Chapter 2
Primary Cilia, Sonic Hedgehog Signaling, and Spinal Cord Development

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Contents

2.1 Introduction 56
2.2 Shh Signaling in the Developing Spinal Cord 57
2.2.1 Patterning the Neural Tube 57
2.2.2 Shh Signaling Pathway Components 59
2.2.3 Maintaining Neural Patterning 61
2.3 Molecular Mechanisms of Shh Signaling in Cilia 63
2.3.1 Requirement of Cilia for Sonic Hedgehog Signaling 63
2.3.2 Cilia-Dependent Sonic Hedgehog Effectors 64
2.4 Cilia Proteins Essential for Shh Signaling: Lessons from Mutants 68
2.4.1 Intraflagellar Transport 68
2.4.2 Other Regulators of Shh Signaling in Cilia 73
2.4.3 Spatiotemporal Regulation of Shh Signaling 75
2.5 Conclusions and Perspectives 76
References 76

Abstract Embryonic spinal cord development requires Sonic hedgehog (Shh) signaling to define ventral motor neuron and interneuron progenitor domains during neural patterning. Shh signaling is inextricably linked to primary cilia, and mutations that disrupt cilia structure and/or function lead to abnormal Shh signaling. The embryonic spinal cord is highly sensitive to perturbations in Shh activity and displays abnormal patterning phenotypes when Shh signaling is up- or downregulated.

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K.L. Tucker and T. Caspary (eds.), *Cilia and Nervous System Development and Function*,
DOI 10.1007/978-94-007-5808-7_2, © Springer Science+Business Media Dordrecht 2013

Mutations in a variety of different cilia genes lead to neural tube patterning phenotypes that provide useful information about the role of different proteins in transducing Shh signals. Here we discuss Shh-dependent spinal cord development and describe what is currently known about the molecular mechanisms regulating Shh signaling in the neural tube.

Keywords Primary cilia • Sonic hedgehog • Patched • Smoothened • Gli • Sufu • Kif7 • PKA • Neural tube patterning • Mouse genetics • Forward genetic screens • Intraflagellar transport • Protein trafficking

2.1 Introduction

Primary cilia are linked to a variety of biochemical pathways, with the Sonic hedgehog (Shh) signaling pathway especially notable for its integral connection to cilia structure and function (reviewed in: Berbari et al. 2009; Eggenschwiler and Anderson 2007; Wong and Reiter 2008). Without primary cilia, Shh signaling cannot occur (Huangfu et al. 2003; Huangfu and Anderson 2005), and genetic mutations that disrupt ciliogenesis or other important cilia functions typically cause defects in Shh signaling, as well. Many such mutations are incompatible with life, because Shh is so important for embryonic development, but in animal models they have revealed much about the complex interaction between cilia and developmental signaling.

Shh plays many roles in the developing nervous system (reviewed in: Sánchez-Camacho and Bovolenta 2009; Hatten and Roussel 2011), where it was first shown to possess an essential function in patterning the embryonic spinal cord, also known as the neural tube (Echelard et al. 1993). During development, there is a gradient of Shh activity in the neural tube that confers different cell fates upon neural progenitors at distinct positions along the dorsoventral axis. The resulting progenitor domains are highly consistent among embryos and also highly sensitive to changes in Shh activity (reviewed in: Jessell 2000; Briscoe and Ericson 2001; Lupo et al. 2006; Dessaud et al. 2008), making the pattern of the ventral neural tube an informative readout of any perturbations to the Shh signaling pathway. Indeed, such perturbations are what provided the initial link between cilia and Shh signaling (Huangfu et al. 2003).

In recent years, descriptive in vivo studies on the relationship between cilia and Shh activity in the neural tube were complemented by cellular and molecular experiments that give a more mechanistic explanation of the role of cilia in Shh signaling. We now know that Shh signals are transduced via a series of proteins that dynamically enter and exit the primary cilium in a ligand-dependent manner (Corbit et al. 2005; Haycraft et al. 2005; Rohatgi et al. 2007), ultimately resulting in either the activation or repression of Shh target genes. If the primary cilium is absent or abnormal, Shh signaling is adversely affected (Huangfu et al. 2003; Huangfu and Anderson 2005; Liu et al. 2005). Furthermore, an entire suite of specialized molecules regulates the

trafficking and transport of Shh pathway components and other ciliary proteins into and within cilia (Haycraft et al. 2005; Kovacs et al. 2008; Liu et al. 2005; May et al. 2005; Seo et al. 2011). Disruption of any aspect of this molecular system can lead to Shh signaling defects and abnormal neural development.

In the first part of this chapter, we explore how Shh signaling patterns the neural tube under normal conditions, as well as how this system allows for particularly elegant investigation of Shh signaling in vivo. We focus our discussion primarily on mammalian models, since much of the work on cilia and developmental signaling has been conducted using genetic techniques in the mouse. We also describe in more detail how Shh signaling occurs at the level of individual cells and how primary cilia are essential for the proper regulation of the proteins that constitute the Shh pathway. Finally, we survey the effects of mutations that affect ciliogenesis, ciliary protein transport, and other important processes in primary cilia function to summarize what is currently known and what remains to be explored in terms of the complex interactions between cilia and the Shh pathway.

2.2 Shh Signaling in the Developing Spinal Cord

2.2.1 Patterning the Neural Tube

In vertebrates, the nervous system arises through a process known as neurulation, during which the undifferentiated neuroepithelium of the ectoderm thickens into the neural plate and folds in upon itself to form the neural tube (reviewed in: Colas and Schoenwolf 2001; Copp et al. 2003). The most rostral portion of the neural tube develops into the brain; the more caudal portion gives rise to the spinal cord. As the cells of the neural tube begin to differentiate, the tissue is patterned by opposing gradients of biochemical signaling activity across the dorsoventral axis. Dorsal patterning is primarily governed by BMP and Wnt cues secreted from the roof plate (reviewed in: Lee and Jessell 1999; Caspary and Anderson 2003; Chizhikov and Millen 2005; Liu and Niswander 2005), whereas ventral patterning is regulated by Shh secreted from the notochord and floor plate. Shh is one member of the Hedgehog family of proteins. In mammals, this family also includes Indian hedgehog, which is essential for skeletal development (reviewed in: Mackie et al. 2011; Whitfield 2008) and Desert hedgehog, which is essential for development and maintenance of the male germ line (Bitgood et al. 1996). Although the vertebrate Hedgehog ligands play distinct roles in development, they use similar mechanisms: Indian hedgehog has been shown to act via primary cilia and signals through many of the same downstream molecules as Shh (Whitfield 2008).

Shh signaling is necessary for the formation of six distinct domains in the ventral neural tube (floor plate, p3, pMN, p2, p1, p0) (Fig. 2.1). The floor plate is the most ventral part of the neural tube and serves as a developmental signaling center, providing a secondary source of Shh ligand during neural patterning, as well as cues that mediate axon guidance later in development, along with Slit and Netrin.

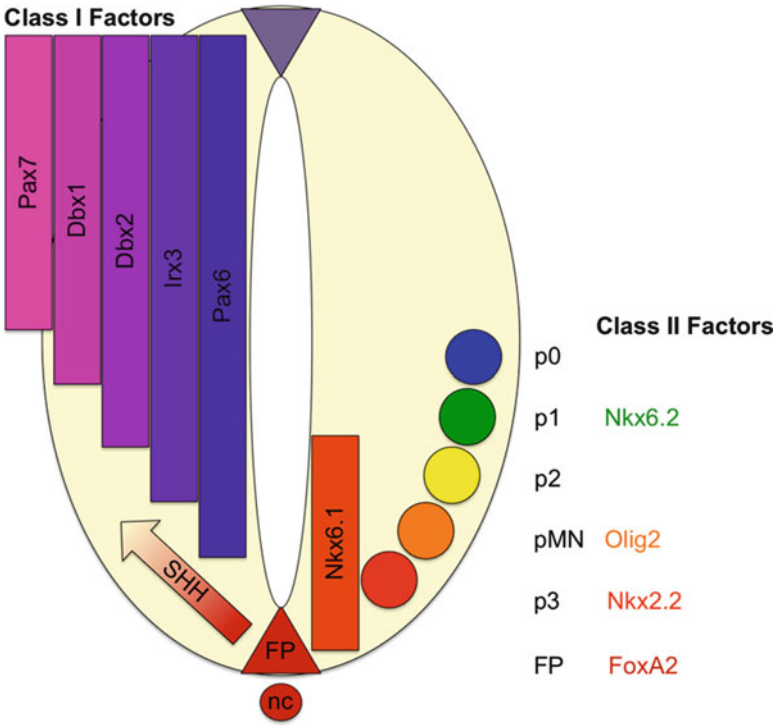

Fig. 2.1 Specification of ventral neural progenitor domains by Shh. Shh produced in the notochord (*nc*) and floor plate (*FP*) leads to the repression of Class I transcription factors and the expression of Class II transcription factors in well-defined dorsoventral domains across the neural tube (*rectangles*). Combinatorial expression of these factors defines the six distinct progenitor domains of the ventral neural tube: FP, p3, pMN, p2, p1, and p0. Nkx6.1 is expressed across multiple ventral progenitor domains; other Class II factors (Nkx6.2, Olig2, Nkx2.2, FoxA2) are restricted to individual domains and can thus be used as molecular markers for specific progenitor cell types (*circles*)

The progenitor cells in the pMN domain give rise to motor neurons (MNs) and subsequently oligodendrocytes. Cells in the other ventral progenitor domains differentiate into different classes of spinal interneurons.

