat-containing protein (β -TrCP), the vertebrate homolog of Slimb (Figure 2A) (Wang et al. 2000; Wang and Li 2006). β -TrCP recruits the SCF ubiquitin ligase complex, which targets Gli3 to the proteasome for partial proteolysis into a truncated form that functions as a repressor (Tempé et al. 2006). Gli1 and Gli2 are also phosphorylated by these kinases prior to binding by β -TrCP, but are mainly targeted for complete degradation by the proteasome (Kaesler et al. 2000; Pan et al. 2006). In some cases, Gli2 has been observed to be processed into truncated forms that might serve as transcriptional repressors (Aza-Blanc et al. 1997; Ruiz i Altaba 1999), although the significance of these less abundant species is currently unclear.

SCF is not the only ubiquitin ligase complex that regulates Ci and Gli activity. In *Drosophila*, a second complex which includes HIB/Roadkill and Cullin 3 (Cul3) also binds full-length Ci proteins in the eye and wing discs to target them for proteasome-mediated degradation (Kent et al. 2006). A mammalian homolog of HIB, SPOP, can functionally substitute for HIB in flies, suggesting that the same complex may also regulate Gli stability (Zhang et al. 2006). Additionally, Gli1, but not Gli2 or Gli3, can interact with Numb, which recruits another E3 ubiquitin ligase, Itch, leading to degradation (Marcotullio et al. 2006).

Upon activation of Hh signaling, both degradation of Gli2 and proteolytic processing of Gli3 into its repressive form are inhibited (Wang et al. 2000; Pan et al. 2006), thereby permitting Gli2 to function as a strong activator of the Hh transcriptional program and allowing full length Gli3 to serve as an activator in some circumstances (Figure 2B) (Bai et al. 2004; Wang et al. 2007a). Overexpression of full length Gli2 causes weak activation of the Hh pathway, whereas overexpression of a truncated form of Gli2 lacking its amino-terminal repressor domain leads to constitutive activation of Hh signaling both *in vitro* and *in vivo* (Sasaki et al. 1999; Sheng et al. 2002; Meyer and Roelink 2003). Overexpression of a truncated Gli3 lacking its carboxy-terminal activator domain causes constitutive repression of downstream signaling (Ruiz i Altaba 1999; Meyer and Roelink 2003). Although these artificial constructs may not faithfully recapitulate the normal post-translational regulation of these transcription factors, these results suggest that the presence of activator and repressor domains critically determines the functions of Gli2 and Gli3, and that modulating the activity of these domains may be one mechanism by

which the same proteins may exert both positive and negative effects on the Hh transcriptional program.

Functions of Gli proteins in development

Of the three Gli transcription factors, Gli2 plays the most important role in mediating the effects of Hh signaling in the neural tube. *Gli2*-deficient mouse embryos display loss of ventral neuronal subtypes normally specified by high levels of Hh signaling (the floor plate and some V3 interneurons), and exhibit an expansion of more dorsal cell types normally specified by lower levels of Hh signaling (Figure 3A) (Ding et al. 1998; Matisse et al. 1998). In contrast, *Gli1*-deficient mice manifest no obvious developmental defects (Park et al. 2000), and *Gli3*-deficient animals exhibit only a subtle expansion of V0, V1 and V2 neural subtypes (Figure 3B) (Persson et al. 2002). These differences suggest that, in the neural tube, cells rely primarily on Gli2 to activate the Hh pathway in response to Shh, whereas Gli1 and Gli3 play supportive roles. Consistent with this, both *Gli1*; *Gli2*, and *Gli2*; *Gli3* double mutant embryos display more severe deficits in ventral neuronal subtype specification than do embryos lacking *Gli2* alone (Park et al. 2000; Motoyama et al. 2003; Bai et al. 2004; Lei et al. 2004).

A different situation occurs in the limb buds, where absence of *Gli2* has no effect on digit patterning, but loss of *Gli3* leads to polydactyly (Hui and Joyner 1993), a phenotype also caused by increased or ectopic Shh in the limb bud (Figure 3A, B) (Riddle et al. 1993). Embryos lacking both *Shh* and *Gli3* exhibit polydactyly similar to mutants lacking only *Gli3*, suggesting that Shh patterns the limb bud by suppressing Gli3 repressor formation (Litington et al. 2002; Welscher et al. 2002). Indeed, a gradient of the repressor form of Gli3 inverse to that of Shh forms in the limb bud (Wang et al. 2000; Litington et al. 2002). At posterior sites proximal to the source of Shh secretion (the future site of digit five), Gli3 repressor levels are lowest, whereas in the anterior limb bud farthest from the source of Shh (the future site of digit one), Gli3 repressor levels are highest. Thus, tissues can respond to Hh signals in two fundamentally different ways. In the first way, exemplified by the neural tube, the principle action of Shh is to induce the activator function of Gli2. In the second way, exemplified by the limb bud, Shh acts primarily to inhibit the formation of Gli3 repressor.

In many tissues, Hh signaling activates the expression of *Ptch1* and *Gli1*, which, in addition to being integral components of the Hh signal transduction pathway, are direct transcriptional targets of Hh signaling (Goodrich et al. 1996; Marigo and Tabin 1996; Bai et al. 2002). As mentioned previously, the developmental role of Gli1 is limited, although double mutant, gain-of-function and gene replacement experiments indicate that Gli1 can act as a positive mediator of the Hh transcriptional program (Park et al. 2000; Bai and Joyner 2001; Bai et al. 2002). Induction of *Ptch1* expression has more complex effects on Hh patterning. Increased Ptch limits the movement of Hh proteins in a developmental field, presumably by directly binding and internalizing Hh proteins (Hepker et al. 1997; Jeong and McMahon 2005). Given its role in Smo inhibition, upregulation of Ptch may also be an important feedback by which cells become desensitized to Hh signals (Casali and Struhl 2004; Dessaud et al. 2007).

It is currently unclear the degree to which the functions of the *Drosophila* Ci interacting proteins Fu, Cos2 and Sufu are conserved in vertebrates. In zebrafish, inhibition of the Cos2 homolog Kif7 induces ectopic Hh signaling in the myotome and neural tube, consistent with data from flies that Cos2 acts as a suppressor of the Hh pathway (Tay et al. 2005). The role of Cos2 homologs in mammals, however, remains unclear, as inhibition of Kif7 and its paralog Kif27 in cell culture assays did not affect Hh signaling (Varjosalo et al. 2006). Mice deficient for *Fu* also do not display overt Hh-related defects (Chen et al. 2005; Merchant et al. 2005), although inhibition of *Fu* in zebrafish suppresses the specification of myotome subtypes that rely on high level Hh signaling (Wolff et al. 2003). In contrast, Sufu functions as a negative

regulator of Hh signaling in both zebrafish and mice (Cooper et al. 2005; Koudijs et al. 2005; Svård et al. 2006). This is somewhat unexpected, given that Sufu plays only a minor role in *Drosophila* Hh signaling (Préat 1992; Ohlmeyer and Kalderon 1998). The molecular mechanism by which Sufu inhibits vertebrate Hh signaling is unclear, although it may both restrict Gli nuclear import and inhibit the activity of Gli transcriptional activators in the nucleus (Cheng and Bishop 2002; Paces-Fessy et al. 2004; Ding et al. 1999; Pearse et al. 1999; Stone et al. 1999).

Thus, the homologs of the Ci interacting proteins appear to manifest varied functions in vertebrate Hh signaling. Fu, a critical regulator of Hh signaling in *Drosophila*, is important for Hh signaling in zebrafish but not in mouse. On the other hand, Sufu, a minor regulator of Hh signaling in fly, is a critical repressor of Hh signaling in both zebrafish and mouse. Together, these observations indicate that whereas much of the Hh signal transduction pathway is remarkably conserved, the mechanisms by which Hh signals are transduced between Smo and Gli have diverged considerably during metazoan evolution (Huangfu and Anderson 2006).

