Development 133, 3-14 doi:10.1242/dev.02169

Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from *Drosophila* to vertebrates

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Although the framework of the Hedgehog (Hh) signaling pathway is evolutionarily conserved, recent studies indicate that fundamental differences exist between *Drosophila* and vertebrates in the way signals are transduced from the membrane protein Smoothened (Smo) to the Ci/Gli transcription factors. For example, Smo structure and the roles of *fused* and *Suppressor of fused* have diverged. Recently, many vertebrate-specific components have been identified that act between Smo and Gli. These include intraflagellar transport proteins, which link vertebrate Hh signaling to cilia. Because abnormal Hh signaling can cause birth defects and cancer, these vertebrate-specific components may have roles in human health.

Introduction

Genetic studies of the signaling pathways that regulate development have led to the view that a few evolutionarily conserved signaling pathways – the bone morphogenetic protein (Bmp), Wnt, receptor tyrosine kinase, Notch and Hedgehog (Hh) pathways – are used repeatedly in different contexts to control many cell fate decisions in all animals. Although cellular context controls the final output of a signaling pathway, most experiments suggest that the core components of each pathway – ligand, receptor, cytoplasmic signal transduction machinery and transcription factor – have been conserved in evolution. The Hh pathway, first elucidated by genetic studies in *Drosophila*, has been considered to be such a conserved cassette. However, recent genetic studies have defined a surprisingly large number of proteins required for Hh signaling in vertebrates that have no apparent role in *Drosophila* Hh signaling.

Loss of activity of the Hh pathway can cause a variety of human birth defects, including holoprosencephaly, polydactyly, craniofacial defects and skeletal malformations (McMahon et al., 2003). Inappropriate activation of Hh signaling is responsible for nearly all basal cell carcinomas, some medulloblastomas and rhabdomyosarcomas, and has been implicated in other tumors (Pasca di Magliano and Hebrok, 2003). In addition, recent findings about the relationship between primary cilia and the mouse Hh pathway (Huangfu et al., 2003) suggest that Hh signaling may be affected in human syndromes caused by defects in cilia, including Bardet Biedl syndrome, Kartagener syndrome, polycystic kidney disease and retinal degeneration (Pan et al., 2005).

Because of these roles of Hh signaling in human biology, it is important to understand both the similarities and differences in the Hh signal transduction pathways in *Drosophila*, the zebrafish and the mouse. Here, we focus on the comparison of the *Drosophila* and vertebrate cytoplasmic signal transduction pathways from Smo to the Ci/Gli transcription factors, the step affected by most of the newly identified, vertebrate-specific components. We describe the evolutionarily conserved core of the cytoplasmic signal transduction pathway, the cases in which the core components of the pathway have changed structure or function in evolution, and explore the functions of the vertebrate-specific components of the pathway. We point out those cases where the observed differences between the fly and vertebrate pathways reflect genuine changes in mechanism and those that may reflect an incomplete understanding of both the fly and vertebrate pathways.

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The conserved scaffold of the Hh signaling pathway

The Hh family of secreted proteins regulates many developmental processes in both vertebrates and invertebrates (McMahon et al., 2003). The *hh* gene was first identified in *Drosophila* because of its role in embryonic segment polarity (Nüsslein-Volhard and Wieschaus, 1980) and was later shown to act in other aspects of *Drosophila* development, such as patterning of the imaginal discs (Basler and Struhl, 1994; Mohler, 1988; Tabata et al., 1992). Soon after the molecular identification of the *Drosophila hh* gene, which showed that it encodes an unusual secreted protein (Lee et al., 1992; Mohler and Vani, 1992; Tabata et al., 1992; Tashiro et al., 1993), vertebrate homologs of Hh were identified in chick and mouse, and were implicated in patterning of the limb and the neural tube (Echelard et al., 1993; Riddle et al., 1993; Roelink et al., 1994).

Hh signaling has been studied in greatest depth in *Drosophila*, where forward genetic screens have identified the components of the Hh signaling pathway and revealed its unconventional nature (Hooper and Scott, 2005). *Drosophila* has a single Hh ligand, which binds to its receptor, the multiple membrane-spanning protein Patched (Ptc). Unlike most receptors, Ptc activity turns off the downstream signaling pathway in the absence of ligand, and binding of Hh relieves that repression. Smoothened (Smo), another transmembrane protein, acts downstream of Ptc and is an essential positive mediator of the Hh signal. Active Smo regulates the bifunctional transcription factor Cubitus interruptus (Ci). Full-length Ci protein can be modified in response to Hh to become a transcriptional activator. In the absence of Hh ligand, Ci is proteolytically processed into a shorter form (Ci^R) that acts as a transcriptional repressor of target genes. Both the proteolytic processing and the nuclear translocation of Ci are tightly regulated processes that involve a protein complex containing the atypical kinesin protein Costal 2 (Cos2; Cos – FlyBase), the serine threonine kinase Fused (Fu) and the novel protein Suppressor of fused [Sufu; Su(fu) – FlyBase].

The genetics of vertebrate homologs of *Drosophila* Hh pathway genes has been studied by gene targeting in the mouse and by forward genetic screens and morpholino knockdown in the zebrafish. These studies show that the scaffold of the Hh pathway is largely conserved in vertebrates: Hh negatively regulates Ptc, which negatively regulates Smo; Smo controls both activation of Gli transcription factors and proteolytic processing events that