During neural development, Shh is first produced by the notochord, a rod-shaped structure derived from mesoderm that runs the length of the ventral face of the neural tube. Diffusion of Shh ligand from the notochord to the ventral midline of the neural tube induces the formation of the floor plate, which also begins to produce Shh (Martí et al. 1995; Roelink et al. 1995). In both the notochord and the floor plate, Shh protein must be activated through catalytic cleavage, N-terminal palmitoylation, and C-terminal cholesterol modification (Bumcrot et al. 1995; Chen et al. 2004; Pepinsky et al. 1998; Porter et al. 1996), before it is released into the extracellular space through a process that depends on the transmembrane protein Dispatched1 (Caspary et al. 2002; Kawakami et al. 2002; Ma et al. 2002; Tian et al. 2005).

In the simplest terms, Shh can be conceived of as a classic morphogen, meaning the ligand confers spatial information onto differentiating cells via a concentration gradient: cells in the most ventral part of the neural tube are close to the source of Shh, and are therefore exposed to higher concentrations of ligand than more dorsal cells. Proper patterning of the ventral neural tube depends on the variable level of Shh signaling activity along the dorsoventral axis, corresponding to the graded concentration of Shh ligand (Ericson et al. 1997). Ventral cell types (floor plate, p3) require a high level of Shh signaling to induce their differentiation, mediolateral cell types (pMN, p2, p1, p0) require less Shh activity, and dorsal cell fates are repressed by Shh activity.

2.2.2 Shh Signaling Pathway Components

Differentiating neural progenitors respond to Shh through a multi-step molecular signaling cascade that results in the expression of distinct target genes in different classes of Shh-responsive cells. Ultimately, Shh signaling regulates target gene expression via the Gli family of transcription factors, which can act either as transcriptional activators or repressors (Bai et al. 2004; Ding et al. 1998; Lei et al. 2004; Litingtung and Chiang 2000; Matise et al. 1998). Other critical regulators of Gli transcriptional activity include the Shh receptor, Patched1 (Ptch1) (Denef et al. 2000; Marigo et al. 1996; Taipale et al. 2002); the major effector of Shh activity, Smoothened (Smo) (Alcedo et al. 1996; Stone et al. 1996; van den Heuvel and Ingham 1996); the Gli inhibitor, Suppressor of Fused (Sufu) (Ding et al. 1999; Kogerman et al. 1999; Svärd et al. 2006); an atypical kinesin, Kif7 (Cheung et al. 2009; Endoh-Yamagami et al. 2009; Liem et al. 2009); and protein kinase A (PKA) (Epstein et al. 1996; Hammerschmidt et al. 1996; Pan et al. 2009; Tuson et al. 2011) (Fig. 2.2). As the Shh signal is transduced, all of the aforementioned proteins localize to primary cilia in a highly regulated manner.

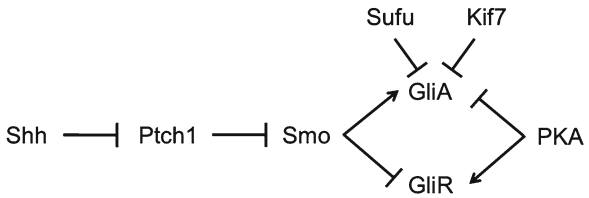

Fig. 2.2 Genetic interactions between core Shh signaling pathway components. Genetic experiments ablating individual components of the Shh pathway have shown whether these proteins primarily activate or inhibit downstream Shh signaling. Shh ligand serves to inhibit the activity of its ligand Ptch1, which in turn inhibits the major Shh effector Smo. Activated Smo leads to the production of GliA, which in turn transcribes Shh target genes. In the absence of Shh, Smo is inhibited and GliR is formed. Other major inhibitors of GliA include Sufu and Kif7. PKA is required for formation of both GliA and GliR, but its most prominent function in Shh signaling is to inhibit Gli activity in the absence of ligand

In the absence of Shh, Ptch1 inhibits Smo, thereby acting as a brake on Shh signaling (Denef et al. 2000; Taipale et al. 2002). When Shh binds to Ptch1, the inhibition of Smo is relieved, triggering activation of Gli proteins and transcription of Shh target genes. The opposing functions of Ptch1 and Smo in Shh signaling become clear when these proteins are ablated in mice. *Ptch1*$^{-/-}$ embryos exhibit a ventralized neural tube in which cells throughout the tissue express markers of the floor plate, indicating a maximal level of Shh signaling in the absence of the inhibitory receptor (Goodrich et al. 1997). *Smo*$^{-/-}$ embryos, on the other hand, are insensitive to Shh ligand and fail to develop any Shh-dependent cell types in the neural tube (Wijgerde et al. 2002). Similar experiments in mouse genetics have used neural tube patterning phenotypes to examine the role of many other proteins in the Shh pathway.

Under normal conditions, Shh-dependent neural patterning arises from differing levels of Gli activator and repressor activity along the dorsoventral axis of the neural tube (Lei et al. 2004; Motoyama et al. 2003; Sasaki et al. 1999). In vertebrates, the Gli family of transcription factors consists of three members: Gli1 functions solely as a transcriptional activator, while Gli2 and Gli3 contain both activator and repressor domains (Bai et al. 2002; Ding et al. 1998; Park et al. 2000; Persson et al. 2002). Gli2 serves as the primary activator and cleaved Gli3 the primary repressor of Shh target genes in the mammalian neural tube (Sasaki et al. 1999). The regulation of Gli2 and Gli3 is critical for the proper transduction of Shh signaling. In the absence of Shh activity, Gli3 is proteolytically cleaved into its repressor form (GliR) through a PKA-dependent mechanism (Wang et al. 2000), and Gli2 is targeted for degradation. Sufu binds to Glis in the absence of Shh signaling, serving both to stabilize the Gli proteins and inhibit their activation (Chen et al. 2009; Tukachinsky et al. 2010; Wang et al. 2010). When Shh is present, the ensuing Shh signaling cascade blocks the cleavage of Gli3 into GliR and stabilizes full-length Gli2, which can then be converted into its activator form (GliA) through an as-yet-unknown mechanism. The atypical kinesin Kif7 was recently revealed to be necessary for the formation of both GliA and GliR via a cilia-dependent process (Cheung et al. 2009; Endoh-Yamagami et al. 2009; Liem et al. 2009). In addition, PKA regulates not just the processing of Gli3 into GliR, but also the activation of Gli2 (Tuson et al. 2011). Ultimately, since Shh pathway activity mirrors the gradient of Shh ligand across the dorsoventral axis, the end result of this tightly regulated signaling cascade is that GliA levels are high in the ventral neural tube, whereas GliR predominates in the dorsal neural tube. These opposing gradients of activator and repressor establish the progenitor domains in the ventral neural tube.