Defective intraflagellar transport disrupts mammalian Hh signaling

A recent screen for *N*-ethyl-*N*-nitrosourea (ENU)-induced mouse mutants that display neural tube defects yielded the surprising observation that mammalian Hh signaling relies on intraflagellar transport (IFT) (Huangfu et al. 2003). First visualized over a decade ago in *Chlamydomonas* flagella, IFT consists of large, multi-subunit complexes that commute bi-directionally between the flagellar base and tip (Kozminski et al. 1993). IFT proteins are conserved among ciliated organisms, and IFT movement along cilia has also been observed in *C. elegans* chemosensory neuronal cilia (Orozco et al. 1999; Snow et al. 2004; Ou et al. 2005) and in mammalian primary cilia (Tran et al. 2008).

IFT plays a critical role in assembling flagella and cilia, as protein synthesis does not occur within these organelles (Rosenbaum and Witman 2002). Thus, the building blocks required for generating these structures must be transported from the cell cortex to the distal end of the cilium. In *Chlamydomonas*, IFT is mediated by at least 17 proteins organized into two complexes, A and B (Cole et al. 1998). IFT from the base of the cilium to its tip (anterograde IFT) is powered by Kinesin II (Kozminski et al. 1995; Cole et al. 1998), whereas IFT from the ciliary tip to the base (retrograde IFT) is mediated by a Dynein motor (Pazour et al. 1998; Porter et al. 1999). Disruption of either *Chlamydomonas* Kinesin II or one of several IFT complex B proteins prevents flagellar assembly (Pazour et al. 2000; Deane et al. 2001). In contrast, mutation of genes encoding components of the *Chlamydomonas* retrograde Dynein motor or several IFT complex A proteins do not completely abrogate flagellum formation. Instead, these mutants have short flagella that accumulate flagellar proteins at the distal end due to defective retrograde IFT (Pazour et al. 1998; Piperno et al. 1998; Porter et al. 1999).

Consistent with their involvement in *Chlamydomonas* flagellum biogenesis, mutations that inhibit the function of mouse IFT also disrupt ciliogenesis (Nonaka et al. 1998; Marszalek et al. 1999; Pazour et al. 2000). The first cilia known to form during mouse development are those of the ventral node. These motile cilia generate a leftward flow in the node that is essential for proper left-right axis determination (Nonaka et al. 2002). Disruption of cilia blocks establishment of this flow, leading to left-right axis defects (Nonaka et al. 1998; Marszalek et al. 1999). Similarly, defects in ciliogenesis in the zebrafish embryo disrupt flow in Kupffer's vesicle, the ventral node equivalent, randomizing left-right axis formation (Essner et al. 2005). Paralysis of motile cilia also presumably causes the situs inversus that often accompanies human primary ciliary dyskinesia (Carlén and Stenram 2005; Storm van's Gravesande and Omran 2005).

In addition, mutations in ciliary proteins are linked to human polycystic kidney disease (PKD), a common genetic disorder characterized by the formation of fluid-filled renal cysts (Pan et al. 2005; Simons and Walz 2006). Although disruption of ciliogenesis in mice can cause mid-gestation lethality, preventing analysis of kidney phenotypes, animals bearing a hypomorphic allele of *Ift88*, which encodes a Complex B protein, survive longer and develop stunted primary cilia in the kidneys and PKD (Pazour et al. 2000; Yoder et al. 2002). Similarly, kidney-specific deletion of *Kif3a*, which encodes an essential component of the Kinesin II motor required for ciliogenesis, also causes PKD (Lin et al. 2003). Together, these results establish links between defects in IFT function and the pathogenesis of important human diseases.

In addition to left-right axis defects, mutant mouse embryos lacking *Kif3a* display an open neural tube, as do embryos lacking the IFT complex B components *Ift188* or *Ift172* (Marszalek et al. 1999; Huangfu et al. 2003; Huangfu and Anderson 2005). *Kif3a*, *Ift88* and *Ift172* mutants also display loss of ventral cell types in the neural tube, including loss of the floor plate, V3 interneurons and motor neurons (Figure 4A). These deficiencies are accompanied by an expansion of lateral neural markers into more ventral domains. Unlike *Shh* mutant embryos (Chiang et al. 1996), neural subtypes such as the V2 precursors are specified in *Ift88* and *Ift172* mutants, and the dorsal neural progenitor marker *Pax7* is not expanded (Huangfu et al. 2003). Thus, the neural tube phenotypes of *Kif3a*, *Ift88* and *Ift172* mutants are reminiscent of those caused by abrogated Hh signaling, but are more severe than those caused by loss of *Gli2* and less severe than those observed in the absence of *Shh*.

Analyses of additional mice defective for various complex B components have yielded concordant results. For example, disruption of *Ift57* or *Ift52* causes ventral neural tube patterning defects resembling those observed in *Ift88* and *Ift172* mutants (Liu et al. 2005; Houde et al. 2006). In the cases in which it has been examined, loss of complex B proteins is associated with an absence of cilia in the ventral node. These results suggest that all IFT complex B proteins may be essential for mammalian Hh signaling and that their loss perturbs this pathway through a common mechanism.

Disruption of retrograde IFT also affects mammalian Hh signaling. Loss of the atypical dynein heavy chain *Dnchc2* (also known as *Dynch1*), a component of the retrograde IFT motor, causes neural tube closure defects and loss of the floor plate and V3 precursors (Huangfu and Anderson 2005; May et al. 2005). Loss of a different dynein subunit, *D2lic*, also likely interferes with retrograde IFT and is associated with phenotypes indicative of impaired Hh signaling (Rana et al. 2004). In neither case are cilia completely lost; as with *Chlamydomonas* mutations affecting retrograde IFT transport, the *Dnchc2* and *D2lic* mutant embryos both display stunted, bulged cilia. Defects in retrograde IFT in *C. elegans* have also been observed to cause a similar bulging (Schafer et al. 2003).

The involvement of IFT proteins in mammalian Hh signaling is surprising, given that mutations in homologous genes do not perturb *Drosophila* Hh signaling (Han et al. 2003; Sarpal et al. 2003). Indeed, many *Drosophila* tissues patterned by Hh are not known to possess cilia. It is interesting that epistatic analyses have suggested that IFT functions at a point downstream of *Smo* and upstream of *Gli3* (Huangfu et al. 2003; Huangfu and Anderson 2005). As noted previously, analyses of the vertebrate homologs of *Sufu*, *Fu* and *Cos2* have suggested that this part of the pathway has diverged most extensively between arthropods and mammals. The contrasting reliance of *Drosophila* and mouse Hh signaling on cilia appears to be part of this evolutionary divergence. Analysis of whether Hh signals are also transduced through cilia in organisms such as zebrafish, tunicates, and Cnidaria will help reveal whether *Drosophila* have lost ciliary involvement in Hh signaling or whether mammals have gained this capability.

In addition to neural tube defects, disruption of mouse IFT proteins *Dnhc2*, *Ift88* or *Ift52* causes preaxial polydactyly, which is typically associated with increased Hh signaling in the limb bud (Figure 4A) (Haycraft et al. 2005; Liu et al. 2005; May et al. 2005). As discussed previously, *Shh* mutants form only a single digit (Chiang et al. 1996; Litingtung et al. 2002; Welscher et al. 2002). In contrast, *Shh*; *Ift88* double mutants display polydactyly similar to that of *Ift88* single mutants and *Shh*; *Gli3* double mutants (Liu et al. 2005). Thus, as in the neural tube, IFT functions downstream of *Shh* in limb patterning. However, in contrast to neural tube patterning, loss of IFT results in a limb bud phenotype characteristic of Hh gain of function. How do IFT proteins regulate Hh signaling in this seemingly paradoxical manner? Is it their role in ciliogenesis that mediates Hh signaling, or some alternate activity? Fortunately, recent insights into ciliary function have shed light on these important questions.