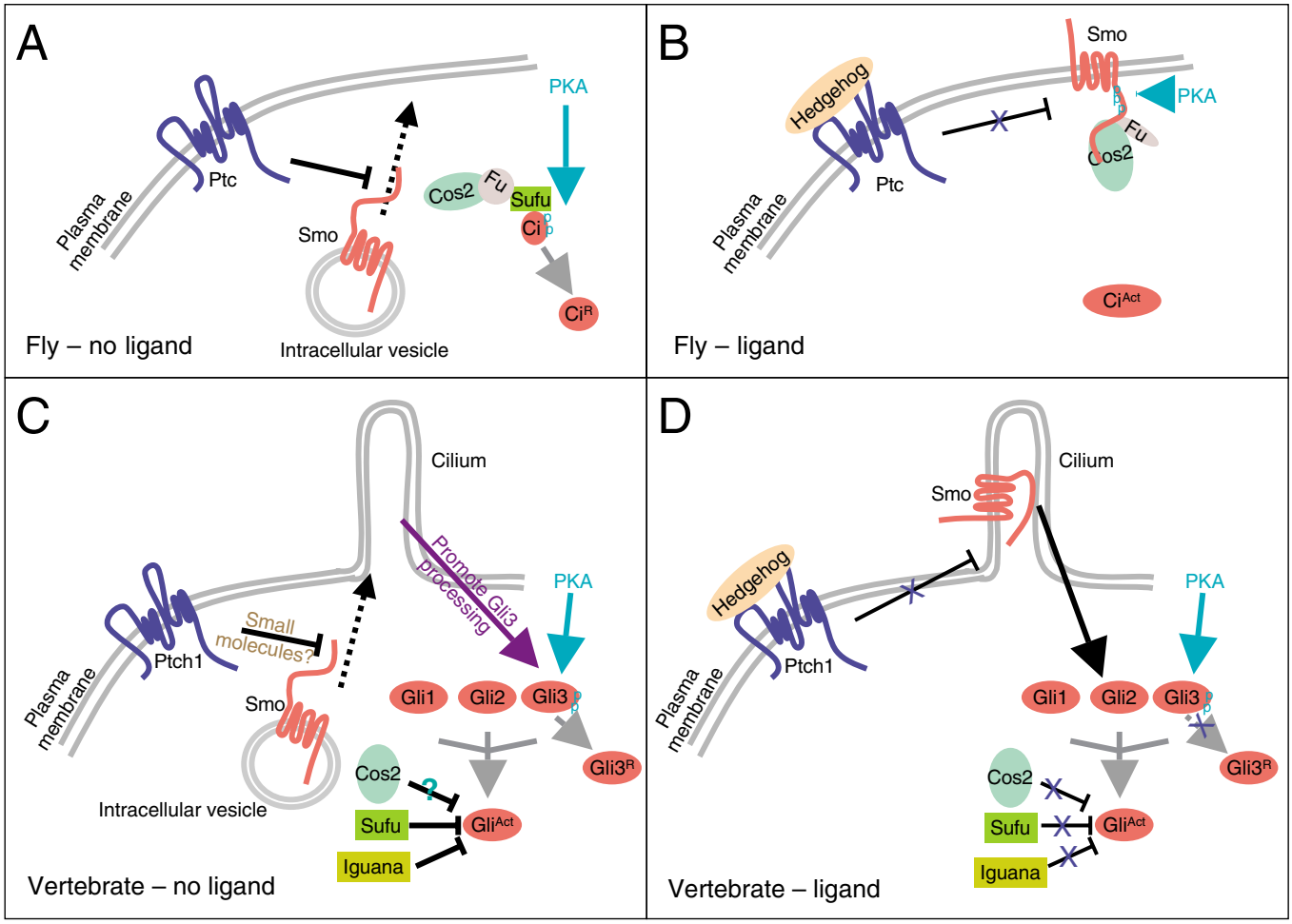

Fig. 1. The Hedgehog pathway in *Drosophila* and vertebrates. The Hedgehog (Hh) pathway in *Drosophila* (A,B) and in vertebrates (C,D) in the absence (A,C) or presence (B,D) of the Hh ligand. (**A**) In the absence of Hh, Ptc prevents the cell-surface localization of Smo, and Ci forms a complex with Cos2, Fu and Sufu, which targets Ci for proteolytic processing into the repressor form (Ci^R). (**B**) In the presence of high levels of Hh ligand, Ptc inhibition is relieved; Smo accumulates at the plasma membrane and forms a complex with Cos2 and Fu through its C-terminal tail; Ci is activated. (**C**) In the absence of Hh, Ptch1 prevents the accumulation of Smo in cilia, possibly through the action of a small molecule. Gli3 is processed into a repressor form ($Gli3^R$) in a cilia-dependent manner. The activation of all Gli proteins is inhibited by Sufu, Iguana (for zebrafish) and probably Cos2. (**D**) In the presence of high levels of Hh ligand, Ptch1 inhibition is relieved; Smo is targeted to cilia and activates Gli proteins in a cilia-dependent manner. Gli3 processing is also inhibited. p, phosphorylation; PKA, protein kinase A.

generate the Gli repressor (Fig. 1). The obvious difference between the *Drosophila* and fish/mouse pathways is that genes encoding specific pathway components have been duplicated in vertebrates. In the mouse, there are three Hh homologs, sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*) and desert hedgehog (*Dhh*); *Shh* and *Ihh* play important roles in embryonic development, and *Dhh* regulates spermatogenesis (Bitgood et al., 1996; Chiang et al., 1996; St-Jacques et al., 1999; Zhang et al., 2001). There are five Hh homologs in zebrafish (http://zfin.org), and three of them [*sonic hedgehog* (*shh*), *echidna hedgehog* (*ehh*) and *tiggywinkle hedgehog* (*twhh*)] play important roles in embryonic patterning (Currie and Ingham, 1996; Ekker et al., 1995). Both mice and zebrafish have two *Ptc* homologs: *Ptch1* appears to be the major receptor during embryonic development (Goodrich et al., 1997; Wolff et al., 2003), and zebrafish *ptc2* mutants have a relatively mild phenotype (Koudijs et al., 2005). Downstream of Ptch1, a single Smo protein mediates all vertebrate signaling (Zhang et al., 2001) by regulating the three homologs of Ci, Gli1, Gli2 and Gli3 (Bai et al., 2004; Motoyama et al., 2003). Gli1 and Gli2 act primarily as activators, while Gli3 acts both as an activator and repressor, like Ci.

The tasks of the Hh signaling pathway

Hh signals have different effects in different contexts. Hh can act as an on/off switch that regulates the fate of immediately adjacent cells, as in the ventral ectoderm of the *Drosophila* embryo (Ingham and Hidalgo, 1993). Alternatively, Hh can act as a short-range morphogen (over 10-15 cell diameters, ~20 μm) that controls three alternative fates as a function of its concentration, as in the *Drosophila* wing imaginal disc (Strigini and Cohen, 1997). Hh can also act as a long-range morphogen that controls several cell fates, as in the vertebrate neural tube, a field that spans many cell diameters over ~200 μm. Components of the signaling pathway can be used differently in these distinct contexts. For example, *Drosophila* Fused is required for Hh signaling in some cell types but not in others; it is required in the wing disc, where Hh acts as a morphogen, but not in dorsal embryonic ectoderm, where Hh acts as a switch (Therond et al., 1999).