The functions of individual Gli proteins in neural tube patterning have also been studied through genetic ablation experiments. *Gli1*$^{-/-}$ mice are phenotypically normal, indicating that Gli1 is dispensable for Shh signaling in mammals. In fact, *Gli1* is itself a Shh target gene, and it has been shown that all Gli1 expression requires Shh signaling (Bai et al. 2002). In the absence of Gli1, it is likely that the other Gli proteins (primarily Gli2) compensate for its function. *Gli2*$^{-/-}$ mouse embryos, in contrast, display reduced Shh activity: they fail to form a floor plate and show a reduction in the number of v3 progenitors, indicating that the

highest levels of Shh activity cannot be achieved without Gli2. This indicates Gli2's role as the primary activator of Shh target gene transcription in the neural tube (Ding et al. 1998; Matise et al. 1998; Park et al. 2000). Finally, *extra toes* (Xt^J) mouse mutants, which lack Gli3 function due to a genomic deletion in the *Gli3* locus, exhibit mediolateral progenitors in more dorsal regions of the neural tube. These embryos show an expansion of the Shh-responsive domain in the absence of the primary repressor (Persson et al. 2002). Epistasis experiments indicate that Gli3 does have some role as an activator; however, $Gli2^{-/-};Gli3^{Xt/Xt}$ double mutant embryos completely lack v3 cells, suggesting the v3 progenitors that are present in $Gli2^{-/-}$ embryos are induced by GliA derived from Gli3 (Motoyama 2003). The neural tube's sensitivity to such slight changes in the balance of GliA and GliR makes this an excellent system for testing hypotheses about Shh signaling through genetic manipulations.

2.2.3 Maintaining Neural Patterning

Progenitor domains in the developing spinal cord are defined by the expression of two classes of Shh-responsive transcription factors (Briscoe 2000). Class I factors, such as Pax7, Pax6, Dbx1, Dbx2, and Irx3, are constitutively expressed by neural progenitors, but are repressed by Shh signaling. Class II factors, such as FoxA2, Nkx6.1, Nkx6.2, Nkx2.2, and Olig2, require Shh signaling for their expression (Briscoe and Ericson 2001). The proteins in each class differ in their sensitivity to Shh activity, such that unique combinations of these factors are expressed in each progenitor domain depending on the ratio of GliA and GliR to which cells are exposed. For example, Nkx6.1, Pax6, and Olig2 are all expressed in the pMN domain, but in the more dorsal p2 domain, Olig2 expression does not occur, while Irx3 expression is permitted. Cross-repressive interactions between pairs of class I and class II proteins establish the boundaries between progenitor domains (for example, Irx3 and Olig2 repress each other's expression). As development proceeds, these unique transcription factor expression profiles regulate the expression of cell type-specific genes that promote differentiation into distinct classes of spinal cord neurons. In this way, class I and class II transcription factors form a gene regulatory network that solidifies the pattern within the neural tube.

Recent work provides further evidence for a model in which graded Shh activity initiates patterning, but other mechanisms refine and maintain the ventral progenitor domains. Studies using a GFP reporter of Gli activity show that the level of signaling in individual progenitor domains changes during the course of development, while their transcriptional profile remains constant. This indicates that proper patterning depends on more than the absolute level of Shh signaling in a cell. Indeed, a model describing the cross-repressive gene regulatory network between the Shh-responsive transcription factors Nkx2.2, Olig2, and Pax6 can explain how these factors remain confined to particular dorsoventral domains, even in the face of variable levels of Shh ligand and GliA/GliR activity during development. At any

given time, neural progenitor cells are influenced both by their current level of Shh signaling and their current transcriptional profile. For instance, due to its status as the strongest repressor in the gene regulatory network, once Nkx2.2 is expressed, it is able to prevent the expression of other Shh-responsive genes (e.g., Olig2), despite fluctuations in levels of Shh signaling. Conversely, cells that might have initially expressed Olig2 at lower levels of Shh activity are able to alter their transcriptional profiles to express Nkx2.2 in response to increased Shh signaling (Balaskas et al. 2012).

Although a classic morphogen model would suggest that cell fates in the ventral neural tube are defined solely by the amount of Shh in the extracellular environment (which in turn determines the levels of GliA and GliR), recent studies reveal that Shh-dependent neural patterning is more complex. This is not so surprising, however, when one considers the inherent complexity of the developing spinal cord. Throughout development, the tissue of the neural tube grows and expands, meaning progenitor cells are constantly shifting their position relative to the source of Shh in the notochord and floor plate. Furthermore, the Shh gradient does not remain constant: the overall amount of ligand increases over time (Chamberlain et al. 2008), such that Shh levels that would be sufficient to induce ventral progenitors earlier in development can be found at more dorsal positions later in development, without inducing a change in dorsal cell fate. Thus, other mechanisms beyond the interpretation of the Shh ligand gradient seem necessary to ensure that proper patterning is established and maintained in the ventral neural tube.

One such mechanism is temporal adaptation. Shh-responsive cells create negative feedback by upregulating Ptch1, the major inhibitor of Shh activity, so cells exposed to a constant concentration of Shh reduce their GliA response over time. Higher concentrations of Shh induce higher initial GliA activity, meaning the duration of GliA-dependent signaling is longer in cells exposed to the highest levels of Shh. Importantly, the duration of Shh signaling is critical for proper specification of progenitor domains. In vivo, the p3 domain has been shown to transiently express Olig2, a marker of the more dorsal pMN domain, before expressing Nkx2.2. This result is also seen in vitro, where neural tube explants exposed to Shh express Olig2 and Nkx2.2 sequentially, and the normal duration-dependent response to Shh is found to require Ptch1 upregulation (Dessaud et al. 2007).

In summary, the mammalian neural tube makes a remarkable system for studying the regulation of Shh signaling in vivo. Since neural progenitor cells along the dorsoventral axis express molecular markers in a highly stereotyped fashion, and we have a solid understanding of the connections between levels of Shh ligand, signal transduction through the primary components of the pathway, and the ultimate patterns of gene expression that define neural progenitor domains, the assessment of neural patterning in mutant embryos gives us many informative clues as to how a given gene regulates Shh signaling, especially when combined with epistasis experiments to show whether the gene of interest acts upstream or downstream of known Shh signaling components. In recent years, genetic experiments like these have shown that primary cilia are essential

for the proper function of the Shh pathway and that the perturbation of cilia leads to a variety of Shh signaling defects that can be assessed via their effects on neural tube patterning.

2.3 Molecular Mechanisms of Shh Signaling in Cilia

2.3.1 Requirement of Cilia for Sonic Hedgehog Signaling

Primary cilia are seen on virtually every type of vertebrate cell and are required for Shh signaling. The connection between primary cilia and Shh signaling is unique to vertebrates (reviewed in: Huangfu and Anderson 2006; Ingham et al. 2011) and was first revealed through in vivo analysis of mouse mutants with defects in cilia genes. More recently, however, new experiments have told us much about the molecular biology of cilia-dependent Shh signaling within individual cells. All major components of the Shh signaling pathway are known to localize in or near primary cilia, and Shh pathway proteins are trafficked in and out of cilia depending on the activation state of the pathway (Fig. 2.3). In general, disrupting ciliogenesis or ciliary protein transport also leads to disruption of Shh signaling.

Cell biology experiments point to an extremely complex mechanism through which a large number of effectors regulate Shh signaling in cilia. These molecules can be disrupted independently of one another, leading to abnormal Shh signaling activity through similar overlapping but ultimately distinct mechanisms. In such cases, the neural tube makes an appealing system for analyzing the roles of individual molecules in the regulation of the Shh pathway, since we can observe shifts in the patterning of ventral neural progenitor domains when a gene is manipulated in mouse embryos and use the patterning phenotype to make inferences about the effects of the mutation on Shh signaling.

Shh signaling activity depends on the proper localization of the pathway's component proteins to primary cilia: Ptch1, Smo, Gli2, Gli3, Sufu, and Kif7 are all known to move into and out of the cilium during Shh signaling (Corbit et al. 2005; Haycraft et al. 2005; Liem et al. 2009; Rohatgi et al. 2007; Tukachinsky et al. 2010). Indeed, without primary cilia, no Shh signaling can occur. Cilia are required for the formation of both GliA and GliR (Haycraft et al. 2005; Huangfu and Anderson 2005; Liu et al. 2005; May et al. 2005); cells without cilia receive no transcriptional signals from the Shh pathway. Despite this, mediolateral progenitors requiring only low levels of Shh signaling are still observed in some mutants with no cilia (Huangfu and Anderson 2005). The progenitors form due to the loss of GliR-dependent repression of mediolateral cell fates. This situation is distinct from *Shh*$^{-/-}$ embryos, for example, in which ventral neural progenitors do not develop because GliA is never induced by Shh signaling, but GliR is still produced (Chiang et al. 1996). Epistasis experiments show that *Shh*$^{-/-}$;*Gli3*$^{-/-}$ double mutants are still capable of producing some ventral progenitors; the loss of GliR derepresses the Shh pathway under these conditions (Persson et al. 2002). In contrast, mutants that lack cilia are much less sensitive to *Gli3* ablation (Huangfu et al. 2003) (Fig. 2.4).