The primary cilium as a cellular signaling center

First described by Zimmerman over a hundred years ago, cilia are found in many tissues across diverse phyla (Zimmerman 1898). The recent renewed interest in primary cilia comes from the recognition that these organelles function as critical signaling centers in the cell (Eggenschwiler and Anderson 2007). Indeed, the varied metaphors used to describe cilia in the literature over the past few years—as “cellular antennae,” as “watchtowers,” as “global positioning systems”—underscore the prominent role primary cilia are now regarded to play in the detection and integration of a variety of signaling cues (Benzing and Walz 2006; Marshall and Nonaka 2006; Singla and Reiter 2006; Mans et al. 2008).

Unlike the more familiar motile cilia that abundantly line such surfaces as the airway and oviduct, primary cilia are solitary microtubule-based extensions that protrude from the plasma membrane of most cells and, with the exception of cilia found in the embryonic node, are thought to be non-motile (Satir and Christensen 2007). They are also dynamic structures that are extended during interphase and later resorbed during cell cycle progression (Quarby and Parker 2005). This regulated assembly and disassembly is thought to be necessary for proper mitosis, as the base of the cilium, known as the basal body, contains a centriole that must be released from the cell membrane to move to the spindle pole.

The formation of a primary cilium initiates during interphase when the centrioles move to the plasma membrane. Vesicles budding from the Golgi deliver proteins that encapsulate the distal end of the mother centriole, leading to an accumulation of pericentriolar material that nucleates microtubule polymerization (Salisbury 2004; Satir and Christensen 2007). These microtubules, which exist as triplets in the basal body, extend as doublets in a 9+0 configuration along the length of the cilium, forming a core structure known as the ciliary axoneme. This microtubule configuration differs from that of most motile cilia, which possess an additional central microtubule pair in a 9+2 arrangement.

At the interface between the axoneme and the basal body are the terminal plate and transition fibers, structures which may function as gateways that regulate the trafficking of proteins into and out of the cilium (Arima et al. 1984). Within the overlying plasma membrane, a condensed lipid zone encircles the base of the cilium, which may also serve to restrict the entry of membrane proteins (Gilula and Satir 1972; Vieira et al. 2006). The basal body, in coordination with these gatekeeper structures, is thought to regulate the loading of ciliary proteins onto IFT particles and their entry into the cilium.

A variety of human diseases are associated with dysfunctional primary cilia. In addition to left-right axis defects and PKD, as discussed above, dysfunctional cilia have been linked to polymorphic diseases such as Bardet-Biedl syndrome (BBS) (Ansley et al. 2003). BBS is characterized by a range of phenotypes including polycystic kidney disease, polydactyly, diabetes, obesity, situs inversus, retinal degeneration, anosmia, sensory defects and cognitive

deficits. To date, mutations in at least twelve different *Bbs* genes have been linked to this disorder, and many of these genes encode proteins that localize to cilia and basal bodies (Nachury et al. 2007; Tobin and Beales 2007). Defective ciliary function may also underlie other multisystem disorders, including Meckel-Gruber syndrome and Joubert syndrome (Adams et al. 2007). Interestingly, like mouse embryos with defects in IFT or Gli3, many BBS- or Meckel-Gruber syndrome-affected individuals exhibit polydactyly. Whether this is a manifestation of altered Hh signaling is currently unclear.

Mammalian Smo activates the Hh pathway at the primary cilium

As discussed previously, double mutant analyses have indicated that IFT proteins function downstream of Smo and upstream of Gli proteins in the Hh pathway. Mechanistic insights into how IFT mediates Hh signal transduction have been provided by recent studies which show that both Ptch and Smo can localize to primary cilia, and that localization of these proteins is mutually exclusive (Corbit et al. 2005; Rohatgi et al. 2007). Thus, in the absence of Hh ligand, Ptch localizes to cilia and inhibits the Hh pathway by keeping Smo out of the cilium. To activate signaling, Hh binds Ptch at the cilium (Rohatgi et al. 2007) and becomes localized to a juxtaciliary region (Chamberlain et al. 2008). Consequently, Ptch is internalized, allowing Smo to move into the cilium, where it promotes downstream pathway activation by shifting the processing of the Gli transcription factors in favor of activator forms (Figure 2B). How Ptch regulates Smo localization, and how Smo, once in the cilium, transduces signals to the Gli transcription factors remain unclear.

Loss of *Ptch1* is sufficient to confer constitutive ciliary localization for Smo (Rohatgi et al. 2007; Ocbina and Anderson 2008). Recent work has focused on determining whether Ptch inhibits Smo localization and activity through small molecule intermediates (Taipale et al. 2002; Rohatgi et al. 2007). Suggestive evidence for this hypothesis comes from the fact that Ptch acts catalytically to inhibit Smo, and is structurally related to the resistance-nodulation-cell division (RND) family of bacterial pumps and the Niemann-Pick C1 cholesterol transporter (Taipale et al. 2002). Thus, Ptch may modulate the concentration of a small molecule, possibly related to cholesterol, which affects Smo trafficking into and/or out of the cilium.

Further support for this hypothesis comes from the fact that sterols affect cellular response to Shh. Treatment of cultured fibroblasts with specific oxysterol derivatives of cholesterol promotes localization of Smo into the cilium (Rohatgi et al. 2007) and can induce differentiation of pluripotent mesenchymal cells into osteoblasts, an effect also observed when these cells are exposed to Shh (Dwyer et al. 2007). Conversely, depleting cholesterol by cyclodextrin or HMG-CoA reductase inhibitor can inhibit pathway response to Shh (Cooper et al. 2003). Furthermore, human genetic disorders such as Smith-Lemli-Opitz syndrome and lathosterolosis are caused by deficiencies in cholesterol biosynthesis, and are characterized by features such as holoprosencephaly and polydactyly, phenotypes that can result from alterations in Hh signaling (Chiang et al. 1996; Kelley et al. 1996; Krakowiak et al. 2003). Pharmacological inhibitors of the sterol synthesis pathway have also been shown to limit the Hh pathway activity and growth of murine *Ptch1*^{+/-}; *p53*^{-/-} medulloblastoma cells (Corcoran and Scott 2006). Together, these data suggest that Ptch suppresses Hh signaling either by transporting inhibitory sterols to Smo, or by preventing activating sterols from reaching Smo (Bijlsma et al. 2006; Corcoran and Scott 2006).

It is tempting to speculate that sterols may also affect Smo activity indirectly by altering the membrane dynamics that regulate the trafficking of proteins into and out of the cilium. Another possibility is that oxysterols might act directly on Ptch to inhibit its function. It also currently unclear how Smo reaches the cilium upon activation of Hh signaling. In the presence of Shh, Smo can be phosphorylated by G protein coupled receptor kinase 2 (GRK2), which induces

Smo binding to β -arrestin and Kif3a in NIH-3T3 cells (Chen et al. 2004; Meloni et al. 2006; Kovacs et al. 2008). Both β -arrestin and Kif3a are required for Smo to traffic into the cilium, suggesting that Kinesin II may directly transport Smo along the axoneme (Kovacs et al. 2008).

Proper ciliary function is required for processing of Gli proteins

The finding that Smo localization to the cilium is required for activating mammalian Hh signal transduction has provided mechanistic insights into the embryonic phenotypes observed upon disruption of IFT complex B components. In the absence of IFT complex B components, cilia are lost or structurally compromised, likely preventing Smo from signaling through Gli2 and activating the Hh transcriptional program. Although this explanation can account for why disruption of IFT complex B components leads to Hh loss-of-function phenotypes observed in the neural tube, it does not explain why polydactyly is also observed in the limb buds of IFT mutants.