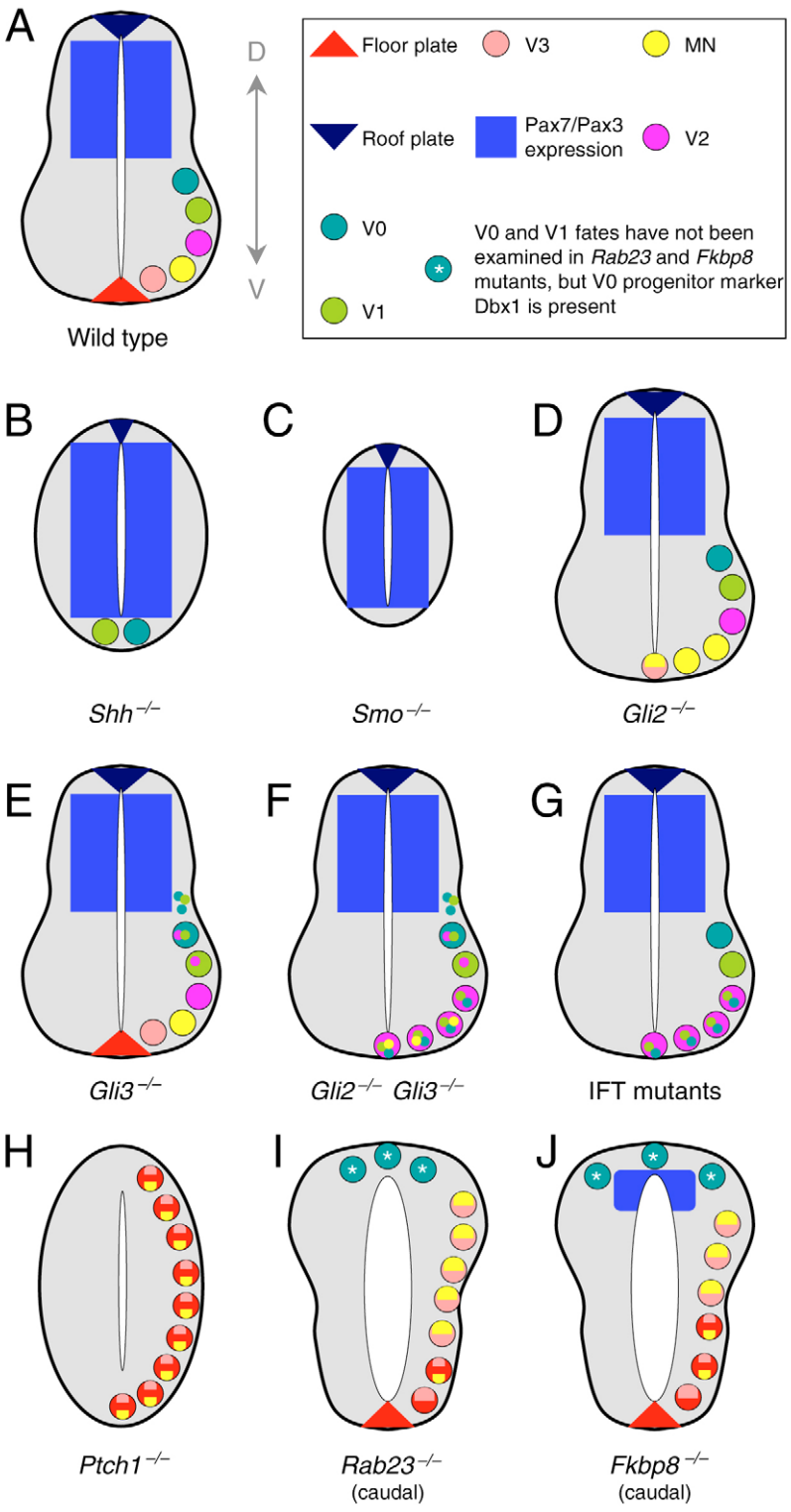

Fig. 2. Neural tube phenotypes in mouse Hh pathway mutants. (**A**) In the wild-type E10.5 embryo, roof-plate cells are specified at the dorsal (D) midline (dark blue), and dorsal neural progenitors express Pax3 and Pax7 (blue). Six ventral (V) neural cell types are specified, each marked in a different color (Jacob and Briscoe, 2003). (**B**) In E10.5 *Shh* mutants, ventral neural cell types are absent, V0 and V1 interneurons are present at the ventral midline, and dorsal progenitor markers are expressed throughout the neural tube (Chiang et al., 1996; Wijgerde et al., 2002). (**C**) Smo is required cell autonomously for specification of floor plate, V3, motoneurons (MNs) and V2 cells; for restriction of dorsal fates; and for the correct positioning of V1 and V0 interneurons (Wijgerde et al., 2002). (**D**) *Gli2* mutants lack floor-plate cells and have reduced number of V3 cells (Ding et al., 1998; Matise et al., 1998). (**E**) V2, V1 and V0 interneurons are expanded dorsally in *Gli3* mutants (Persson et al., 2002). (**F**) *Gli2/Gli3* double mutants lack both floor-plate cells and V3 interneurons (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003). (**G**) IFT mutants (*Ift172*, *Polaris* and *Dnchc2*) lack floor-plate cells, V3 interneurons and nearly all MNs; V2, V1 and V0 cells are expanded ventrally (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). (**H**) *Ptch1* mutants have a ventralized neural tube (Goodrich et al., 1997; Motoyama et al., 2003). No roof-plate or dorsal progenitor cells are specified, and ventral cell types are expanded dorsally. (**I**) *Rab23* mutants have a ventralized caudal neural tube (Eggenschwiler et al., 2005; Eggenschwiler et al., 2001). No roof-plate or dorsal progenitor cells are specified, and ventral cell types are expanded dorsally. (**J**) *Fkbp8* mutants have a ventralized caudal neural tube (Bulgakov et al., 2004). No roof-plate cells are present; ventral cell types are expanded more dorsally than in *Rab23* mutants. Unlike in *Rab23* mutant, dorsal progenitor cells are present.

A simple model for the short-range morphogen activity of Hh in the *Drosophila* wing disc has been suggested based on the bifunctional nature of the Ci transcription factor (Hooper and Scott, 2005; Méthot and Basler, 2000). In the absence of Hh, Ci^{R}, which is generated by proteolytic processing of full-length Ci, represses Hh target genes. Low levels of Hh block this processing of Ci, which causes derepression of some target genes, but activator Ci (Ci^{Act}) is not made. At higher Hh concentrations, Ci is fully converted to the activator form and, with the help of Fused, moves to cell nuclei (Méthot and Basler, 2000). Accordingly, a gradient of Hh can specify at least three distinct fates.

Vertebrate Hh signaling has been studied in greatest depth in the mouse neural tube, where different concentrations of Shh appear to specify a series of cell fates (Jacob and Briscoe, 2003). The source of Shh is at the ventral pole of the neural tube, first in the notochord and then in the floor plate. Mutants that lack all Hh signaling fail to

specify correctly six distinct cell types in the ventral half of the neural tube (Wijgerde et al., 2002) (Fig. 2). As in the fly wing disc, Gli3 repressor is essential for the fates of cells that experience low concentrations of Shh (lateral neural cell types) (Persson et al., 2002) and Gli2 activator is essential for the ventral neural cells that respond to highest levels of Shh, the floor plate (Ding et al., 1998; Matise et al., 1998) (Fig. 2). The intervening neural cell types can be specified by different levels of Gli activator (Stamataki et al., 2005), which suggests that different levels of Shh are directly translated into different ratios of Gli activator/Gli repressor, which control cell fate. However, the specification of multiple neural cell types in response to different amounts of Shh over a large field could place constraints on this signaling pathway that would require new components or strategies.

Smo: the nodal point of the pathway

The single *Smo* gene in *Drosophila*, mouse and zebrafish is responsible for all responses to Hh ligands (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Varga et al., 2001; Zhang et al., 2001). Because different ligand concentrations can cause different cellular responses, Smo must be able to relay different levels of activity. Thus, both its regulation and function are complex.