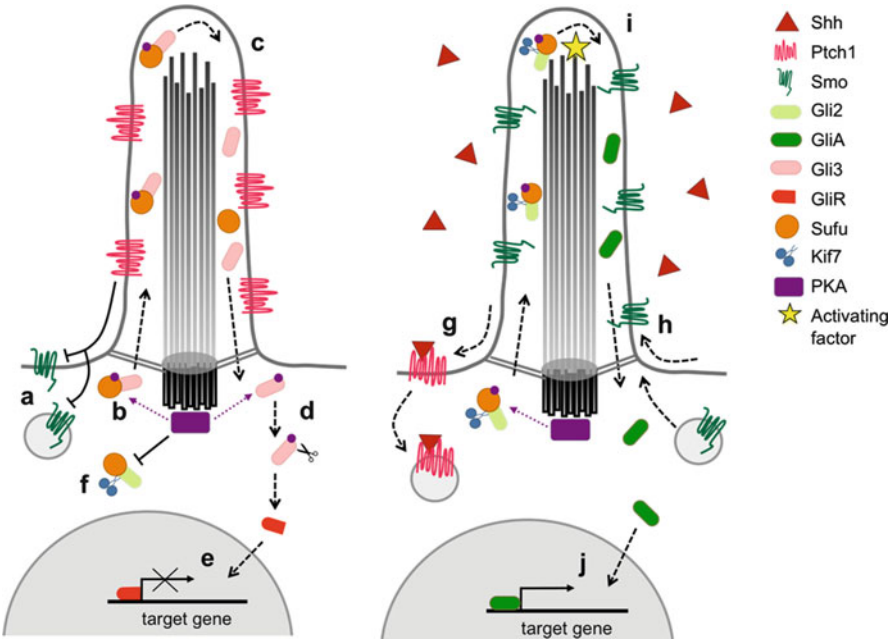

Fig. 2.3 Localization of major Shh pathway proteins within cilia. In the absence of Shh ligand (*left panel*), the Shh receptor Ptch1 is localized to the ciliary membrane, which inhibits the ciliary entry of Smo. Studies have reported that Smo is localized either to the plasma membrane or to intracellular vesicles (or, perhaps, both) in the absence of Shh signaling (**a**). Without Shh stimulation, Gli transcription factors, particularly Gli3, are converted to their repressor form. Gli3 is bound to Sufu, which is regulated via phosphorylation (*purple circles*) by PKA to promote its ciliary localization (**b**). The Sufu/Gli3 complex must be trafficked to the tip of the cilium in order for Gli3 to dissociate from Sufu and promote the formation of GliR (**c**), although it is not known whether this dissociation occurs within the cilium or after the complex exits the cilium. Gli3 itself is also phosphorylated by PKA (*purple circles*) at the base of the cilium to promote its cleavage by the proteasome into GliR (**d**). Processed GliR then translocates to the nucleus, where it represses the transcription of Shh target genes (**e**). At the same time, PKA and Kif7 together inhibit the ciliary localization and activation of Gli2 (**f**). In the presence of Shh (*right panel*), Ptch1 binds to the ligand and is internalized (**g**). The removal of Ptch1 from the ciliary membrane allows Smo to enter the cilium via lateral transport, targeted vesicle fusion, or both (**h**). Activated ciliary Smo promotes the enrichment of Gli proteins at the tip of the cilium, where they are activated to form GliA through a currently unknown mechanism (**i**). GliA then translocates to the nucleus where it initiates transcription of Shh target genes (**j**)

2.3.2 Cilia-Dependent Sonic Hedgehog Effectors

Under baseline conditions, cilia-dependent mechanisms are required to properly regulate the Shh pathway. In the absence of Shh, Ptch1 is enriched in the membrane of the primary cilium (Rohatgi et al. 2007). The presence of Ptch1 serves to inhibit the accumulation of Smo in ciliary membrane, although a small amount of Smo

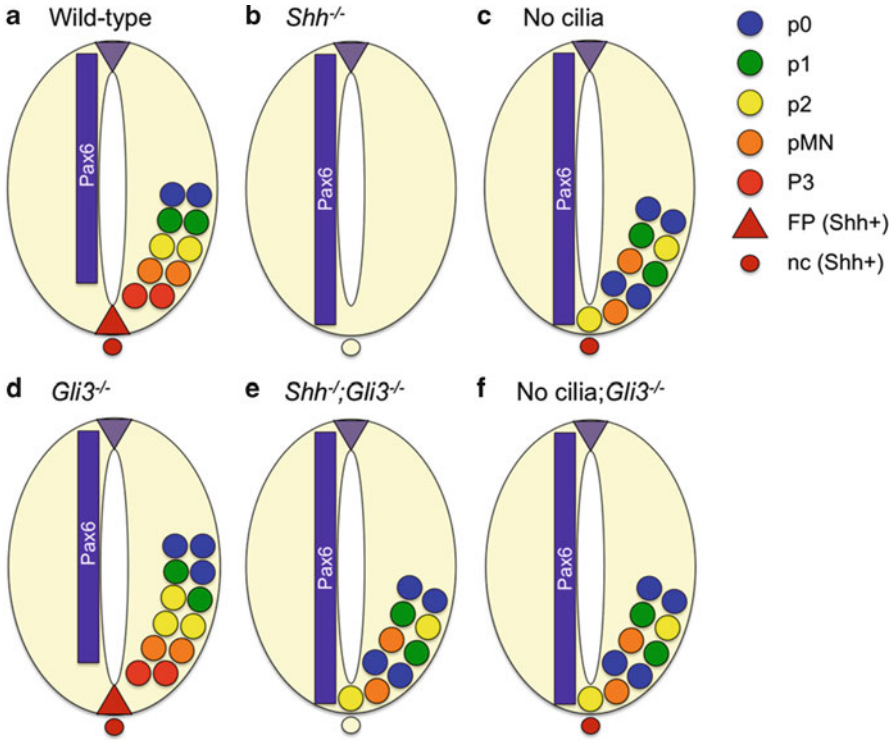

Fig. 2.4 Neural tube patterning requires cilia and Shh. Progenitor domains in the neural tube reveal changes in the balance between GliA and GliR in different mutant embryos. Wild-type embryos (**a**) exhibit six ventral progenitor domains based on graded Shh signaling along the dorsoventral axis. In *Shh*$^{-/-}$ embryos (**b**), no GliA is produced, but GliR still forms, inhibiting all six classes of ventral progenitors and dorsalizing the neural tube. Embryos that lack cilia (**c**) due to mutations in a variety of genes are deficient in both GliA and GliR; the result is that the highest levels of activation and repression cannot be achieved, and the neural tube displays mediolateral progenitor cells throughout the ventral region. These cells are ligand-insensitive; the notochord (nc) continues to produce Shh in cilia mutants even though the floor plate (FP) is not established. Mutations in *Gli3* help reveal more about the mechanism of Shh signaling in various mutants. *Gli3*$^{-/-}$ single mutants (**d**) show a mild phenotype in which mediolateral progenitor domains are slightly expanded and overlapping. Loss of *Gli3* in a *Shh*$^{-/n}$ embryo (**e**) derepresses Shh signaling, allowing mediolateral progenitors to form even in the absence of ligand. In a cilia mutant embryo, however, loss of Gli3 has little effect (**f**) because GliR formation requires cilia

constantly shuttles through the cilium, even in the presence of Ptch1 (Kim et al. 2009; Ocbina and Anderson 2008). Gli2 and Gli3 are also found in cilia in the absence of Shh ligand, and Gli proteins must travel into and out of primary cilia to be properly converted into GliR (Haycraft et al. 2005).

When Shh binds Ptch1, an unknown process causes Ptch1 to exit the cilium and leads to Smo becoming enriched there (Corbit et al. 2005; Rohatgi et al. 2007). The exact mechanism of Smo's movement into cilia remains a matter of debate, although

there is some evidence that Smo moves from the plasma membrane into the cilium via lateral transport (Milenkovic et al. 2009), and other studies point to a mechanism by which intracellular Smo is targeted to the cilium (Wang et al. 2009). Another possibility is that Smo uses several mechanisms for its transport into cilia. A recent study supporting the multiple-mechanisms model identified a novel protein, LZTFL1, as a negative regulator of ciliary trafficking of Smo (Seo et al. 2011). LZTFL1 mediates the interaction between Smo and a protein coat complex known as the BBSome. The BBSome is responsible for targeting a variety of membrane-associated receptors to the ciliary membrane (for more about the BBSome, see Chap. 6 by Berbari, Pasek, and Yoder and Chap. 9 by Baker and Beales). When LZTFL1 is knocked down, the ciliary localization of BBSome proteins and of Smo increases, irrespective of Shh signaling activity. However, further ciliary enrichment of Smo can still be seen under LZTFL1-depleted conditions upon treatment with a Shh pathway agonist. This implies that, while some Smo trafficking is mediated by the BBSome and repressed by LZTFL1 in the absence of Shh, Smo is also targeted to the cilia membrane through LZTFL- and BBSome-independent mechanisms. Other experiments have also revealed that the ciliary enrichment of Smo upon Shh stimulation depends on kinesin-based transport (specifically, Kif3a) and β-arrestins (Kovacs et al. 2008). Further study will determine whether these data can be reconciled into a single model of Smo transport.

