Chapter 1
Overview of Hedgehog Signaling Pathway

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Introduction

Initially discovered in *Drosophila* and later found in all vertebrate model organisms, the Hedgehog (Hh) family of secreted proteins plays critical roles in both embryonic development and adult tissue homeostasis [41, 84]. Numerous human genetic disorders and cancer have been associated with aberrant Hh signaling activity [41, 63, 84].

Hh acts through a conserved pathway to influence the balance between activator and repressor forms of the Gli family of zinc finger transcription factors (Gli^A and Gli^R; Fig. 1.1). While *Drosophila* has only one Hh and one Gli protein, Cubitus interruptus (Ci), mammals have three Hh family members (Sonic hedgehog (Shh), Indian hedgehog and Desert hedgehog) and three Gli proteins (Gli1, Gli2 and Gli3). In mice, Gli^R function is mainly derived from Gli3, whereas Gli^A function is primarily contributed by Gli2. Gli1 is a transcriptional target of Hh signaling and acts in a positive feedback to reinforce Gli^A activity. The reception of Hh signals is mediated by a 12-span transmembrane protein Patched (Ptc) that binds directly to Hh, and a 7-span transmembrane protein Smoothened (Smo) that transduces the signal into the cytoplasm. Ptc blocks Smo activity in the absence of Hh, allowing the production of Gli^R/Ci^R that represses a subset of Hh target genes. Binding of Hh to Ptc activates Smo, which blocks Gli^R/Ci^R production and promotes Gli^A/Ci^A activation. The fundamentals of *Drosophila* and mammalian Hh

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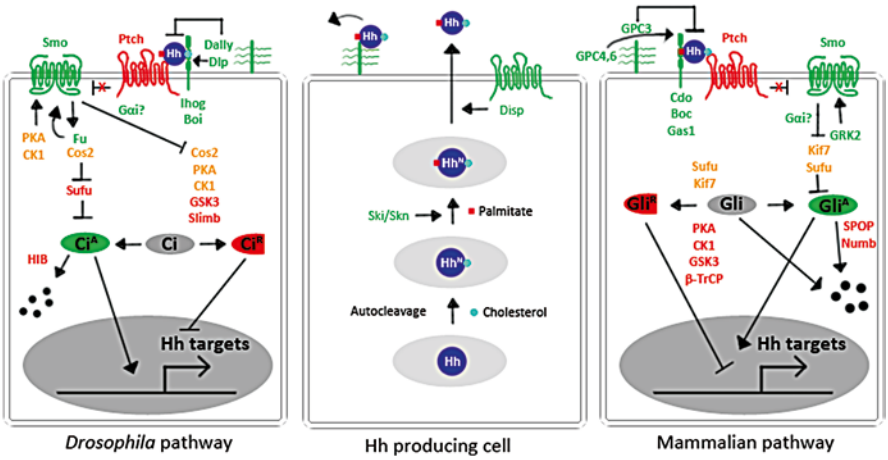

Fig. 1.1 Sending and transducing the Hh signal. In Hh-producing cells, full-length Hh is autocatalytically cleaved to generate an N-terminal fragment (HhN) modified by cholesterol. HhN is palmitoylated by Ski/Skn. Secretion of dual lipid-modified Hh is mediated by Disp. HSPGs facilitate Hh movement. Hh signal reception is facilitated by Ihog/Boi in *Drosophila* and Cdo/Boc/Gas1 in mammals, functioning as essential coreceptors. Dally and its mammalian HSPG counterpart GPC3 inhibit Hh pathway activity, whereas Dlp and related molecules GPC4 and GPC6 promote Hh signaling. In the absence of Hh, Ptc blocks Smo and full-length Ci/Gli2/Gli3 is phosphorylated by multiple kinases and subsequently targeted to ubiquitin/proteasome-mediated proteolysis through Slimb/βTRCP to generate a truncated repressor form (Ci^R/Gli^R). In *Drosophila*, efficient phosphorylation of Ci requires the kinesin-like protein Cos2, which acts as a molecular scaffold to bridge Ci and its kinases. Hh-binding to Ptc blocks its inhibition on Smo. In *Drosophila*, Ptc inhibition triggers Smo phosphorylation by PKA and CKI, leading to the cell surface accumulation and activation of Smo. Smo then recruits Cos2-Fu to activate Fu and dissociates Cos2-Ci-kinase complexes to inhibit Ci phosphorylation and processing. Furthermore, high levels of Hh stimulate Ci^A via Fu-mediated antagonism of Sufu. Hh signaling induces the expression of nuclear HIB that targets Ci^A for degradation. Fu-Cos2 is also involved in a feedback regulation of Smo phosphorylation. In mammalian systems, Kif7 is the mammalian Cos2 homolog but it does not interact directly with mSmo. mSmo phosphorylation requires GRK2. In mammals, Fu homolog is not required for Hh signaling and Sufu is a key negative regulator of Hh signaling. Kif7 and Sufu seem to play dual roles in positive and negative regulation of the Hh pathway. In addition to SPOP, which targets full length Gli2 and Gli3 for degradation, Numb is involved in Gli1 degradation. This figure is adapted from [41]