Smo is an integral membrane protein with a seven-transmembrane (TM) domain structure like that of G-protein-coupled receptors (GPCRs) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). However, only limited evidence indicates that Smo directly couples with G proteins (DeCamp et al., 2000; Kasai et al., 2004) and it is unclear whether there is a ligand that regulates Smo activity. Sequence comparisons among vertebrate and invertebrate Smo proteins show that the TM region of Smo is relatively conserved

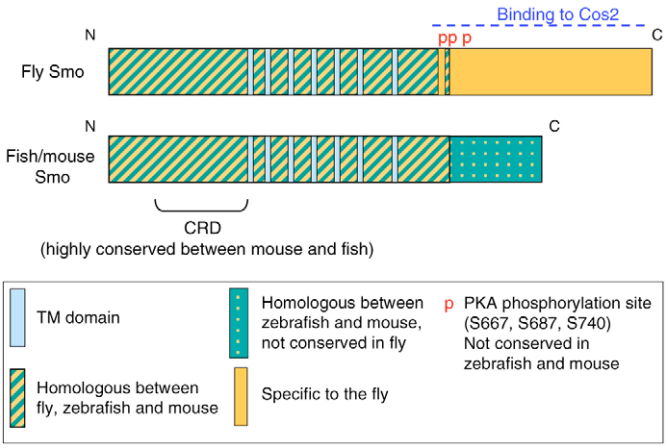

Fig. 3. Alignment of the fly Smo protein with the zebrafish/mouse Smo. The transmembrane (TM) domain and the N-terminal regions of the protein are relatively conserved from fly to mammals. The cysteine-rich domains (CRDs) in fish and mouse smoothened (Smo) are very similar (70% identical, 82% similar), while the CRD in fly Smo is more divergent (43% identical, 56% similar between fly and mouse). C-terminal to the 180 residues adjacent to the 7th TM domain of *Drosophila* Smo, there are only short patches of homology between *Drosophila* Smo and either the zebrafish or the mouse Smo, whereas this region is relatively conserved (31% identity) between zebrafish and mouse Smo. This same region in flies is important for binding to Cos2, indicating that the interaction between Smo and Cos2 is not conserved in vertebrates. The protein kinase A phosphorylation sites in the fly Smo protein are not conserved. PKA, protein kinase A.

across species, while significant divergence exists in the cytoplasmic C-terminal tails (Fig. 3). This divergence is the first clue that Smo may act differently in vertebrates and invertebrates.

Divergence in Smo structure

The seven-TM domain structure of Smo strongly resembles that of the Frizzled (Fz) family of proteins, the receptors of the Wnt signaling pathway, which shares several features with the Hh pathway (Nusse, 2003). Both Smo and Fz have an N-terminal extracellular cysteine-rich domain (CRD), which, in Fz, is required for binding to Wnt family ligands (Bhanot et al., 1996). Its function in Smo is unknown. Smo does not bind to Wnt family ligands, nor does it bind to the Hh ligand directly. Instead, Hh regulates Smo indirectly through the Hh receptor Ptc, which apparently does not directly interact with Smo (see below). Mutations in the *Drosophila* CRD disrupt Smo activity in vivo (Alcedo et al., 2000; Nakano et al., 2004), while deletion of the CRD in mammalian cells does not affect the activity of overexpressed Smo (Murone et al., 1999; Taipale et al., 2002). These differences could be due to divergence of the pathway, but more likely reflect activation by endogenous signals versus overexpression. Nevertheless, while the CRDs in fish and mouse Smo are 70% identical and 82% similar, the CRD in fly Smo is more divergent (43% identical, 56% similar between fly and mouse), which could mean that the vertebrate and *Drosophila* CRDs have distinct functions (Fig. 3).

The heptahelical TM region of Smo is relatively conserved (45% identity) across species. In the fly wing disc, expression of a Fz/Smo chimeric protein, in which the cytoplasmic domain of Fz was replaced with that of Smo, could mediate both low and high Hh responses in response to Wg, instead of Hh (Hooper, 2003). This indicates that the binding of Wg changes the conformation, or multimerization, of the receptor sufficiently to regulate the activity of Smo, and that similar changes occur during the normal activation of both Fz and Smo.

The Fz/Smo chimera experiments indicated that the cytoplasmic domain of *Drosophila* Smo is sufficient to mediate all responses to Hh, when properly regulated. Additional experiments in the fly have confirmed that the cytoplasmic C-terminal domain (CTD) is essential for Smo activity: expression of a membrane-tethered version of the Smo C-terminal tail alone can partially activate Hh signaling (Jia et al., 2003; Nakano et al., 2004).

Despite its important function, the CTD is the most divergent region of the protein: only the 180 amino acid juxtamembrane region is highly related in vertebrate and invertebrate Smo (Fig. 3). The 400 amino acids of the *Drosophila* protein that lie more C-terminally have only short patches of homology with either the zebrafish or the mouse Smo, whereas the entire CTD is relatively conserved (31% identity) between zebrafish and mouse Smo.

Is Smo activated by different mechanisms in flies and vertebrates?

In the absence of the Hh ligand, Ptc inhibits Smo activity. Upon binding of the ligand, Ptc relieves its inhibition of Smo and allows Smo to activate downstream components. This relationship is evolutionarily conserved; in both vertebrates and invertebrates, biochemical data show that mammalian Ptch1 binds to the Shh ligand (Marigo et al., 1996; Stone et al., 1996) and genetic analyses show Smo acts downstream of Ptc (Alcedo et al., 1996; Chen and Struhl, 1996; Zhang et al., 2001). It is, however, not clear how Ptc regulates Smo in any organism. There is little evidence that the endogenous Ptc and Smo proteins interact directly. In fact, activation of the Hh pathway induces opposite changes in the subcellular

localization of Ptc and Smo (Denef et al., 2000; Incardona et al., 2002), and Ptc inhibits Smo activity in a catalytic manner, whereby one molecule of Ptc can regulate ~50 Smo molecules (Taipale et al., 2002), contrary to the prediction of a stoichiometric interaction model.

If Ptc does not regulate Smo activity through direct interactions, there must be intermediate components that relay signals from Ptc to Smo. Some clues suggest that the intermediate component could be a small molecule. The Ptc protein has similar structure to the RND (resistance, nodulation, division) family of bacterial proton gradient driven transmembrane molecular transporters (Hooper and Scott, 1989; Nakano et al., 1989) and to the Niemann-Pick C1 (Npc1) protein, a regulator of cholesterol trafficking (Scott and Ioannou, 2004). Ptc, like Npc1, also has a sterol-sensing domain (Carstea et al., 1997), which suggests that sterols might regulate Ptc activity. These structural features suggest that Ptc may transport hydrophobic molecules that, in turn, regulate Smo activity. The finding that several exogenous small molecules that bind Smo act as either agonists and antagonists of Smo activity (Chen et al., 2002) is consistent with the possibility that an endogenous small molecule regulates Smo.

However, similar effects of these small molecules have not been seen in *Drosophila*. Cyclopamine, for example, inhibits Hh signaling in both zebrafish and mammals through direct interactions with the heptahelical region of Smo, but it has no effect in *Drosophila*, nor does it bind to the *Drosophila* Smo protein (Chen et al., 2002; Chen et al., 2001; Taipale et al., 2000). Although it is possible that subtle changes in the conformation of the *Drosophila* Smo protein render it inaccessible to cyclopamine, it is also possible that the difference in activity of small molecules represents the divergence in the regulation of Smo by upstream components of the pathway.

Correlation of activation and localization of Smo

Cell-surface accumulation of Smo protein correlates with the activation of the Hh pathway in *Drosophila* in vivo (Denef et al., 2000; Nakano et al., 2004; Zhu et al., 2003). In addition, overexpressed membrane-tethered forms of Smo are more potent activators of the pathway in the wing disc than is wild-type Smo, while Smo mutant forms that are trapped in the endoplasmic reticulum (ER) have weaker activities. These results suggest that Hh signaling may regulate the activity of *Drosophila* Smo through regulation of its subcellular localization.