How can IFT mediate Hh pathway activation in certain developmental contexts and Hh pathway inhibition in others? One possible explanation for these dual functions comes from the finding that, in addition to their role in potentiating Gli2 activator formation, IFT components also promote Gli3 repressor activity. This is evidenced by the observation that embryos mutant for genes encoding several IFT components, including *Ift88*, *Ift172*, *Kif3a* and *Dnchc2*, display decreased processing of Gli3 into its repressor form (Haycraft et al. 2005; Huangfu and Anderson 2005; Liu et al. 2005; May et al. 2005).

As mentioned previously, the principle role of Shh during limb bud patterning is to inhibit formation of Gli3 repressor (Wang et al. 2000). In the absence of Gli3 repressor formation, Shh-dependent genes such as *Gremlin* and *Hoxd13* are ectopically expressed in the limb (Litingtung et al. 2002; Welscher et al. 2002). Consistent with the observed defects in Gli3 repressor formation, *Gremlin* and *Hoxd13* are also ectopically expressed in the limb buds of embryos lacking *Ift88* or *Dnchc2* (Liu et al. 2005; May et al. 2005). In addition, overexpression of full length Gli3 in IFT-deficient limb bud cells is incapable of suppressing Gli1-mediated Hh pathway activation, although overexpression of a truncated Gli3 lacking its carboxy-terminal activator domain can still function as a repressor in the absence of IFT (Haycraft et al. 2005). Together, these results indicate that IFT plays a critical role in Gli3 repressor formation and activity both *in vitro* and *in vivo*.

As also previously mentioned, proper patterning of the neural tube depends primarily on Gli2 activator function, with Gli3 activator playing a supportive role, as evidenced by the observation that *Gli2*; *Gli3* double mutant embryos display more severe ventral neural patterning defects than do *Gli2* single mutants (Motoyama et al. 2003; Bai et al. 2004; Lei et al. 2004). IFT complex B mutants display loss of ventral cell types that largely resemble the phenotypes observed in *Gli2*; *Gli3*-double mutant embryos (Huangfu et al. 2003; Huangfu and Anderson 2005). However, loss of Gli3 partially mitigates the neural patterning defects observed in either *Ift88*- or *Ift172*-deficient embryos by restoring formation of motor neurons (Huangfu et al. 2003). Given the phenotypes observed in the limb bud and neural tube, the most parsimonious explanation for reconciling these results is that, while the cilium is essential for full Gli3 repressor activity, some residual repressor activity remains even in the absence of the cilium.

Finally, all three Gli proteins, as well as Sufu, can localize to the cilium (Haycraft et al. 2005), suggesting that key Gli processing steps do not simply require cilia, but occur within the cilium itself. Interestingly, Gli1, which can localize to cilia, does not require IFT for activity, unlike Gli2 and Gli3 (Haycraft et al. 2005). Proteasomes, which are critical for regulating Gli2 protein levels and for generating Gli3 repressor, have been observed to accumulate near basal bodies (Wigley et al. 1999). Thus, it is possible that Gli2 and Gli3 may be post-translationally

modified at the cilium in a Hh-dependent manner, and that proteasomes at the ciliary base may subsequently interpret these cues, prior to Gli2/3 entry into the nucleus.

Additional ciliary components function in Hh signal transduction

If cilia are in fact integral to the transduction of Hh signals during mammalian development, inhibition of critical ciliary proteins such as those involved with proper functioning of the basal body should also be expected to disrupt Hh signal transduction. One such basal body protein is *Ofd1*, disruption of which causes Oral-facial-digital type I (*Ofd1*), a human syndrome characterized by craniofacial defects, polydactyly and, in a minority of patients, PKD (Romio et al. 2004). Indeed, mouse embryos lacking *Ofd1* display loss of cilia in the node and reduced Hh signaling in the neural tube, as well as anterior expansion of *Hoxd* genes in the limb buds, suggesting that Gli3 processing is defective (Ferrante et al. 2006).

Similar to *Ofd1*, loss of another basal body protein, *Ftm* (also known as *Rpgrip11*), compromises ciliary structure and Hh signaling (Vierkotten et al. 2007). Embryos lacking *Ftm* display fewer cilia in the node and limb buds, and these cilia are bulbous and short (Vierkotten et al. 2007). In addition to left-right axis patterning defects, *Ftm* mutants display loss of ventral cell types in the neural tube, polydactyly and impaired Gli3 processing. Similar to IFT mutants, the *Ftm* neural phenotype is partially rescued by loss of Gli3.

As expected, *Ftm*-deficient fibroblasts are less responsive to Shh compared to wild-type fibroblasts (Vierkotten et al. 2007). Surprisingly, however, both fibroblasts and mesenchymal limb cells cultured from *Ftm* mutant embryos are ciliated *in vitro*. Indeed, in spite of the ciliary defects in the node, other tissues such as the trachea and olfactory epithelium appear to have normal cilia in *Ftm* mutant embryos. These results suggest that some requirements for ciliogenesis may vary among different tissues. For instance, *Rpgrip1*, a basal body protein similar to *Ftm*, is required for stabilizing cilia specifically on photoreceptor cells (Hong et al. 2001). It is possible that *Rpgrip1* and *Ftm* may have partially overlapping functions in some tissues.

In addition to IFT and basal body components, many other proteins are likely to be required for proper cilium formation. *Arl13b* is a small GTPase that localizes to the cilium, and disruption of this protein results in cyst formation in the zebrafish pronephros (Sun et al. 2004). Mouse embryos lacking *Arl13b* possess shortened nodal cilia with defective assembly of the outer doublet microtubules (Casparly et al. 2007). Similar to mutants with defective IFT or *Ofd1*, *Arl13b* mutants display an open neural tube and polydactyly. However, *Arl13b* mutants exhibit a Hh phenotype that appears unique from the simple gain- or loss-of-function defects described earlier: In the neural tubes of *Arl13b* mouse mutants, the gradient of the Hh pathway response appears shallower compared to wild-type. The domain of motor neurons, which is normally specified by intermediate levels of Hh, is significantly expanded both dorsally and ventrally in the neural tube. At the same time, *Arl13b* mutants display defective formation of the floor plate and V3 interneurons, cell fates that require higher levels of Gli activator than do motor neurons. Consistent with a role for *Arl13b* in Hh pathway activation, loss of both *Ptch1* and *Arl13b* fails to activate the Hh pathway to the same extent as loss of only *Ptch1*. Together, these results indicate that both full activation and inhibition of the Hh pathway are partially disabled in the absence of *Arl13b*. Interestingly, however, Gli3 repressor formation is not noticeably diminished in *Arl13b* mutants.

Whereas the phenotypes of the IFT mutants discussed thus far have generally been associated with impaired Hh signaling, loss of Tetratricopeptide repeat-containing Hedgehog modulator-1 (*THM1*) increases Hh pathway activity (Tran et al. 2008). In marked contrast to mutations in *Ifi52*, *Ifi57*, *Ifi88*, *Ifi172*, *Dnchc2* and *Kif3a*, the neural tubes of *THM1* mutants display a Gli2-dependent expansion of ventral cell types, including floor plate, V3 interneurons and

motoneurons (Figure 4B). But like other IFT mutants, *THM1*-deficient embryos exhibit polydactyly and an increased Gli3 activator-to-repressor ratio. In addition, double mutant analysis indicates that THM1 acts downstream of both Shh and Smo. Thus, *THM1* mutants possess phenotypes both similar to and distinct from other IFT mutants.