signal transduction pathways are similar, though major difference can be found in several regulatory steps. There is accumulating evidence suggesting that Hh signaling can also exert Gli-independent non-transcriptional effects [94]. In this chapter, we review the basics of the Hh signaling pathway and highlight some of the recent findings in the field.

Hh Signal Transduction

Lipid Modification and Multimerization of Hh

In Hh-producing cells, full-length Hh precursor undergoes autocleavage to release an N-terminal fragment (HhN) with a cholesterol moiety covalently linked to its C-terminus (Fig. 1.1) [70]. HhN is then palmitoylated near its N-terminus by the acyltransferase Skinny Hedgehog (Ski/Skn) [9]. While cholesterol modification increases the affinity of Hh for cell membranes and restricts its free dispersal [7, 49], dual lipid modifications facilitate the formation of large multimeric Hh complexes, allowing Hh to move over a long distance ([98] and references therein). HhN forms nanoscale oligomers with heparan sulfate proteoglycans (HSPGs), and disruption of HhN oligomerization and HSPGs interaction compromises specifically long-range signaling [86]. Dispatched (Disp), a transmembrane protein structurally related to Ptc, is required for the secretion of lipidated Hh to the extracellular space [2, 7, 55]. A recent study suggested that Disp might also act with Ptc1 to mediate the transport of Shh through tissues [29].

Heparan Sulfate Proteoglycans Regulate Hh Signaling

Genetic studies in *Drosophila* have shown that members of the glypican subfamily of HSPGs, Dally and Dally-like (Dlp), modulate the transport and reception of Hh signals [95]. Mutations in these genes as well as those affecting the biosynthesis of HSPGs impede the spread of Hh signals and reduce Hh pathway activity [50]. HSPGs seem to affect Hh signaling in many different ways (Fig. 1.1). In the absence of HSPGs, cell surface Hh diminishes, suggesting that HSPGs contribute to the stability of Hh. HSPGs appear to be required for Hh movement as a narrow strip of HSPG-deficient cells is sufficient to completely block Hh signaling in wild-type cells behind the mutant clone. In addition, Dlp is critical for Hh signaling activity and may act as an essential coreceptor [96]. A recent study suggested that there are two functional families of glypicans in *Drosophila* and mammals [92]: Dlp and its mammalian counterparts, including GPC4 and GPC6, constitute a group that acts positively and cell-autonomously for Hh signaling, whereas Dally and other glypicans, such as GPC3, form another group that inhibits Hh response. Consistent with this, GPC3 competes with Ptc for Hh binding in vitro and inhibits Hh signaling during mouse development [8]. It is important to note that HSPGs also regu late other signaling molecules, including Wg/Wnt and Dpp/TGF-β [95], and thus these extracellular matrix proteins likely exert differential effects on multiple signaling pathways during development and tumorigenesis.