By contrast, transfected Smo in mammalian cultured cells is internalized after activation of the pathway, instead of accumulating at the cell surface (Incardona et al., 2002). Similarly, the internalization of Smo has been observed when the pathway is activated using a Hh agonist, and can be reversed by treatment with the Hh antagonist cyclopamine (Chen et al., 2004). These findings suggested that Smo localization might be regulated differently in flies and mammals. However, recent findings on the role of cilia in the Hh pathway suggest that vertebrate Smo, like the *Drosophila* protein, is recruited to specialized membranes in response to ligand (see below).

Smo C-terminal tail phosphorylation plays different roles in *Drosophila* and vertebrates

The *Drosophila* Smo protein has a large C-terminal tail that contains a set of sites for phosphorylation by protein kinase A (PKA) and casein kinase I (CKI) (Fig. 3). Smo phosphorylation is coupled with Hh activation in *Drosophila* (Denef et al., 2000; Zhang et al., 2004). Using phosphorylation-mimicking or unphosphorylatable forms of Smo, it was found that Smo phosphorylation is required for both its cell-surface accumulation and its activity in cell culture and in vivo (Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005). In vitro kinase assays show that both PKA and CKI kinases can phosphorylate Smo directly, and modulation of PKA or CKI activities in vivo changes Hh-induced Smo cell-surface accumulation (Jia et al., 2004).

Most of the phosphorylated residues identified in the fly Smo C-terminal tail are not conserved in vertebrates, which argues that vertebrate Smo proteins are regulated differently. Mammalian cell culture experiments show that the mammalian Smo protein is also phosphorylated, and that the phosphorylation depends on a different kinase, the G-protein-coupled receptor kinase 2 (Grk2) (Chen et al., 2004). Phosphorylation by Grk2 causes internalization of Smo, a process that involves β-arrestin 2 (Arrb2).

In the zebrafish, *arrb2* morpholino knockdown experiments indicate that *arrb2* acts as a positive regulator of the Hh pathway (Wilbanks et al., 2004), which suggests that Smo phosphorylation and internalization promotes its activity. This contrasts with the case in *Drosophila*, where Smo phosphorylation also activates Smo but leads to cell surface accumulation. Mouse mutants that lack *Arrb2* are viable, but mutants that lack both *Arrb1* and *Arrb2* are embryonic lethal (Kohout et al., 2001), although the double-mutant embryos have not been examined for defects in Hh signaling. Mouse mutants that lack *Grk2* die between embryonic day (E) 9.0 and E15.5 with heart abnormalities (Jaber et al., 1996), but similarly have not been examined for Hh mutant phenotypes.

Downstream of Smo: cytoplasmic Hh signal transducers

Analysis of the protein complexes that associate with *Drosophila* Smo has led to an understanding of the signal transduction mechanism and has provided information about how different concentrations of Hh ligand can mediate different responses. However, recent studies show that some of these downstream events may not be conserved in vertebrates.

Cos2 bridges Smo and Ci in *Drosophila*

Cos2 is a kinesin-related protein that regulates the production of both Ci^{R} and Ci^{Act} (Sisson et al., 1997; Wang, G. et al., 2000; Wang and Holmgren, 2000). Overexpression of the CTD of Smo activates low levels of Hh signaling in the wing disc. This phenotype is suppressed by expression of an additional copy of wild-type Cos2, which suggests that the CTD of Smo antagonizes Cos2 activity (Hooper, 2003).

This idea was supported by evidence showing that direct physical interactions occur between *Drosophila* Smo and Cos2 (Jia et al., 2003; Lum et al., 2003b; Ogden et al., 2003; Ruel et al., 2003). In the absence of Hh signaling, Cos2 forms a complex with Fu, Sufu and the Ci transcription factor that both promotes cleavage of the full-length Ci to Ci^{R} and keeps full-length Ci out of the nucleus. In response to low levels of Hh, the Smo-Cos2 complex is recruited to the membrane, and this relieves the inhibitory effect of Cos2 on Ci, which may lead to dissociation of Ci from the Smo-Cos2 complex (Ruel et al., 2003). However, Ci is not fully activated and cannot enter the nucleus, because Ci is tethered by Sufu in a complex that also includes Cos2. At high levels of Hh signaling, this final restriction is removed, and Ci^{Act} can move into the nucleus to activate the pathway to a high level.

The identification of homologs of *Cos2* was challenging because of the large number of kinesin-related genes in vertebrate genomes. Two mammalian Cos2-like proteins, Kif7 and Kif27, both share

considerable sequence homology with *Drosophila* Cos2 (22-23% identical, 37-38% similar) (Katoh and Katoh, 2004). Morpholino knockdown experiments have indicated that a zebrafish homolog of mammalian Kif7 acts like Cos2 as a negative regulator of the Hh pathway (Tay et al., 2005). The major Cos2 interaction domain of Smo is in the long C-terminal tail present in the fly protein that is not conserved in zebrafish (Jia et al., 2003). A more membrane-proximal segment that interacts with Cos2 has limited sequence conservation in the vertebrate Smo protein (Lum et al., 2003b), although it has not been tested whether this domain can bind Kif7. The roles of Kif7 and Kif27 in the mammalian Hh pathway have not yet been tested.

The role of Fused

In *Drosophila*, the response to high levels of Hh specifically requires the serine/threonine kinase Fused (Preat et al., 1990). Recently, a zebrafish homolog of *fused* (*stk36*) was identified, and morpholino knockdown experiments indicate that zebrafish *fused* is required for responses to high levels of Hh (Wolff et al., 2003). Morpholino knockdown of both *fused* and *Sufu* restored the specification of muscle cell types that depend on high levels of Hh signaling, which suggests that zebrafish Fused antagonizes Sufu, similar to the role of Fused in the fly pathway (Wolff et al., 2003). However fish Fused also appears to play a role in the response to submaximal, as well as high, Hh signaling, unlike the *Drosophila* protein (Wolff et al., 2003).

The mouse Fused homolog (Stk36) is broadly expressed in the developing embryo, including in Hh-responsive tissues (Chen et al., 2005). However mice that lack all activity of this protein survive beyond birth and have no apparent defects in hedgehog signaling (Chen et al., 2005; Merchant et al., 2005). This striking discrepancy between the mouse and the zebrafish/fly functions of this protein could be explained if another distantly related kinase overlaps in function with mouse Fused homolog. Alternatively, the function of Fused may have diverged between fish and mice.

Sufu: a major repressor of the vertebrate pathway

Drosophila Sufu was identified through its ability to suppress the phenotype of *fused*. Sufu helps to tether full-length Ci in the cytoplasm, and therefore helps keep the pathway switched off in the absence of ligand (Méthot and Basler, 2000). Sufu also appears to play a role in the inhibition of Ci^{Act} in the nucleus (Kogerman et al., 1999; Pearse et al., 1999; Wang et al., 1999). *Sufu*-null mutant flies are viable and fertile, and show a phenotype only when *fused*, or another pathway component, is also mutated (Preat, 1992). Thus, *Drosophila* Sufu has a minor role in negative regulation of the *Drosophila* Hh pathway.