Pharmacological evidence shows that, although Smo moves into the cilium as a consequence of Shh pathway activation, the mere presence of Smo in the cilium is not sufficient to transduce the Shh signal and trigger the formation of GliA: Smo must also be activated (Wang et al. 2009). The two-step process of Smo-dependent Shh signaling (translocation and activation) has yet to be fully explained. Smo shares many characteristics with G protein-coupled receptors (Ayers and Thérond 2010) and is known to respond to several small molecules in pharmacological screens. Therefore, one appealing model posits that a yet-unknown endogenous molecule – possibly a cholesterol-derived oxysterol (Nachtergaele et al. 2012) – regulates Smo activation, and that Ptch1 inhibits Smo by blocking its access to this molecule, as well as blocking its accumulation in cilia.

The presence of activated Smo in the cilium causes enrichment of Gli proteins at the tip of the cilium, inhibits GliR formation, and triggers the formation of GliA (Chen et al. 2009; Wen et al. 2010). Studies have shown that the processing of Gli proteins into GliA and GliR requires transport of Gli proteins into and out of the primary cilium. It is not known how trafficking through the primary cilium regulates the conversion of full-length Gli proteins into activator or repressor, but experiments thus far have indicated that the process involves the core Shh pathway proteins Sufu, Kif7, and PKA (Chen et al. 2009, 2011; Tukachinsky et al. 2010). Because the balance of GliA and GliR ultimately determines the output of the Shh signaling pathway, proper regulation of Gli proteins is key, and the disruption of Gli processing and/or activation leads to signaling defects that cause abnormal neural patterning in vivo.

Sufu, Kif7, and PKA act at the primary cilium to regulate Gli processing and activation. Sufu binding to Gli proteins inhibits their transcriptional functions.

Sufu can act in a cilia-independent manner to inhibit Gli proteins by sequestering them from the nucleus, but it also moves into and out of cilia with the Glis, becoming enriched in cilia upon Shh stimulation. For GliR or GliA to form, Gli proteins must first dissociate from Sufu. This dissociation requires kinesin-dependent trafficking of the Sufu/Gli complex to primary cilia (Humke et al. 2010; Tukachinsky et al. 2010). In addition to inhibiting the formation of GliR and GliA, Sufu serves to stabilize Gli proteins: genetic ablation of Sufu leads to degradation of Gli2 and Gli3 via the ubiquitin E3 ligase adaptor Spop. Gli3 is more affected by loss of Sufu than Gli2; *Sufu* null embryos exhibit a ventralized neural tube phenotype due to the alteration in the balance of GliA and GliR that results from Gli3 degradation (Wang et al. 2010).

Kif7, an atypical kinesin, normally localizes to the base of the cilium, where it is ideally positioned to regulate the access of Shh pathway proteins to the ciliary compartment. Kif7 serves as a negative regulator of Shh signaling downstream of Smo. Upon activation of the Shh pathway, Kif7 moves to the tip of the cilium concomitantly with Gli proteins, indicating that it may regulate the transport and activation of Gli2, as well as the processing of Gli3 into GliR (Endoh-Yamagami et al. 2009; Liem et al. 2009). One model suggests that, as a motor protein, Kif7 may inhibit Gli activation by moving Gli proteins away from the cilium in the absence of signals from Shh (Liem et al. 2009). Further experiments are needed to test this model. Notably, the role of Kif7 in Shh signaling was initially disputed, as cell-based assays of Kif7 knockdown did not show a significant effect on Shh pathway output (Varjosalo et al. 2006). Thus, in vivo analysis of neural tube patterning proves to be a more sensitive method for examining subtle effects of perturbations to the Shh pathway.

The stepwise nature of the Shh signaling cascade allows for regulation of the pathway by the same effectors at multiple discrete steps. For example, PKA is known to phosphorylate Sufu, thereby promoting Sufu's ciliary localization (Chen et al. 2011). However, analysis of mouse mutants deficient in PKA reveals a more severe neural tube patterning phenotype than *Sufu*$^{-/-}$ mutants, indicating that PKA is affecting Gli activity and Shh signaling through Sufu-independent mechanisms, in addition to regulating Sufu. Specifically, PKA acts as a negative regulator of the Shh signaling cascade (Epstein et al. 1996; Hammerschmidt et al. 1996; Concordet et al. 1996). PKA is localized at the base of the primary cilium, where it regulates the ciliary entry of Gli proteins, the production of GliR from Gli3, and the activation of Gli2 (Tuson et al. 2011). Although PKA regulates a wide variety of processes in adult vertebrate cells, its most notable function during development seems to be regulation of the Shh pathway in the neural tube as well as in other Shh-responsive tissues (Huang et al. 2002). When a Shh signaling component plays multiple roles in regulating the pathway, as PKA does, it complicates the interpretation of experiments in which this molecule is perturbed. Yet it is not surprising for a single effector to interact with multiple steps of a signaling cascade, especially when all the major signaling components localize to a small organelle like the cilium.

The dynamic regulation of Shh pathway proteins in primary cilia points to the central nature of cilia and ciliary protein trafficking in Shh signaling. In addition to

the core Shh pathway proteins described above, a variety of other effectors contribute to the proper function of Shh signaling in cilia and can lead to in vivo neural patterning defects when disrupted.

2.4 Cilia Proteins Essential for Shh Signaling: Lessons from Mutants

2.4.1 Intraflagellar Transport

The link between cilia and Shh signaling was first discovered in a forward genetic screen that identified several mouse mutants with defects in intraflagellar transport (IFT) (Huangfu et al. 2003; Huangfu and Anderson 2005). IFT relies on a highly conserved family of proteins to move cargoes along the microtubules that form the ciliary axoneme. Anterograde IFT relies on the kinesin-2 motor as well as the 11 proteins that constitute the IFTB complex. Retrograde IFT uses the six proteins in the IFTA complex and the cytoplasmic dynein motor (reviewed in: Pedersen and Rosenbaum 2008; Rosenbaum and Witman 2002) (Fig. 2.5). When anterograde IFT is disrupted, ciliogenesis does not occur. (For a more detailed discussion of IFT and ciliogenesis, see Chap. 1, Sect. 4.3). When retrograde IFT is disrupted, IFT cargoes accumulate at the distal end of the cilium and interfere with the normal flow of ciliary protein traffic.

As mentioned before, mutant mouse embryos that lack cilia due to anterograde IFT defects have no Shh signaling; they fail to produce either GliA or GliR (Huangfu et al. 2003; Huangfu and Anderson 2005). In contrast, defects in retrograde IFT can lead to either elevated Shh signaling activity or loss of the Shh response, depending on the causative mutation. Analysis of mouse mutants has revealed the distinct roles of the dynein retrograde motor and the IFTA complex in Shh signaling, as well as several regulatory steps in between them.

Loss of the dynein component Dync2h1 (also called Dnchc2) leads to a reduction in ventral cell types in the neural tube (Huangfu and Anderson 2005; May et al. 2005). These dynein mutant embryos completely lack retrograde IFT and have extreme defects in cilia structure, with very short, almost spherical cilia. Neural tube patterning in *Dync2h1* mutants resembles IFTB mutants, which fail to generate cilia altogether, and loss of dynein also causes reduced levels of cleaved Gli3 compared to wild-type. These phenotypes indicate that the cilia-dependent processes required to generate GliA and GliR are likely to be lost in dynein mutants. Past studies have also proposed that the lack of Shh signaling in *Dync2h1* mutants is due to a failure of Smo to localize to their abnormal cilia (May et al. 2005); however, these interpretations were based on the analysis of motile cilia in the embryonic node, where the role of Shh signaling has not been characterized in as much detail. Later experiments in Shh-responsive cell types have confirmed that Smo does traffic into dynein-deficient primary cilia (Kim et al. 2009; Ocbina and Anderson 2008), so this cannot explain the Shh signaling defects in these mutants.