Modulation of Pathway Activity by Multiple Hh-Binding Proteins

In addition to Ptc, there are multiple Hh-binding proteins identified in *Drosophila* and mammals. Some of them might act as coreceptors of Hh (Fig. 1.1). Genetic analysis in *Drosophila* revealed that the Ihog family of immunoglobin/fibronectin repeat-containing proteins, Ihog (Interference hedgehog) and Boi (Brother of Ihog), are essential for Hh pathway activity [96, 105]. Mammalian homologs of Ihog/Boi, Cdo and Boc, are also positively involved in Shh signaling [81, 97, 102]. The Ihog/Cdo family proteins bind Hh through fibronectin domains [81, 97], and Ihog can enhance Hh binding to Ptc [97], suggesting that they act as Hh coreceptors. Indeed, Ihog promotes surface presentation of Ptc, and both Ihog and Ptc are required for high-affinity Hh binding, supporting the notion that Ihog and Ptc constitute the Hh receptor in *Drosophila* [105].

Hip1 and Gas1 are two vertebrate-specific Hh-interacting proteins. *Hip1* encodes a membrane-bound glycoprotein that acts as a negative regulator of Hh signaling by competing with Ptc for Hh binding [18]. *Hip1* expression is induced by Hh signaling and restricts Hh signaling through a negative feedback mechanism [17, 36]. On the contrary, *Gas1* encodes a GPI-anchored membrane protein that promotes Shh signaling [1, 58]. Since Gas1 acts cooperatively with Cdo in the positive regulation of Hh response [1], it might function as a coreceptor of Hh.

Ptc Inhibits Smo Catalytically

Being the core Hh-binding receptor, Ptc paradoxically functions as an inhibitor of Hh signaling and blocks pathway activation in the absence of Hh. The precise mechanism by which Ptc regulates Smo remains a mystery. Recent studies suggest that Ptc and Hh reciprocally regulate Smo subcellular localization and conformation. Ptc and Smo are largely segregated in *Drosophila* imaginal discs [22] and they do not form stable protein complexes [43, 79]. Cultured cell experiments suggested that Ptc inhibits Smo at a substochiometrical concentration [79]. Ptc is homologous to the resistance-nodulation-division (RND) family of prokaryotic proton-driven transporter, and might function by transporting an endogenous small molecule Smo agonist or antagonist across membranes, as conserved residues in RND-like transporters are essential for Ptc function [79]. Indeed, Ptc regulates trafficking of lipoproteins through endosomes [44]. Several natural and synthetic small molecules can inhibit or activate Hh pathway at the level of Smo [10, 11]. In cultured cells, Ptc induces the secretion of pro-vitamin D3, and both pro-vitamin D3 and vitamin D3 inhibit Hh signaling at high concentrations [6]. Oxysterols, which lie downstream of vitamin D3 in the cholesterol biosynthetic pathway, act as positive regulators of Hh signaling at a level upstream of Smo [21, 25]. Whether oxysterols or related molecules function as physiological Smo regulators remains to be determined. A recent genetic study in *Drosophila* suggested that the phospholipid, phosphatidylinositol-4 phosphate (PI4P), is a target of Ptc action. In *Drosophila* cells, PI4P promotes Smo accumulation and Hh pathway activation, and Ptc restricts the production of PI4P by

regulating its kinase/phosphatase directly or indirectly [98]. Exactly how Ptc regulates PI4P levels and whether oxysterols or lipoprotein-derived lipids are linked to the effects of PI4P on Smo await further investigation.

Regulation of Smo Trafficking and Conformation

In *Drosophila*, Ptc restricts Smo cell surface expression by promoting endocytosis and degradation of Smo. Hh induces opposite changes in the subcellular distribution of Ptc and Smo, with Smo accumulating on the cell surface and Ptc entering the cytoplasm [22, 39, 106]. How Hh and Ptc reciprocally regulate Smo trafficking is not clear, but it is mediated at least in part by Smo phosphorylation. Phosphorylation-deficient Smo variants fail to accumulate on the cell surface in response to Hh, whereas phospho-mimicking Smo variants constitutively accumulate on the cell surface [39, 104].

A similar reciprocal trafficking relationship is observed for mammalian Ptc1 and Smo but this occurs in the primary cilium, a microtubule-based cell surface protrusion present in most mammalian cells (Fig. 1.2). Genetic studies in mice have implicated primary cilia as essential cellular organelles for mammalian Hh signaling.