The zebrafish *dreumes* gene was recently shown to encode Sufu. Although the *dreumes* mutant does not show any obvious Hh-related phenotypes (Koudijs et al., 2005), morpholino knockdown of *sufu* in zebrafish causes a gain of Hh signaling phenotype in muscle patterning (Wolff et al., 2003). The stronger effect of the morpholino knockdown is probably due to a maternal Sufu contribution that is not inactivated in homozygous *dreumes* mutants (Koudijs et al., 2005). The *sufu* morpholinos produced an effect on muscle patterning even when wild-type *fused* was present, which suggests that Sufu has a different, and more crucial, role in negative regulation of the vertebrate pathway than in *Drosophila*.

A targeted allele of mouse Sufu has a more dramatic effect on Hh signaling. Null embryos die at midgestation and show a strong Hh gain-of-function ventralization of cell types in the neural tube (Cooper et al., 2005). Like patched 1 (*Ptch1*) null mutants, all cells in the neural plate of *Sufu* embryos express markers of the most ventral neural cell type, the floor plate, and express *Ptch1*

1, a direct Hh target gene. It is possible that the stronger phenotype of the mouse *Sufu* null than the zebrafish morphant is due to residual Sufu function in the fish that is resistant to morpholino knockdown.

Both the zebrafish and mouse results demonstrate that vertebrate Sufu plays a crucial role in negative regulation of the pathway, in contrast to its subtle role in *Drosophila*. In keeping with these findings, humans that are heterozygous for *SUFU* mutations have a predisposition to medulloblastoma, as seen with mutations in *PTCH1* (Pasca di Magliano and Hebrok, 2003; Taylor et al., 2002). It remains to be determined which aspect of Sufu function is crucial in vertebrates – regulation of Gli nuclear localization, regulation of Gli-activator activity or some other role in the pathway.

PKA is a conserved negative modulator of the pathway

In *Drosophila*, PKA is required for the proteolytic cleavage of Ci to generate Ci^{R} (Chen et al., 1999; Chen et al., 1998; Price and Kalderon, 1999; Wang et al., 1999). The phosphorylation of specific Ci residues by PKA primes the phosphorylation of additional sites by CK1 and GSK3β that are also necessary for processing (Price and Kalderon, 1999); fully phosphorylated Ci can be recognized by Slimb, a component of the SCF ubiquitin ligase that allows proteasome-dependent processing of Ci. In both *PKA* and *Slimb* mutant clones, full-length Ci accumulates owing to lack of proteolytic cleavage; however, Ci becomes a transcriptional activator only in *PKA*, but not in *Slimb*, mutant clones because PKA is also required to prevent activation of the full-length Ci protein (Wang et al., 1999). In support of this idea, loss of PKA phosphorylation sites not only blocks the proteolytic cleavage of Ci, but also makes it constitutively active (Chen et al., 1999; Price and Kalderon, 1999).

In addition to its repressor function, PKA has also been proposed to act as an activator of the *Drosophila* Hh pathway. Unphosphorylatable forms of Ci can be further activated by PKA overexpression (Chen et al., 1998), which suggests PKA may phosphorylate other factors that regulate Ci activity. As described above, PKA directly phosphorylates Smo, and overexpression of the unphosphorylatable form of Smo does not rescue the *Smo* mutant phenotype, which suggests that the activator role of PKA is due to its ability to phosphorylate Smo.

No genetic analysis has been carried out in vertebrates to assess the in vivo functions of Btrc (β-TrCP; β-transducin repeat-containing protein, the Slimb homolog), CK1 and GSK3β, in part because of the existence of more than one homologous gene. However, PKA has been shown to function as a repressor, but not as an activator, of the zebrafish and mouse Hh pathways. Dominant-negative and constitutively active forms of PKA expressed in zebrafish embryos indicate that PKA is a cell-autonomous negative regulator of Hh signaling in the fish (Concordet et al., 1996; Hammerschmidt et al., 1996). Two mouse genes encode the PKA catalytic subunits Cα and Cβ (*Prkaca* and *Prkacb*). Although both Cα and Cβ single mutants develop normally, Cα homozygous mutants that have only a single copy of wild-type Cβ, or Cβ homozygous mutants with only a single copy of wild type Cα, survive to E12.5, and show dorsal expansion of ventral markers in the neural tube, consistent with a role for PKA as a negative regulator of Hh signaling (Huang et al., 2002). As the PKA phosphorylation sites are not conserved in vertebrate Smo proteins (Fig. 3), it is unlikely that PKA activates the vertebrate pathway by phosphorylating Smo.

Smo and downstream proteins may require localization to cilia for activity

Intraflagellar transport (IFT) is a process that is required for the assembly and maintenance of all cilia and flagella in plants, protists and animals (Rosenbaum and Witman, 2002). Seventeen IFT proteins have been identified that comprise two large complexes (A and B) that transport cargo along the axonemal microtubules of the cilia. We and others have found that several components of the mouse IFT machinery, including three IFT complex B proteins (Ift172/Wimple, Polaris/Ift88/Ttc10, Ngd5/Ift52), an IFT anterograde motor subunit (Kif3a) and an IFT retrograde motor subunit (Dnchc2) are all required for Hh signaling at a step between Smo and the Gli transcription factors (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). The neural tubes of these IFT mutants lack most ventral neural cell types, but lateral and dorsal neural cell types are specified normally, a phenotype similar to that of mutants that lack both *Gli2* and *Gli3* (Bai et al., 2004; Lei et al., 2004; Motoyama et al., 2003) (Fig. 2). *Ift172* mutants also do not form a lung (D.H. and K.V.A., unpublished), like *Gli2 Gli3* double mutants (Motoyama et al., 1998) and unlike *Shh* mutants (Litingtung et al., 1998), which suggests that IFT is required for both Shh and Ihh signaling. Mouse IFT proteins are required for all modulation of both Gli activator and Gli repressor in response to Hh ligands (Huangfu and Anderson, 2005; Liu et al., 2005); as a result, IFT mutants display a loss of Hh signaling phenotype in the neural tube, where Gli activators play the major role in pattern formation, and a gain of Hh signaling phenotype in the limb, where Gli3 repressor plays the major role.

Although *Drosophila* has IFT proteins, fly mutants that lack the homologs of *Ift88*, *Ift172* and *Kif3a* are viable and do not have the patterning defects expected in Hh pathway mutants (Avidor-Reiss et al., 2004; Han et al., 2003; Ray et al., 1999). Instead, these mutants have defects in sensory behavior; this correlates well with the requirement for these proteins to make cilia in ciliated sensory neurons.

The function of the IFT components in mammals has some parallels with that of *Drosophila* Cos2. Both have dual positive and negative functions in the pathway, as both are required for Ci/Gli3 processing and both are required for Ci/Gli activator functions. Cos2 is a kinesin-like protein and IFTs are microtubule associated, although the Kif3a kinesin-subunit of the anterograde IFT motor is not the ortholog of Cos2 (Katoh and Katoh, 2004). Because of the similar functions of Cos2 and IFT proteins, one possibility is that IFT proteins substitute for the function of Cos2 in mammals and provide the missing link between Smo and the Gli proteins.