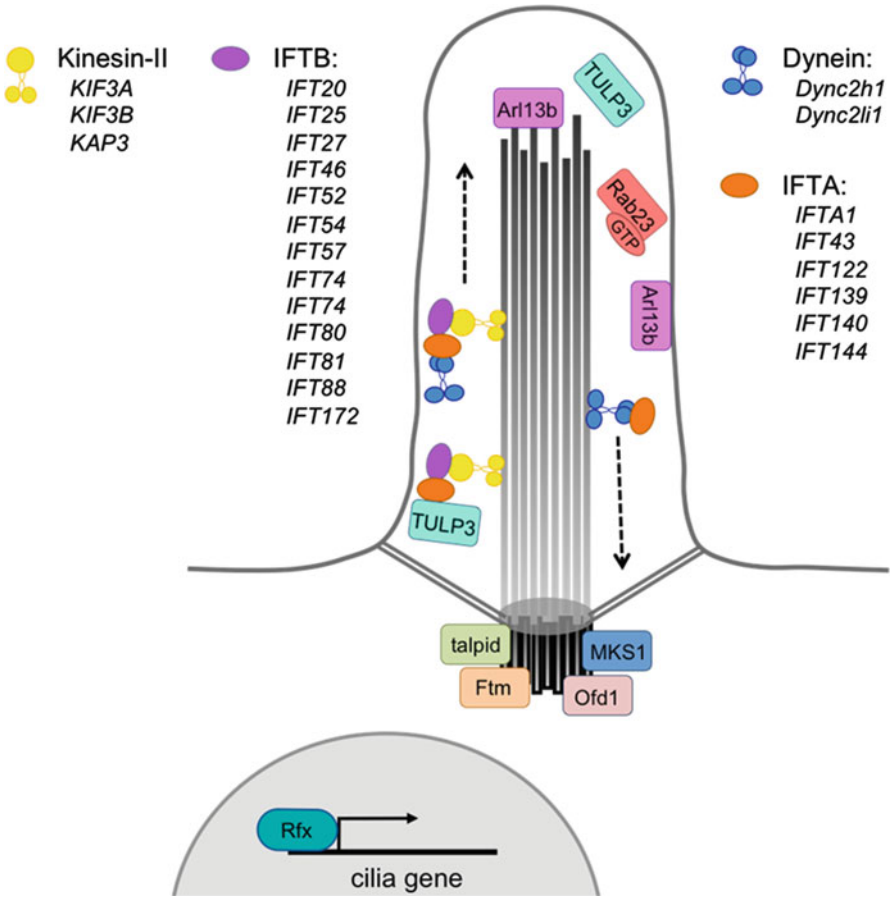

Fig. 2.5 Intraflagellar transport (*IFT*) proteins and other proteins that regulate Shh signaling through ciliogenesis or ciliary protein trafficking. A variety of mutations affecting ciliary proteins have been shown to disrupt Shh signaling. These include proteins associated with intraflagellar transport (the kinesin-II and dynein motors and the IFTA and IFTB complexes) as well as others with functions that are not as well understood. Some of these proteins are found in cilia: TULP3 requires the IFTA complex to enter the cilium and localizes to ciliary tips in the absence of Shh signaling; it is not known whether it associates with the axoneme or the membrane. Rab23 is found in cilia in its GTP-bound state, but not its GDP-bound state. Arl13b is primarily associated with the ciliary membrane but is seen at the base and the tip of the cilium even after the membrane is removed with detergent. Other mutations affecting ciliogenesis and Shh signaling disrupt proteins associated with the basal body: Ofd1, MKS1, Ftm, and talpid are among these. The Rfx transcription factors are not found in cilia, but regulate the transcription of a variety of ciliary genes, thus regulating ciliogenesis and Shh signaling

The neurodevelopmental effects of IFTA complex mutations have been studied using several mouse lines generated in forward genetic screens. In contrast to dynein mutations, the genetic ablation of the IFTA proteins IFT139 (also called THM1 or Ttc21b) or IFT122 results in elevated Shh activity in the neural tube (Qin et al.

2011; Tran et al. 2008). Unlike dynein mutants, IFTA mutants still exhibit some retrograde IFT, albeit with reduced efficiency. Their cilia are typically longer than dynein mutants', with bulges due to accumulated proteins at the distal ends. In *alien (aln)* mutant embryos, ventral cell types, including floor plate and p3 progenitors, expand dorsally beyond their normal domains due to a null mutation in *IFT139*. Epistasis experiments show that IFT139 acts upstream of Gli2 to regulate Shh signaling, and thus neural patterning. Although loss of IFT139 activates the Shh pathway, it does not completely suppress the phenotype of a *Shh*$^{-/-}$ or *Smo*$^{-/-}$ embryo. Furthermore, *IFT139*^{aln} embryos that are also heterozygous for a null allele of *Shh* show a partial rescue of their abnormal neurodevelopmental phenotype (Stottmann et al. 2009). These genetic data indicate that the ventralized neural tube of *IFT139*^{aln} mutant embryos is caused by overactivation of Gli2 and that IFT139-dependent retrograde IFT regulates Gli2 activation in a manner that is partially, but not completely, dependent upon Shh signaling through Smo. Previous experiments have confirmed that Smo can localize to nodal cilia in *IFT139*^{aln} mutants and that overexpressed Gli1 protein can localize to cilia in mutant mouse embryonic fibroblasts (Tran et al. 2008); the Shh dependence of Smo and Gli enrichment in *IFT139*^{aln} primary cilia has yet to be shown, however. Because the IFTA complex mediates protein transport out of the cilium, it is plausible that the overactivation of Gli2 in *IFT139*^{aln} mutant embryos arises at least in part from Gli proteins' constitutive localization to primary cilia in the *IFT139*^{aln} mutant.

As in *IFT139*^{aln} embryos, loss of IFT122 causes an expansion of ventral progenitors in the neural tube. In fact, *sister of open brain (sopb)* mutant embryos with a null mutation in *IFT122* exhibit a more severe ventralization phenotype than *IFT139*^{aln} mutants. In *IFT122*^{sopb} embryos, cells expressing markers of the floor plate are found within the mediolateral regions of the neural tube, and pMN progenitors extend into even the most dorsal part of the neural tube. Analysis of *IFT122*^{sopb}*;Shh*$^{-/-}$ double mutants reveals neural patterning almost identical to that of *IFT122*^{sopb} single mutants, indicating most of the ventralization phenotype caused by loss of IFT122 is ligand-independent. *IFT122*^{sopb}*;Gli2*$^{-/-}$ mutants show that, as in *IFT139*^{aln} mutants, the ventralization of the *IFT122*^{sopb} neural tube is dependent upon GliA derived primarily from Gli2. Cell biology experiments in *IFT122*^{sopb} mouse embryonic fibroblasts show that Gli2 and Gli3 are both enriched at the tips of mutant cilia in a ligand-independent manner, but the ligand-dependent ciliary localization of Smo and Sufu are largely unaffected by loss of IFT122 (Qin et al. 2011). It is clear that transport of Gli proteins to the tip of the cilium is required for their activation, and that Glis must dissociate from Sufu to become activated (Haycraft et al. 2005; Liu et al. 2005; Tukachinsky et al. 2010). Perhaps differential regulation of the ciliary trafficking of Sufu and Glis by the IFTA complex contributes to the overactivation of the Shh pathway in these mutants.

Several recent studies examined the interplay between retrograde and anterograde IFT components in ciliogenesis and Shh signaling (Ocbina et al. 2011; Liem et al. 2012). The results suggest that mutations in *IFT172*, which codes for a member of the IFTB complex, are able to rescue defects in *Dync2h1* mutants. Cilia structure and neural tube patterning in *Dync2h*$^{lln/lln}$;*IFT172*$^{avc/+}$ double mutants

were similar to wild-type. The ability of a mutation in an anterograde trafficking protein to rescue structural defects caused by abnormal retrograde trafficking implies that a balance between anterograde and retrograde IFT regulates cilia formation, and that the Shh signaling defects in *Dync2h1* mutants arise primarily from abnormal cilia structure. Intriguingly, the same study also found that *IFT122*^{sopb} can partially rescue neural patterning, cilia structure, and Shh pathway protein trafficking defects in *Dync2h1* mutants and that this rescue is ligand-dependent. This result was interpreted to mean that IFT122 has a role in anterograde IFT as well as retrograde IFT, such that loss of IFT122 can restore the balance between the two that is required for proper ciliogenesis. It also implies that the ligand-independent gain of Shh signaling activity in *IFT122*^{sopb} mutants arises from an undescribed IFT-independent function of IFT122, and perhaps the IFTA complex in general, in regulating the Shh pathway (Ocbina et al. 2011). It would be interesting to test whether loss of IFT139 is also able to rescue neural patterning defects in dynein mutants, or whether this additional Shh regulatory function is specific to IFT122.