The *Chlamydomonas* homolog of THM1 is the complex A protein IFT139 [D. Cole, personal communication]. Disruption of *Chlamydomonas* complex A IFT components partially impairs retrograde IFT without affecting anterograde IFT, resulting in shortened cilia with bulges at their tips (Piperno et al. 1998). Consistent with this, *THM1*-deficient mouse embryos possess stunted, bulbous nodal cilia similar to those observed in *Dnchc2* mutants (Huangfu and Anderson 2005; May et al. 2005), and ablation of THM1 in cultured cells diminishes retrograde IFT (Tran et al. 2008). It is interesting that more profound defects in retrograde IFT, as presumably found in *Dnchc2* and *mD2lic* mutants, do not result in increased Gli2 activity (Rana et al. 2004; May et al. 2005).

Even in the absence of *THM1*, Smo displays normal localization to the tips of nodal cilia (Tran et al. 2008). Similarly, overexpressed Gli proteins also localize to cilia in *THM1*-deficient fibroblasts. These data suggest that whereas complete disruption of retrograde IFT, as seen in *Dnchc2* or *D2lic* mutants, inhibits Hh pathway activity, reduced retrograde IFT velocity can augment Hh signaling. One possibility is that partial abrogation of retrograde IFT prevents Gli repressor activity but preserves some Gli activator activity. Alternatively, IFT complex A members may be required for Gli repressor activity through a function distinct from their role in retrograde IFT. It will be interesting to determine whether loss of other IFT complex A proteins affects the Hh pathway similarly to loss of *THM1*, and whether increased pathway activation in these IFT complex A mutants also depends on Gli2.

Given the litany of ciliary genes involved in Hh signaling, it might appear as if any defect in ciliary structure is sufficient to perturb Hh pathway activity. However, two cases suggest that this supposition is incorrect. *Rfx3* is a transcription factor involved in the expression of many ciliary genes, including *D2lic* (Bonnafe et al. 2004). Mice mutant for *Rfx3* possess shortened nodal cilia but can survive to adulthood, suggesting that Hh signal transduction is not dramatically altered (Bonnafe et al. 2004). Similarly, loss of *Stumpy*, a highly conserved ciliary protein, causes ependymal and kidney epithelial cilia to be short and reduced in number (Town et al. 2008). Despite these defects in ciliary morphology, *Stumpy* mutants lack recognized Hh-associated phenotypes.

Additional proteins that have previously been found to modulate the Hh pathway may also have roles in ciliary function. Among these is Rab23, a Rab family GTPase that negatively regulates Hh signaling downstream of Smo and upstream of Gli2 (Eggenchwiler et al. 2001). The immunophilin family member FK506 binding protein 8 (FKBP8) is another intriguing negative regulator of Hh signaling that functions downstream of Smo and upstream of Gli2 in neural tissues (Bulgakov et al. 2004; Cho et al. 2008). Sil1, a cytosolic protein of unknown function, is a positive mediator of Hh signaling that acts downstream of Ptch (Izraeli et al. 2001). *Evc*, which localizes to the base of the cilium, potentiates Indian Hh-regulated bone development (Ruiz-Perez et al. 2007). Finally, genetic analyses of *Tectonic* have suggested that this protein may potentiate Hh signaling in the presence of Hh ligands, but may also suppress the pathway in the absence of upstream signals (Reiter and Skarnes 2006). In summary, these studies have provided tantalizing clues that suggest the involvement of additional novel components in ciliogenesis and Hh signaling.

Are cilia involved in Hh pathway-mediated tumorigenesis?

During many developmental processes, activation of the Hh pathway is mediated through paracrine interactions between the epithelium and mesenchyme. In the adult, however, Hh

signaling plays a more limited role, although upregulation of Hh pathway activity, either through autocrine signaling or mutations that act cell-autonomously, can give rise to cancer. Indeed, a diversity of human tumors express targets of the Hh pathway, and there is evidence that inappropriate activation of Hh signaling may contribute to the development of lung cancer, pancreatic cancer, prostate cancer and glioma (Ruiz i Altaba et al. 2002; Pasca di Magliano and Hebrok 2003; Rubin and Sauvage 2006). However, for two human cancers in particular—basal cell carcinoma (BCC) and medulloblastoma—there is extensive evidence that increased Hh signaling causes these malignancies (Athar et al. 2006; Tang et al. 2007).

Humans diagnosed with Basal cell nevus syndrome (also known as Gorlin's syndrome) are prone to developing widespread BCCs. These patients have been found to be heterozygous for *Ptch1*, and *Ptch1*^{+/-} mice exhibit many features of this disease, including a propensity for developing medulloblastomas and sarcomas, and BCCs after irradiation (Hahn et al. 1998; Aszterbaum et al. 1999). Mutations in the *Ptch1* locus are also detected in 10-20% of sporadic medulloblastomas in humans, and in 50-60% of sporadic BCCs (Tang et al. 2007). BCCs without *Ptch1* mutations often harbor mutations in *Smo* (Xie et al. 1998; Lam et al. 1999; Crowson 2006). These mutations likely render Smo insensitive to the inhibitory action of Ptch, leading to constitutive activation of downstream signaling.

A homolog of at least one oncogenic form of Smo isolated from a human patient with BCC localizes constitutively to the cilium in the absence of Hh ligand (Corbit et al. 2005). This is in contrast to wild-type Smo, which moves to the cilium only upon Hh stimulation. In addition, cyclopamine, which has been used as a chemotherapeutic agent for BCC and medulloblastoma in animal models, removes Smo from the cilium (Corbit et al. 2005). Given the preponderance of data indicating that cilia are required for proper Hh pathway activity downstream of Smo, one important question might be, Are cilia necessary for the growth of tumors, particularly those initiated and sustained by Smo activation? And are human tumors ciliated?

Although there are currently no published reports that have examined ciliary distribution across a panel of different tumor types, we have observed that cilia are frequently absent from established human cancer cell lines (S.Y.W. and J.F.R., unpublished results). However, these cells have been selected for growth under tissue culture conditions and may not be representative of tumors arising in human patients. Indeed, we have recently examined biopsies from human BCCs and found that cells from these tumors are frequently ciliated (Wong et al., manuscript submitted). In addition, we have observed that cilia are also present on cells from BCC-like lesions that arise in transgenic mice induced to express a constitutively active form of Smo (SmoM2) specifically in the skin. Others have recently observed that cells from a subset of human medulloblastomas can also be ciliated (Han et al., manuscript submitted).

Because cilia play critical roles in modulating Hh signaling during development, it is likely that these organelles also serve important functions during tumorigenesis, particularly for Hh-dependent cancers such as BCC and medulloblastoma. As discussed previously, while cilia are necessary for propagating signals initiated by Smo and transduced downstream through Gli activators, these organelles are also required for Gli3 repressor formation, which may inhibit the expression of a host of genes, including those that promote cell proliferation. Thus, the functions of cilia during tumorigenesis are likely to be complex (Figure 5). In addition, cilia may have roles in modulating a multitude of other signaling pathways important for cancer, including Wnt and PDGF (Schneider et al. 2005; Gerdes et al. 2007; Corbit et al. 2008).

Lingering Questions

The identification of the primary cilium as a critical modulator of both positive and negative signals of the Hh pathway has placed this organelle at the crossroads of Hh signal transduction. While many new questions have been raised, many old ones remain to be addressed. For

instance, it remains unclear how Ptch restrains Smo activity or how Smo triggers Gli activation. The central involvement of the cilium in these processes suggests that understanding how Ptch alters the cilium, what proteins move Smo into the cilium, and what Gli processing components function within the cilium may provide significant insights into these difficult problems.