Alternatively, several lines of evidence suggest that IFT components have a different function from Cos2 and are required because of the role of cilia in the Hh pathway. Both the IFT anterograde and retrograde motors are required for both Gli activator functions and formation of Gli3 repressor (Huangfu and Anderson, 2005), which is consistent with a requirement for normally structured cilia in transduction of the signal from Smo to Gli proteins rather than vectorial transport of pathway components. In addition, the presence of cilia is correlated with Hh responsiveness. Non-motile primary cilia are present on most vertebrate cells, including every Hh-responsive cell type that has been examined (Pazour and Witman, 2003). Cultured cells become Hh responsive only when they are confluent (Bailey et al., 2002), and also have cilia only when they are confluent (Quarmby and Parker, 2005).

Recent data have shown that mouse Smo becomes localized to cilia in response to Hh signaling (Corbit et al., 2005). Smo is approximately threefold enriched in the cilia relative to other parts of the cell in the mouse node, where Hh signaling is active. When Smo was expressed in MDCK cells, it was not localized to cilia unless the pathway was activated by treatment with Shh. Expression of an activated allele of Smo in these cells led to its constitutive localization to cilia, even in the absence of Hh. This signal-dependent localization is reminiscent of the situation in *Drosophila*, where Smo activity correlates with its cell-surface localization, although Hh-responsive cells in *Drosophila* do not have cilia. The localization of Smo to cilia depends on a short motif immediately C-terminal to the last TM domain that is present in other 7-TM receptors that localize to cilia. This motif is required for localization of mouse Smo to cilia, and appears to be required for Smo activity (Corbit et al., 2005). The same motif is present in *Drosophila* Smo; if that motif is required for membrane localization of fly Smo, it will be interesting to explore how *Drosophila* has retained this targeting mechanism even in the absence of cilia.

As IFT proteins are required for both Gli3 processing and Gli activation (Huangfu and Anderson, 2005; Liu et al., 2005), both positively and negatively acting components of the pathway may be localized to cilia. It will be of particular interest to learn whether mammalian Gli, Sufu and Fu are also localized to cilia. If these proteins are enriched in cilia, it would suggest that cilia act as signaling centers where pathway components can be concentrated and their interactions enhanced. It will also be of interest to determine whether IFT-mediated transport is required for pathway components to interact correctly.

There are currently no data indicating that IFT proteins are required for Hh signaling in zebrafish, but several pieces of data are missing in the IFT puzzle. At this point, there are two possibilities: IFT proteins may have been substituted for Cos2 in the pathway at some point after the divergence of fish and mammals; or fish and mammals may use both Cos2 and IFT proteins in parallel processes downstream of Smo. The morpholino data indicate that Cos2 is an important negative regulator in zebrafish, while there are no data about Cos2 function in mammals. Nor is it known whether IFT proteins are required for Hh signaling in the zebrafish. There are zebrafish mutations in several IFT components, but these mutants or morpholino knockdown experiments have not shown Hh-related phenotypes (Sun et al., 2004; Tsujikawa and Malicki, 2004). However, zygotic null mutants of IFT components still have cilia, because of maternally contributed components (Sun et al., 2004), so it is possible that IFT proteins play a role in zebrafish Hh signaling. Although data to resolve these issues should become available soon, the similarity of the fish and mammalian Smo proteins (and their differences from *Drosophila* Smo) suggests to us that vertebrates will use both Cos2 and IFT proteins in Hh signal transduction.

Additional cytoplasmic components of the vertebrate pathway

Genetic screens and targeted mutations have identified several other genes that appear to act downstream of Smo and upstream of Gli proteins in the vertebrate Hh pathway, based on loss-of-function phenotypes that resemble those of other Hh pathway mutants and on genetic and physical interactions with known pathway components. These mutants have diverse phenotypes (Fig. 2) and may have distinct effects on Gli proteins (Fig. 4), suggesting that there are additional fundamental differences between the mechanisms that transduce Hh signals in *Drosophila* and vertebrates.

A new component of the zebrafish pathway, *iguana* (*igu*; *dzip1* – Zebrafish Information Network), has both positive and negative effects on the pathway (Sekimizu et al., 2004; Wolff et al., 2004). Igu acts downstream of PKA, interacts genetically with Sufu and

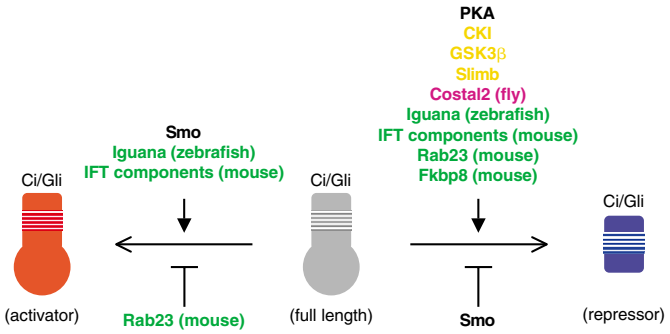

Fig. 4. The regulation of Ci/Gli proteins. The full-length Ci protein (gray) can be proteolytically cleaved to generate a repressor form (blue) or activated to generate an activator form (red). The vertebrate Gli homologs share similar domain structures with Ci, but only Gli3 is known to be cleaved into a functional repressor form. The conserved and diverged aspects of the regulation of Ci/Gli activation and cleavage are shown. The zinc-finger domains are indicated by stripes. Components that have been shown either to promote or to prevent these processes are indicated in the figure. Components shown in black are common to both vertebrates and invertebrates; those in yellow are likely to be conserved, but there is insufficient in vivo data to support their conserved roles; those in purple can play a role in invertebrates; and those in green function in mouse and zebrafish, but not in fly. CKI, casein kinase 1; GSK3β, glycogen synthase kinase 3β; PKA, protein kinase A.

appears to control the nuclear localization of Gli proteins. *igu* has homologs of unknown function in both *Drosophila* and mammals. Human *DZIP1*, the homolog of *igu*, was initially identified in a yeast two-hybrid screen as a protein that interacts with DAZ, a protein involved in spermatogenesis (Moore et al., 2003); however, the functions of *DZIP1* in spermatogenesis and Hh signaling have not been determined.

Several other mouse genes have been identified that act in the cytoplasmic signaling pathway, including *Sil*, *Rab23*/open brain and *Fkbp8*. Mutations in all these genes cause striking effects on neural patterning, but the phenotypes are distinct (Fig. 2).

Rab23 (previously *opb*) mutants show a gain of Hh signaling phenotype in the caudal spinal cord and other cell types (Eggenschwiler and Anderson, 2000; Eggenschwiler et al., 2001). Rab23 belongs to the Rab family of GTPases that regulate vesicular transport. Although overexpressed Rab23 colocalizes with Ptch1 in endosomes (Evans et al., 2003), double mutant analysis indicates that Rab23 acts downstream of both Ptch1 and Smo (Eggenschwiler et al., 2005). Loss of Gli2 almost completely suppresses the Rab23 phenotype (Eggenschwiler et al., 2005), which argues that the Rab23 acts primarily as a negative regulator of Gli2 activity. There is a *Drosophila* ortholog of *Rab23*, but mutants in this gene are viable and fertile (J. Sierra and I. Guererro, personal communication).

Both *Ift172* and *Polaris/Ift88* are required for the activity of *Rab23* (Huangfu et al., 2003). If we assume that the IFT proteins are required because of their role in cilia, then Rab23 could regulate a trafficking event downstream of Smo that requires cilia or Rab23 could regulate trafficking inside cilia.