A subsequent study showed that a severe mutation in the IFTA gene *IFT144 (IFT144*^{dmhd}*)* can prevent ciliogenesis, further indicating that proteins traditionally associated with retrograde IFT can play a role in anterograde IFT. The hypomorphic allele *IFT144*^{twt} did not significantly affect cilia structure, however. Because cilia structure and Shh signaling are so tightly linked, it is perhaps not surprising that the two *IFT144* alleles show distinct neural patterning phenotypes. The severe structural defects in *IFT144*^{dmhd} cilia lead to a loss of Shh signaling, while *IFT144*^{twt} mutants show ectopic activation of the Shh pathway similar to other IFTA mutants. Furthermore, compound mutants with *IFT144*^{twt} and *IFT122*^{sopb} alleles showed ciliogenesis and patterning defects as severe as *IFT144*^{dmhd}, indicating that the IFTA components IFT144 and IFT122 work together to build cilia. The study also showed that many ciliary membrane proteins, including Smoothened, were mislocalized in IFTA mutant cilia, but that soluble proteins, including Gli2, Sufu, and Kif7, were unaffected by IFTA mutations (Liem et al. 2012). The authors therefore propose that IFTA-dependent trafficking of membrane proteins into cilia may explain the ectopic Shh activation and neural patterning phenotypes in IFTA mutants.

Although both IFTA and IFTB proteins have been linked to ciliogenesis as well as the regulation of Shh signaling, new research on mutant mice indicates that IFT25 (and perhaps IFT27) regulate Shh signaling independently of cilia structure. Mice with a protein null allele of *IFT25* also fail to produce IFT27 and show defects in Shh signaling. Specifically, the mutants show an expansion of mediolateral progenitors at the expense of the most ventral (floor plate) cell types. They also have defects in Ptch1 and Smo localization, Gli processing, and ligand-stimulated enrichment of Gli proteins at the tips of cilia. Despite these defects, their cilia are structurally intact (Keady et al. 2012). These results show that the IFTB complex components IFT25 and IFT27 are required for regulating Shh signaling but are dispensible for ciliogenesis. The exact functions of IFT25 and IFT27 are not known, but they are known to form a complex

(Bhogaraju et al. 2011). As a Rab-like small GTPase, IFT27 may act as a switch to regulate trafficking of Shh pathway components to cilia in a ligand-dependent manner (Qin et al. 2007).

Because IFT complexes and the molecular motors that power IFT are so critical for ciliogenesis and Shh signaling, it is not surprising to learn that factors regulating the expression of IFT genes can affect Shh signaling, as well. The Rfx family of transcription factors is evolutionarily conserved from invertebrates to vertebrates (Dubruille 2002; Swoboda et al. 2000) and is found to regulate the expression of cilia-specific genes, including the dynein component *D2lic* (Bonnafe et al. 2004) and *IFT172* (Ashique et al. 2009). Although genetic manipulations of some Rfx family members, such as *Rfx2* and *Rfx4*, affect both ciliogenesis and Shh signaling (Ashique et al. 2009; Chung et al. 2012), *Rfx3* mutants display no obvious Shh signaling defects, despite having abnormal cilia in the embryonic node and on ependymal cells in the brain (Bonnafe et al. 2004; El Zein et al. 2009). Because Rfx family members are differentially expressed across various tissues, it may be that Rfx3 is not normally expressed in the neural tube, or is expressed redundantly with other Rfx proteins, and is thus dispensable for cilia-dependent neural patterning. Still, it serves as an illustrative example of how factors affecting ciliogenesis and neurodevelopmental Shh signaling may be separable through genetic analysis.

Finally, in addition to the IFT proteins and their motors, a variety of proteins localized to the basal body have been shown to regulate ciliogenesis, such that mutations affecting these proteins leads to phenotypes that resemble anterograde IFT mutants. *Ftm* was discovered as one of six genes deleted in the *fused toes* (*Ft*) mouse mutant, which has defects indicative of reduced Shh signaling activity (Götz et al. 2005; Peters et al. 2002). Targeted knockouts of the individual genes in the *Ft* mutation revealed that *Ftm* is responsible for the majority of defects seen in mutant embryos, including loss of ventral progenitors in the neural tube (Vierkotten et al. 2007). The $Ftm^{-/-}$ phenotype shares many characteristics with anterograde IFT mutants, and indeed, $Ftm^{-/-}$ embryos show reduced numbers of cilia in some tissues such as the node. However, unlike IFTB and kinesin-II mutants, $Ftm^{-/-}$ mutants possess normal motile cilia in some tissues such as the trachea, and $Ftm^{-/-}$ primary fibroblasts can grow cilia in vitro. Thus, Ftm seems to specifically regulate Shh signaling – in particular, the formation of GliA and the processing of GliR – in addition to regulating ciliogenesis and cilia structure in tissues like the node. Ftm is localized to the basal body, placing it in an ideal position from which to regulate the entry of Shh pathway proteins to the cilium. Future experiments are needed to characterize the effects of Ftm ablation on the ligand-dependent ciliary localization of Shh signaling effectors. Other basal body proteins, including Ofd1 (Ferrante et al. 2006; Singla et al. 2010) and talpid (Bangs et al. 2011), affect ciliogenesis and Shh signaling in much the same way as Ftm. Loss of Mks1, another basal body protein, abolishes cilia in most tissues, including the neural tube, but spares some motile cilia. Furthermore, $Mks1^{-/-}$ embryos survive longer than most ciliogenesis mutants and seem to retain a small amount of GliA, suggesting that Mks1 may have functions in Shh signaling outside of ciliogenesis (Weatherbee et al. 2009).

2.4.2 Other Regulators of Shh Signaling in Cilia

Although IFT plays a crucial role in ciliogenesis and Shh signaling, this is not the only mechanism that controls the ciliary localization of Shh pathway proteins or the proper regulation of GliA and GliR transcriptional activity. In recent years, still more effectors of Shh signaling have been identified through genetic manipulations that affect neural tube patterning. Such experiments have revealed positive and negative regulators of Shh signaling that act at multiple distinct steps in the pathway. These Shh signaling effectors come from a variety of different protein families, including some that remain almost completely uncharacterized. In addition, some of these proteins are unique to vertebrates, implying that they may be specific regulators of ciliary Shh signaling (which occurs only in vertebrates, reviewed in: Huangfu and Anderson 2006; Ingham et al. 2011; Varjosalo et al. 2006). This places them in contrast to the IFT complexes, which are more general mediators of ciliogenesis and ciliary maintenance found in organisms ranging from the flagellate alga *Chlamydomonas reinhardtii* to mammals.

While it may be easy to understand why the complete ablation of cilia due to anterograde IFT mutations leads to Shh signaling defects, it is more difficult to conceptualize the complex and sometimes contradictory phenotypes caused by perturbations to retrograde IFT. Recent studies have identified effectors of IFTA that also play a role in regulating Shh signaling (as well as other cilia-dependent signaling pathways; see Chap. 6 by Berbari, Pasek, and Yoder for more details). These proteins are still being characterized, but their further study may shed new light on some of the unsolved mysteries of Shh signaling, including how Smo regulates the formation of GliA.

TULP3, a member of the tubby family of proteins, is one such novel regulator of Shh signaling. Tubby family proteins are conserved across eukaryotes and interact with membrane phosphoinositides to regulate a variety of signaling pathways (reviewed in: Mukhopadhyay and Jackson 2011). Both a targeted null mutation of *TULP3* and the hypomorphic *hitchhiker* mutation result in ventralized neural patterning similar to that seen in IFTA mutants (Norman et al. 2009; Patterson et al. 2009). Epistasis experiments show that TULP3 acts downstream of Shh and Smo, but upstream of Gli2. Loss of TULP3 has no effect on Gli3 processing or Shh-dependent localization of Gli3 to the tips of cilia (Mukhopadhyay et al. 2010; Norman et al. 2009). It is not yet clear, however, how the Shh-dependent ciliary enrichment of Gli2 is affected by mutations in *TULP3*.