It is also currently unknown how general a role cilia play in Hh signaling in other organisms. Although it is clear that mouse Hh signaling relies on cilia and *Drosophila* Hh signaling does not, the involvement of cilia in Hh signaling in other organisms has not been well explored. For example, does Hh signaling act through cilia in ascidians or Cnidaria? Are cilia important for Hh signaling in fish? Although loss of IFT components causes kidney cysts in zebrafish (Sun et al. 2004), Hh-related phenotypes have not yet been observed, possibly because cilia persist in these mutants through early development, presumably due to maternal deposition of wild-type IFT mRNA or protein (Tsujikawa and Malicki 2004). Thus, it will be interesting in future studies to assess the phenotypes of fish lacking both maternal and zygotic IFT contributions.

Even in mammals, it is currently unclear whether all Hh signaling is transduced through the cilium, or whether some cell types or developmental events respond to Hh signals through alternative mechanisms. One possible example of cilium-independent Hh signaling might be in the guidance of commissural axons. Shh functions as a midline signal that guides commissural axons towards their intermediate target, the floor plate (Charron et al. 2003). As Shh presumably acts at the growth cone to induce rapid cytoskeletal changes, it is difficult to imagine how the cilium could mediate this guidance. Intriguingly, the correct projection of commissural axons depends on Boc, a cell surface protein that binds Shh (Okada et al. 2006). Could Ptch mediate cilium-dependent Hh activity, while Boc, or a similar protein, Cdo, mediate cilium-independent Hh activity?

Perhaps a more philosophical question is, Why is the cilium required for Hh signaling at all? As noted above, work from *Drosophila* has shown that organisms can clearly solve the problem of Hh signal transduction through other means. Cilia have evolutionarily ancient roles in sensing and transducing environmental information, extending back to our unicellular ancestors. Upon achieving multicellularity, there would have been new demands for coordinating cellular behaviors. Perhaps there was a low barrier for adapting the signaling machinery already present in the cilium for these purposes. If so, ciliary transduction of Hh signals may represent a truly ancient form of intercellular communication likely to be well-represented throughout the animal kingdom.

ACKNOWLEDGEMENTS

The authors acknowledge the support of grants from the NIH (RO1AR054396), the Burroughs Wellcome Fund, and the Sandler Family Supporting Foundation to J.F.R. S.Y.W. acknowledges the support of the A.P. Giannini Foundation, the Herbert W. Boyer Fund, and the American Cancer Society.

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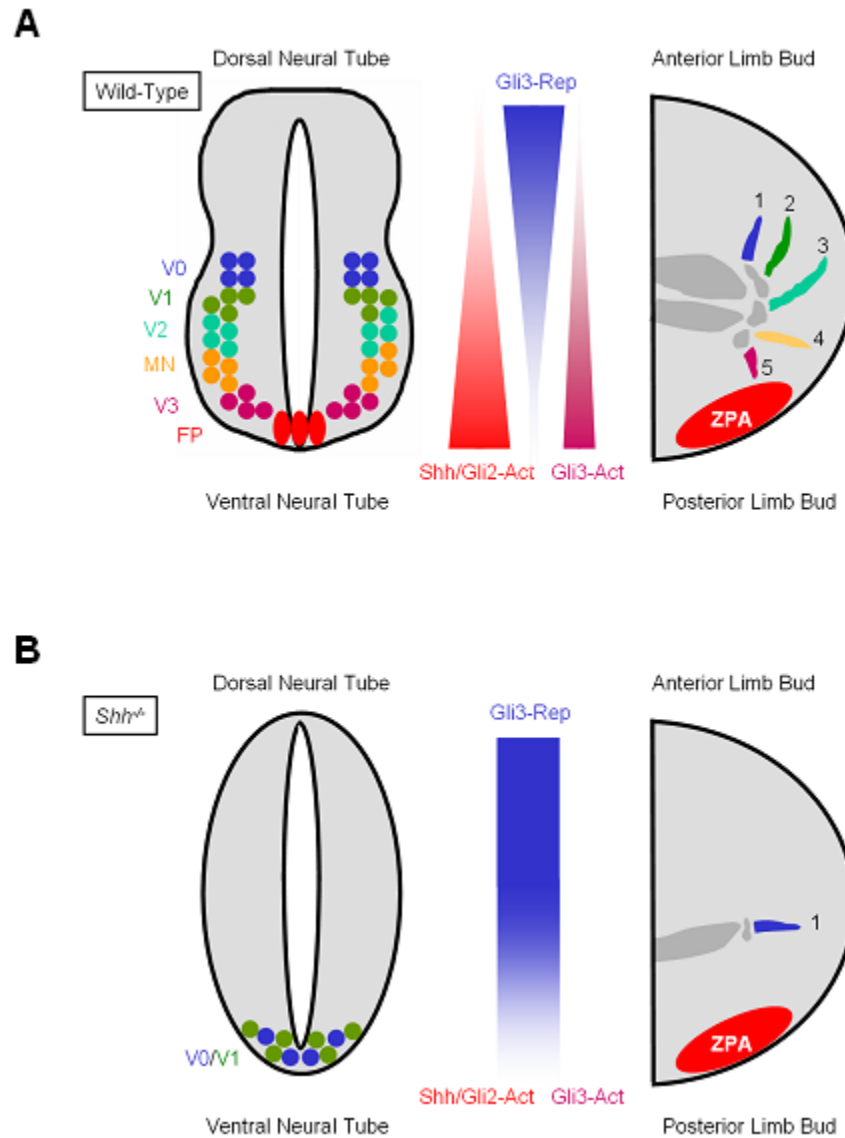


Figure 1. Proper patterning of the neural tube and limb buds is mediated by a gradient of Shh (A) In the neural tubes of wild-type embryos, Shh produced by the notochord, floor plate (FP) and possibly gut forms a gradient along the dorsal-ventral axis. Similarly, Shh released by cells in the zone of polarizing activity (ZPA) in the limb buds forms a gradient along the anterior-posterior axis. Increased Shh concentration and duration of action are associated with increased Gli2 activator (Gli2-Act) and Gli3 activator (Gli3-Act) functions, and reduced Gli3 repressor (Gli3-Rep) formation. High level Hh signaling is critical for specifying the ventral-most cell types in the neural tube, including FP and V3 interneurons (V3). More dorsal cell types (V0, V1, V2 and motorneurons (MN)) are specified by lower levels of Hh signaling. In limb buds, the Shh and Gli3-Rep gradients are interpreted for the specification of digit identity (digits 1-5). (B) In *Shh* mutant embryos, the gradient of Hh pathway activation is absent. Gli3 is mostly converted into Gli3-Rep along the dorsal-ventral axis of the neural tube and the anterior-posterior axis of the limb buds, although *Ihh* may still activate the pathway to some degree. In mutant neural tubes, only the V0 and V1 neuronal subtypes are specified, and in mutant limb buds, only the anterior-most digit (digit 1) is formed.