Fkbp8 is a member of the FK506-binding protein family (the immunophilins) that acts as an antagonist of Hh signaling in the caudal neural tube (Bulgakov et al., 2004) and also acts downstream of Smo and upstream of Gli proteins (Eggenschwiler et al., 2005). The ventralized phenotype of *Fkbp8* mutants is distinct from that of *Rab23* mutants: there is a greater expansion of the most ventral neural cell type, the floor plate, but dorsal cell types are less affected (Fig. 2). Fkbp8 has been shown to inhibit the Ca^{2+}-calmodulin activated serine/threonine-specific protein phosphatase calcineurin (Shirane and Nakayama, 2003), although it is not known whether the function of Fkbp8 in the Hh pathway depends on calcineurin. There are three calcineurin catalytic subunit genes, and mutants that lack any single subunit do not have Hh-related phenotypes (Schulz and Yutzey, 2004), which could be due to overlapping functions. Unlike Rab23, the effect of Fkbp8 appears to be neural specific; this is the first clear example of a tissue-specific regulator of the pathway in vertebrates.

Sil was identified as a gene at a chromosome breakpoint associated with human leukemia (Aplan et al., 1990), although Sil is not the oncogene of the translocation. Targeted deletion of *Sil* causes embryonic lethality at ~E9.0, associated with a loss of ventral neural cell types, such as *Smo* or *Kif3a* mutants (Izraeli et al., 1999). Genetic experiments demonstrated that Sil is required downstream of Ptch1 to turn on Hh targets (Izraeli et al., 2001). The Sil protein does not have any obvious structural domain that indicates its function, and there is no obvious *Drosophila* homolog.

At least one newly defined component may play roles in both the mammalian and *Drosophila* pathways. A targeted mouse mutation in a gene encoding an Ig-family transmembrane protein, Cdon, causes microform holoprosencephaly, a condition associated with loss of Hh signaling (Cole and Krauss, 2003). The *Drosophila* homolog of this gene, *iHog*, was identified in an RNAi screen to be a positive regulator of Hh signaling (Lum et al., 2003a), which is consistent with the mouse mutant phenotype.

For each of the newly identified components, future experiments will test their function in the pathway. Even if *Drosophila* homologs of the new vertebrate genes do not have dramatic phenotypes on their own, they might, like *Sufu*, still modulate the fly pathway in the appropriate double mutant background. The analysis of mouse double mutants that lack both the newly identified players and core pathway components should define the step affected by each new vertebrate gene. The subcellular localization of the proteins in embryonic tissues will also be of central importance. For example, it will be important to learn if any of these proteins are localized to cilia. Bardet-Biedl syndrome (BBS) is a complex human genetic disorder associated with polycystic kidney, polydactyly, situs reversal and obesity (Pan et al., 2005). Both polydactyly and situs reversal are associated with abnormal Hh signaling, which suggests that there could be connections between abnormal Hh signaling, BBS and basal bodies. Some BBS proteins are localized to basal bodies, the centriole-based structure at the base of the cilia, and some BBS proteins undergo IFT (Blacque et al., 2004; Kim et al., 2004; Kim et al., 2005), so some of the newly identified Hh pathway components could act in basal bodies.

Are these new genes just the tip of the iceberg? Given that the genetic screens that identified Rab23 and the IFT mutants have not yet been carried to saturation (García-García et al., 2005; Kasarskis et al., 1998), it is certain that more components remain to be identified that act between Smo and Gli. For example, the chicken *talpid* mutants have phenotypes similar to those of IFT mutants (Buxton et al., 2004; Caruccio et al., 1999; Lewis et al., 1999; Wang, B. et al., 2000), and could well encode additional proteins that act at this step.

Several hundred proteins are required for normal cilia structure and function (Avidor-Reiss et al., 2004; Li et al., 2004), and if normally structured cilia are essential for mammalian Hh signaling, many of those proteins might impact on Hh signal transduction. If this proves to be the case, the challenge will be to understand the physical relations among the ciliary and Hh pathway components that allow efficient Hh signaling.

The Ci/Gli transcription factors

The separation of activator and repressor function into different vertebrate Gli proteins provides the opportunity for additional modulation of the pathway. In *Drosophila*, the bifunctional protein Ci is the only transcription factor at the end of the Hh pathway, while there are three Gli proteins in the mouse that have different biochemical properties and different modes of regulation. Gli3 is bifunctional, like Ci: it can be processed into a repressor form in vivo and also functions as a transcriptional activator (Wang, B. et al., 2000). Gli1 cannot be proteolytically processed and is only a transcriptional activator (Dai et al., 1999; Lee et al., 1997; Ruiz i Altaba, 1998). Gli2 is an important activator in vivo, and because Gli1 can replace Gli2 function in vivo, it is likely that Gli2 does not have a significant repressor function (Aza-Blanc et al., 2000; Wang, B. et al., 2000).

The three Gli proteins and Ci share high homology in the zinc-finger domain, but have limited homology outside this region (Matise and Joyner, 1999), which raises the possibility that other proteins may interact with and modulate the activity of individual Gli proteins. Several vertebrate proteins that modulate Gli activity have been identified, but their specificities are not yet clear. MIM/BEG4, a transcriptional target of mammalian Hh signaling, can associate with Gli1 and Gli2 and potentiate transcriptional activity, at least in vitro (Callahan et al., 2004; Gonzalez-Quevedo et al., 2005). The Ski co-repressor can bind Gli3 and regulate its repressive activity (Dai et al., 2002). The mouse Zic proteins function in dorsal neural patterning and can interact with Gli proteins to modulate their transcriptional activity, but no specificity of interaction was observed in vitro (Aruga, 2004; Koyabu et al., 2001). Specific interactions with other transcription factors or co-factors might help explain how the single morphogen Shh can specify so many different ventral neural cell types (Fig. 2).

Conclusion: evolution and the Hh pathway

Despite the evolutionarily conserved core of the pathway, the data reviewed here highlight, surprisingly, many differences between the *Drosophila* and vertebrate Hh signal transduction pathways. Differences are apparent in the structure of the Smo protein, especially its CTD, in the activity of small molecule agonists and antagonists of Smo, in the subcellular localization of active Smo, in the requirement for IFT and other proteins in the vertebrate but not the *Drosophila* pathway, and in the functions of Fused and Sufu. Although these differences are real, and the identification of vertebrate-specific pathway components has important implications for the human diseases that involve Hh signaling and cilia, additional experiments are required to determine whether the biochemical mechanisms of signal transduction differ fundamentally between *Drosophila* and vertebrates.

Comparisons of the pathway between species should help to define the true core pathway. Did mammals add cilia to the pathway or did *Drosophila* lose the requirement for them? The ancestral roles of Cos2, Fused and Sufu also need to be investigated. More detailed comparisons between the zebrafish and mouse pathways is a first step towards answering these questions. RNAi strategies should also help to define the essential components of the pathway in non-model organisms in order to deduce the structure of the ancestral Hh signaling pathway.

Note added in proof

Recently, Haycraft et al. demonstrated that Gli1, Gli2, Gli3 and Sufu proteins are enriched in cilia (Haycraft et al., 2005).

We thank Jeremy Reiter and Jonathan Eggenschwiler for sharing information prior to publication. Our research is supported by the NIH.

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