TULP3 localizes to cilia and binds to the IFTA complex. The association between TULP3 and IFTA requires some IFTA proteins, including IFT122, but other IFTA proteins in the complex are not required for TULP3 binding (Mukhopadhyay et al. 2010). In $IFT122^{sopb}$ mutants, TULP3 is not present in cilia, suggesting that the association between TULP3 and the IFTA complex is required for its ciliary localization. As $IFT122^{sopb}$ mutants exhibit more severe neural tube ventralization than $IFT139^{aln}$ mutants, it is possible that some of the Shh pathway overactivation in $IFT122^{sopb}$ results from mislocalization of TULP3. It seems clear from the current

data that TULP3 serves as a negative regulator of Gli2 activation in cilia, but the exact mechanism of its action remains to be elucidated. Because TULP3 is known to be required for the proper ciliary trafficking of several G protein-coupled receptors, one model posits that it may regulate a novel receptor involved in Shh signal transduction (Mukhopadhyay et al. 2010).

The small GTPase Rab23 was identified as a regulator of Shh signaling when the *open brain* (*opb*) mouse line was discovered in a forward genetic screen (Eggenschwiler et al. 2001; Eggenschwiler and Anderson 2000). *Rab23*^{opb} embryos exhibit a ventralized neural tube with expansion of the floor plate, v3, and pMN domains. Double mutant analysis indicates that Rab23 acts as a negative regulator of the Shh pathway downstream of Smo but upstream of Gli2; however, slight differences in patterning between *Rab23*^{opb};*Smo*$^{-/-}$ double mutants and *Rab23*^{opb} single mutants (specifically: double mutants do not show expansion of the floor plate) indicate that Rab23 may also have some Smo-dependent functions (Eggenschwiler et al. 2006).

Cell biology studies of Rab23 present a more complex view of how this GTPase may regulate Shh signaling. Rab family GTPases are involved in vesicle transport to various subcellular compartments (reviewed in Grosshans et al. 2006). Thus, Rab23 was hypothesized to regulate the trafficking of Shh pathway proteins to primary cilia. Recent work indicates that wild-type Rab23 (but not the mutant protein Rab23-S23N, which is a constitutively GDP-bound dominant negative) is localized to cilia and that it regulates ciliary trafficking of Smo (Boehlke et al. 2010). Specifically, Rab23 is thought to act by promoting recycling of Smo that is already localized to the cilium, while inhibiting the ability of other Smo molecules to enter the ciliary compartment. How this proposed mechanism would allow Rab23 to inhibit Gli2 activation is not yet clear. Another recent study showed that Rab23 binds directly to Sufu, promoting Sufu-dependent inhibition of Gli1 transcriptional activity (Chi et al. 2012) by sequestering Gli1 in the cytoplasm. These results provide a more compelling explanation for Rab23's role as a negative regulator of Shh signaling, but the experiments were conducted in cell types that lack primary cilia. It is unclear whether the interaction between Rab23 and Sufu can occur in ciliated cells, or whether ciliary Rab23 has some other function that remains to be described.

Another small GTPase, Arl13b, plays a unique role in the regulation of Shh signaling. The *hennin* (*hnn*) mutant mouse has a protein null mutation in *Arl13b* that leads to loss of the most ventral cell types in the neural tube (floor plate) and an expansion of more mediolateral cell types (pMN). Epistasis experiments show that the *Arl13b*^{hnn} phenotype is caused by abnormal regulation of Gli2 (Caspary et al. 2007). Specifically, the highest levels of GliA in the floor plate are never reached, while a moderate level of GliA drives the expression of Shh target genes throughout most of the neural tube. Gli3, meanwhile, appears to be relatively unaffected by loss of Arl13b. An intact gradient of Gli3-derived GliR regulates the residual patterning in the *Arl13b*^{hnn} neural tube, but *Arl13b*^{hnn};*Gli3*$^{-/-}$ double mutants have essentially no dorsoventral patterning (all cells in the double mutants assume either a v3 or pMN fate).

At the cellular level, Arl13b regulates multiple components of the Shh signaling pathway in cilia. First, *Arl13b*^{hnn} cells have short cilia with defects in the

microtubule ultrastructure of the ciliary axoneme (Caspary et al. 2007). Although these defects are not as severe as those seen in IFTB mutants, the abnormal structure of primary cilia could contribute to the misregulation of the Shh pathway in cells lacking Arl13b. Furthermore, many of the proteins that make up the Shh signaling pathway show abnormal localization patterns in *Arl13b*^{hnn} cells (Larkins et al. 2011). Specifically, Ptch1 is found in the cilium even after cells are treated with Shh ligand, and Smo is enriched in cilia even in the absence of Shh. Meanwhile, Sufu, Gli2, and Gli3 fail to become enriched at the tip of the cilium upon Shh treatment in cells lacking Arl13b. Because Gli2 activation requires its transport to the tip of the cilium, the abnormal ligand-dependent trafficking of Shh pathway proteins may explain why GliA function is impaired in *Arl13b*^{hnn} mutants. It is not yet clear whether the abnormal transport of Shh pathway proteins in *Arl13b*^{hnn} cells arises from the structural defects in mutant cilia, or whether Arl13b has a specific function in regulating protein trafficking.

Genetic ablation of Tectonic1, a member of a novel protein family awaiting further characterization, reduces the levels of both GliA and GliR, although the effect seems greater on GliA. Without Tectonic1, the floor plate and v3 progenitors are not specified, indicating a reduction in GliA. Yet, in *Tectonic1*$^{-/-}$*;Shh*$^{-/-}$ double mutants, there are more ventral progenitors than in *Shh*$^{-/-}$ single mutants, implying that less GliR is present to inhibit the specification of these cell types in the double mutant embryos (Reiter and Skarnes 2006). We do not know whether Tectonic regulates Shh signaling from within the cilium, although the related *Tectonic2* gene has been linked to the human ciliopathy Meckel-Gruber syndrome and appears in the cilia proteome database (Shaheen et al. 2011; ciliaproteome.org). (For more on human ciliopathies, see Chap. 6 by Berbari, Pasek, and Yoder and Chap. 9 by Baker and Beales.).

2.4.3 Spatiotemporal Regulation of Shh Signaling

Beyond identifying new effectors of Shh signaling, the analysis of various mouse mutants has revealed unexpected aspects of the mechanism by which Shh signaling patterns the developing neural tube. In particular, many of the mutations described above (*IFT139*^{aln}, *TULP3*$^{-/-}$, *Rab23*^{opb}, *Arl13b*^{hnn}) exhibit patterning defects in the caudal neural tube alone, while the rostral neural tube appears normal despite defects in Shh signal transduction (Caspary et al. 2007; Eggenschwiler et al. 2001; Norman et al. 2009; Tran et al. 2008). Other mutants, like *IFT122*^{sopb} and various alleles of *Dync2h1*$^{-/-}$, show defects throughout the neural tube, but with variable phenotypes along the rostrocaudal axis. One explanation for this puzzling trend may be that the contributions of Gli2 and Gli3 to neural patterning seem to differ along the rostrocaudal axis. Specifically, in the rostral neural tube, ventral cell types still occur in *Gli2*$^{-/-}$ mutants in which Shh signaling has been activated by a *Ptch1* knockout (Motoyama et al. 2003). This indicates that Gli3 can compensate for the loss of Gli2-derived GliA in the rostral neural tube. In the caudal neural

tube, however, loss of Gli2 abolishes ventral cell types even when the Shh pathway is maximally activated by the *Ptch1* mutation. Therefore, mutations in Shh effectors that differentially regulate Gli2 versus Gli3 may result in a variable phenotype along the rostrocaudal axis.

Furthermore, the caudal neural tube develops slightly earlier than the rostral neural tube (Papalopulu and Kintner 1996). It is known that the timing of Shh signaling is just as important as the dose of Gli activity received by different spatial domains of the developing tissue (as described earlier in this chapter under Sect. 2.2.1 "Patterning the neural tube"). Therefore, perturbations of the balance between GliA and GliR may have different effects on neural patterning depending on the duration of the Shh-sensitive window of development at different points along the rostrocaudal axis.

2.5 Conclusions and Perspectives

In summary, the regulation of spinal cord development by Shh is completely dependent upon mechanisms that operate within primary cilia. While many aspects of the molecular mechanisms behind this pathway still remain a mystery, over the past 10 years, genetic experiments in mouse models have taught us much about the role of cilia in regulating Shh signaling. Because the mammalian neural tube is so sensitive to subtle shifts in Shh signaling via genetic manipulation, studies using this system have revealed new levels of complexity in the Shh pathway and discovered many unsuspected effectors. The novel, vertebrate-specific regulators of Shh signaling identified most recently (such as TULP3 and Tectonic1) have opened up new avenues of research that will help us solve some of the mysteries in the field, and there are sure to be still more Shh effectors awaiting our discovery in future unbiased genetic screens.

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