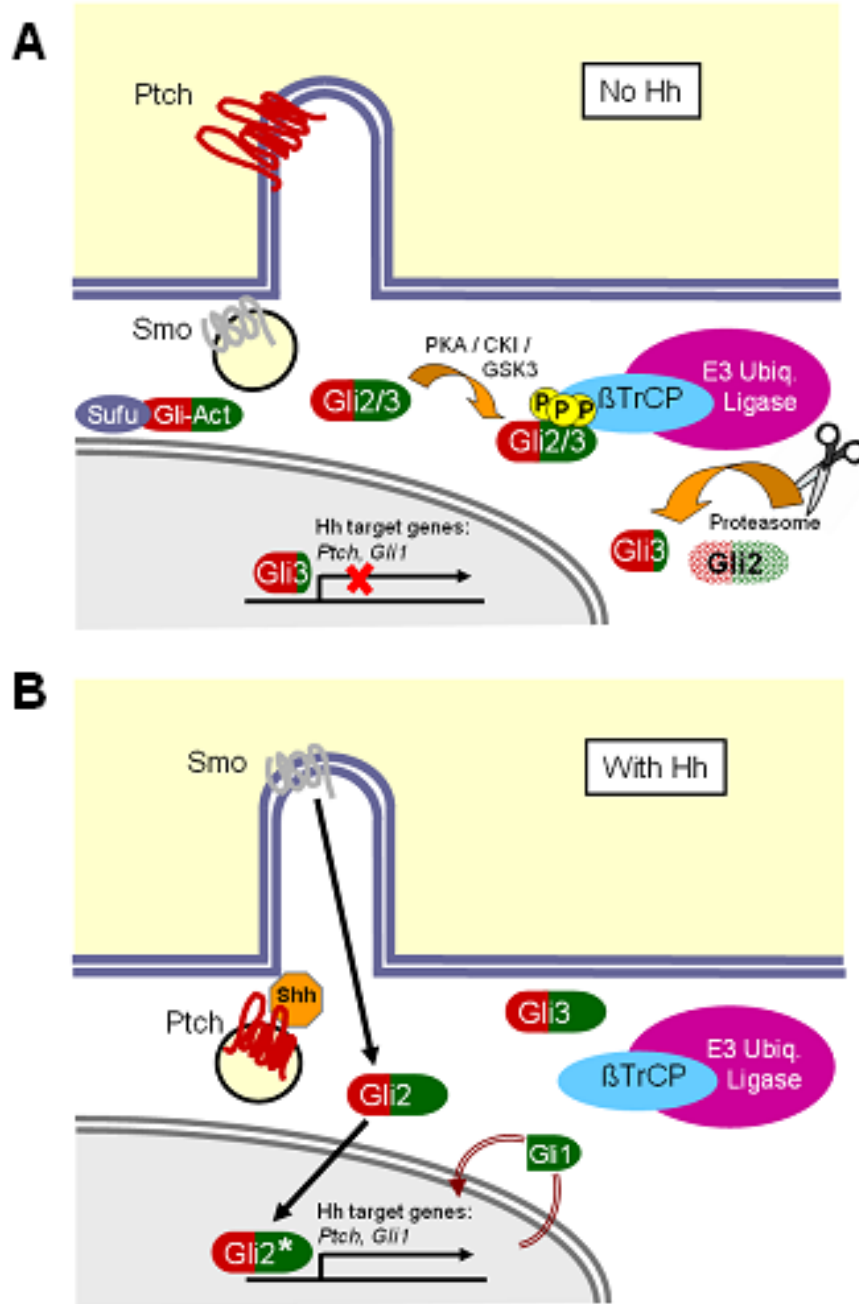


Figure 2. A model of mammalian Hh signal transduction

(A) In the absence of Shh, Ptch localizes to the primary cilium and, through an unknown mechanism, prevents Smo from entering the cilium. Gli2 and Gli3 are phosphorylated by kinases, including PKA, CKI and GSK3, to generate phosphopeptide binding motifs for β -TrCP, which recruits an E3 ubiquitin ligase complex. Ubiquitination of Gli2 and Gli3 targets these proteins to the proteasome, which processes Gli3 into a carboxy-terminal-truncated repressor form and degrades Gli2. Gli3 subsequently enters the nucleus and inhibits transcription of downstream Hh target genes such as *Ptch1* and *Gli1*. (B) In the presence of Shh, Ptch is internalized, allowing Smo to traffic into the primary cilium. It is unclear whether Smo traffics directly to the cilium, or accumulates first in the plasma membrane. At the cilium,

Smo inhibits the formation of Gli3 repressor and activates Gli2, which enters the nucleus to promote transcription of *Ptch1* and *Gli1*, whose protein products negatively and positively feed back on this pathway, respectively.

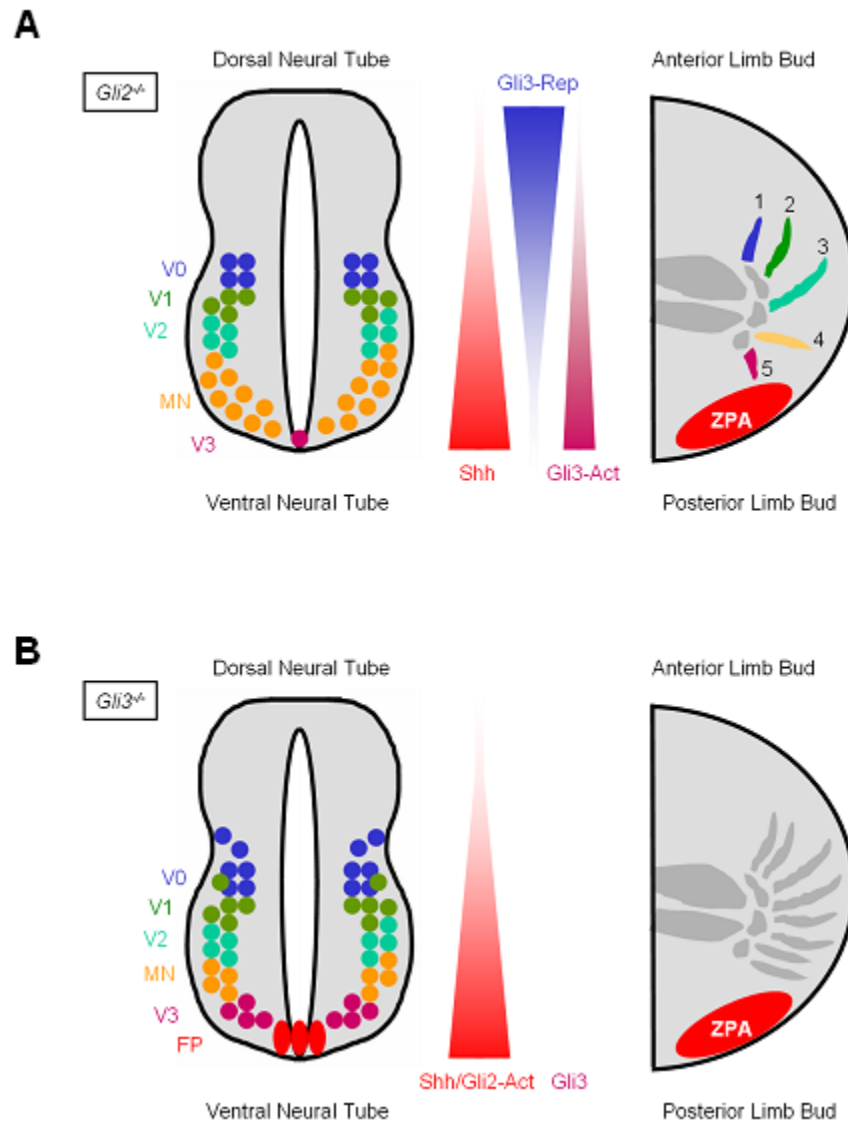


Figure 3. The neural tube and limb buds differ in their reliance on *Gli2* and *Gli3* for proper patterning

(A) In *Gli2* mutant embryos, gradients of Shh and Gli3-Rep/Act are established, but downstream signaling is not properly activated. In the neural tube, which relies predominantly on Gli2 activator function, specification of neuronal subtypes that require the highest levels of Hh pathway activity (FP and V3) is deficient. Because proper patterning of the limb bud depends upon a gradient of Gli3-Act/-Rep, and not on Gli2-Act, digit specification is normal in *Gli2* mutants. (B) In *Gli3* mutant embryos, gradients of Shh and Gli2-Act are established, but formation of Gli3-Rep is absent. *Gli3*-deficient neural tubes display only a subtle expansion of V0 and V1 neuronal subtypes. Because proper limb patterning depends on a gradient of Gli3-Rep, *Gli3* mutant limb buds upregulate genes normally repressed by Gli3-Rep, including *Gremlin* and *Hoxd13*, and exhibit polydactyly.

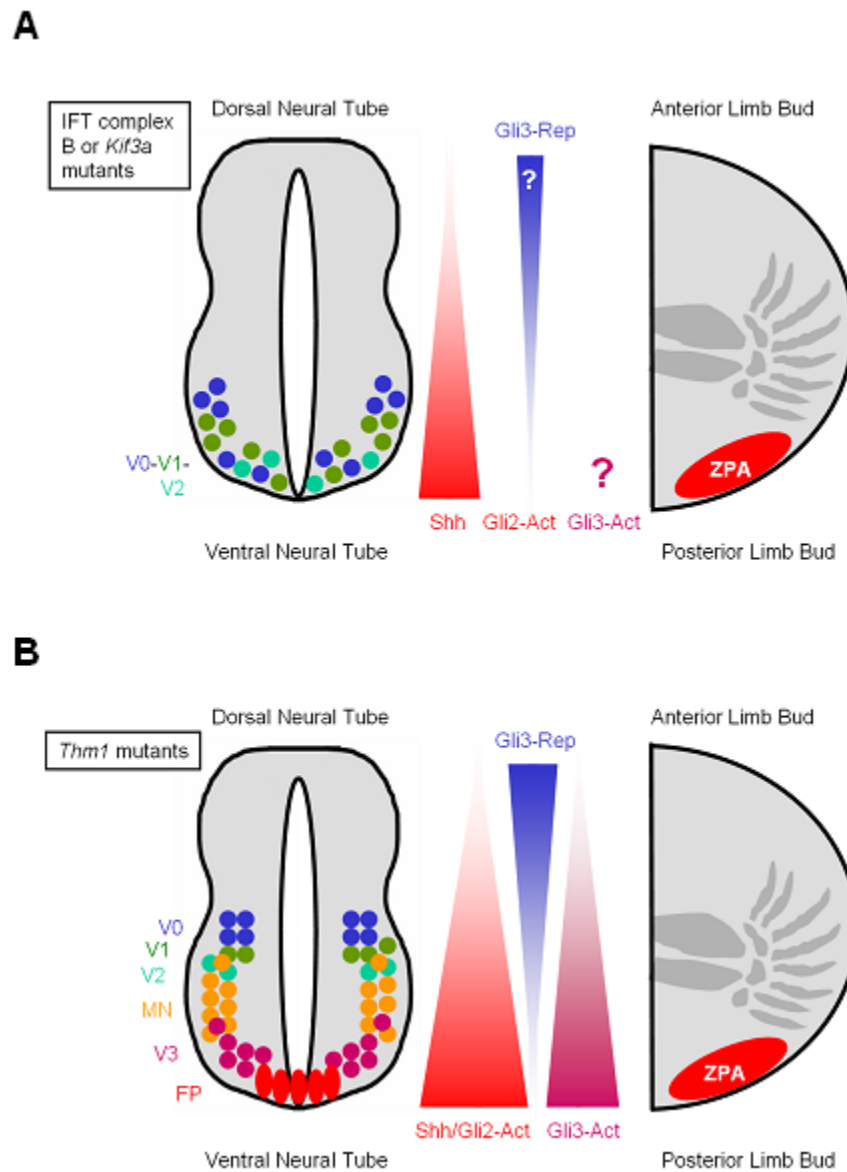


Figure 4. Many IFT mutations cause polydactyly and loss of ventral neural tube fates
(A) In embryos lacking IFT components such as IFT88, IFT172 and *Kif3a*, a gradient of *Shh* is established, but signaling through *Gli2-Act* and formation of *Gli3-Rep* are disrupted. Consequently, these mutants do not properly activate the Hh transcriptional targets, and display loss of ventral cell types (FP, V3, MN) and expansion of more dorsal subtypes (V0, V1, V2) in the neural tube. In IFT mutant limb buds, disruption of *Gli3-Rep* formation leads to polydactyly. **(B)** In contrast to other IFT mutants, *THM1* mutants display increased Hh signaling mediated by *Gli2-Act* and *Gli3-Act*, leading to an increase in ventral subtypes (FP, V3, MN) in the neural tube. *THM1* is also essential for *Gli3* processing, defects of which in *THM1* mutants result in polydactyly.

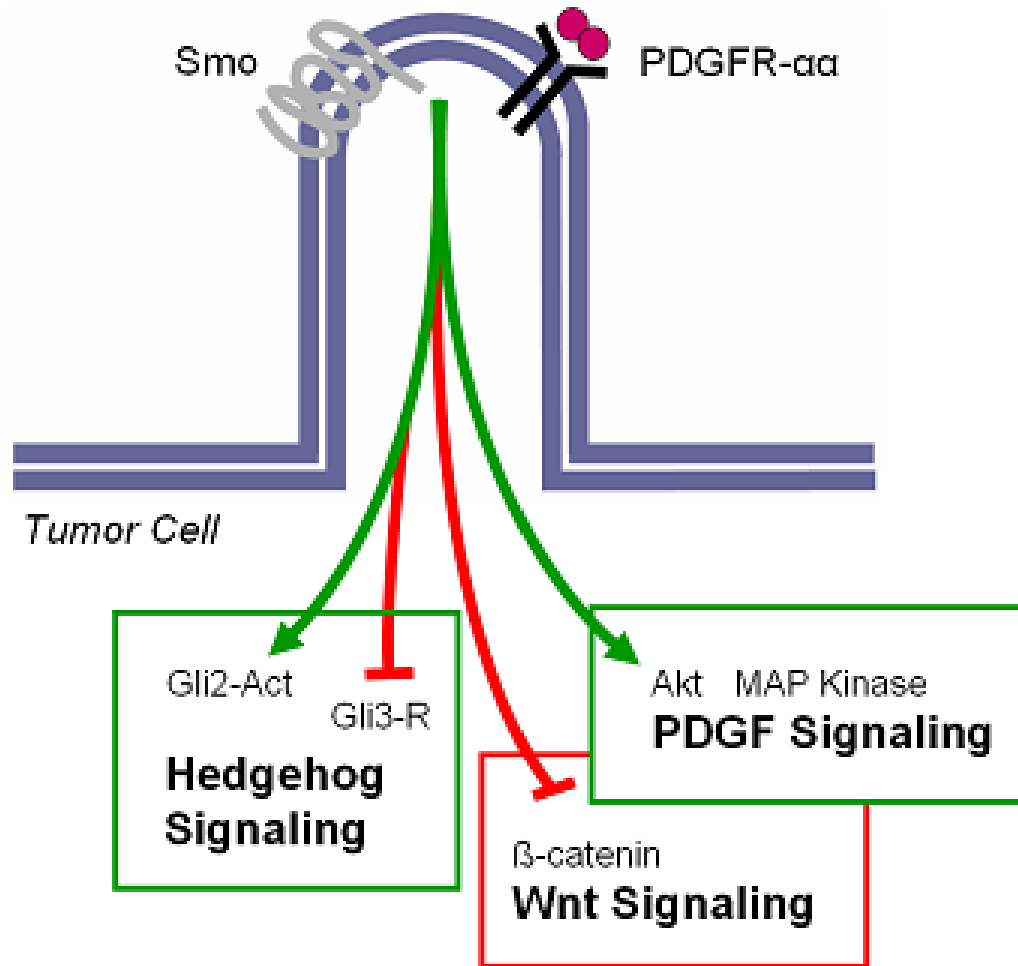


Figure 5. The primary cilium likely coordinates a variety of signaling pathways important for cancer

In Hh-dependent cancers such as BCC and medulloblastoma, the cilium may modulate the activity of signaling pathways in addition to Hh. For instance, localization of PDGF receptor- α to the cilium is important for transducing downstream signals mediated by Akt and MAP kinases. The cilium may also normally restrain Wnt pathway activity by preventing the accumulation of β -catenin. The balance of the downstream effects of these pathways likely governs cellular decisions for proliferation and apoptosis.