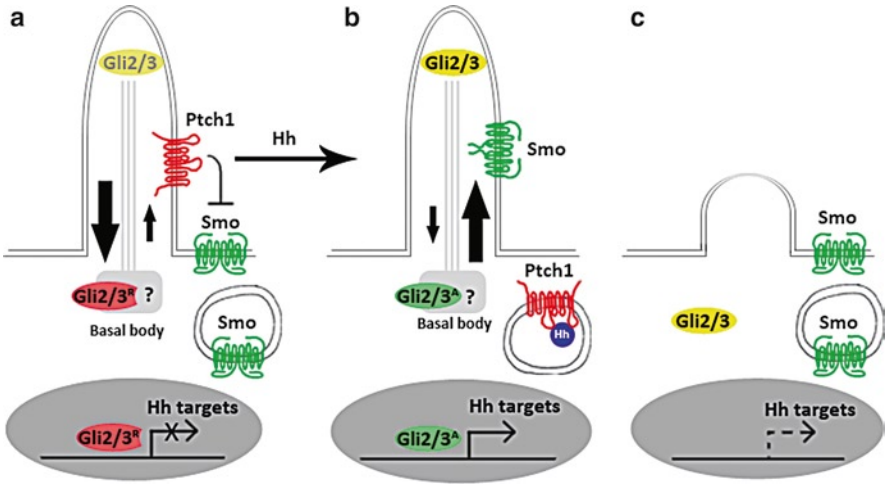

Fig. 1.2 Hh signaling and primary cilia. (**a**) In the absence of Hh, Ptch1 localizes to the primary cilia and inhibits Smo from entering primary cilia. Due to high retrograde transport activities, little or low levels of full length Gli2 and Gli3 are detected at the ciliary tip. Gli3 and Gli2 (to a lesser extent) are processed to form truncated repressors, which enter the nucleus to inhibit a subset of Hh target genes. (**b**) Hh binding of Ptch1 leads to the elimination of Ptch1 from the primary cilia and, subsequently, the entry of Smo into primary cilia. Full length Gli2 and Gli3 are found at the ciliary tip probably due to high anterograde transport activities. By ill-defined processes, Gli2 and Gli3 are converted into active forms, which promote the transcription of Hh target genes. (**c**) Deletion of primary cilia abolishes the processing as well as activation processes of Gli2 and Gli3. In addition to regulating Smo ciliary localization, Hh also induces a conformational change of Smo essential for its activation. See text for details

Mutations affecting the intraflagellar transport (IFT) machinery or other components that are involved in the assembly and function of cilia affect Hh signaling in several developmental contexts [30]. In the absence of Hh, Ptc localizes to cilia and prevents Smo from accumulating in the cilia; binding of Hh to Ptc triggers reciprocal trafficking of Ptc and Smo, with Ptc moving out of and Smo accumulating in the cilia [20, 72]. Ciliary localization of Smo correlates with Hh pathway activation: both an oncogenic Smo mutation and Smo agonists, such as SAG and oxysterols, promoted accumulation of Smo in the cilia [20, 72], and mutation of a conserved ciliary localization motif in Smo prevented its ciliary accumulation and abolished its signaling activity [20]. How Ptc restricts Smo ciliary accumulation is not clear. Smo may constantly move in and out of the cilia in equilibrium by binding to anterograde and retrograde IFT motors and Ptc may tilt this balance. In support of this model, β-arrestins promote Smo ciliary localization by mediating its association with the anterograde IFT motor kinesin-II in response to Hh [48], and Smo is enriched in the cilia of cells defective in retrograde transport [46, 64]. However, recent studies indicated that ciliary entry of Smo does not require microtubule-dependent cytoplasmic motors [46], and that Smo moves through a lateral transport pathway from the plasma membrane to the ciliary membrane [62].

Ciliary localization of Smo is not sufficient for its activation [3, 46, 73, 89], and Smo activation at the cilia likely may involve additional steps including conformational change [104]. FRET analysis demonstrated that both *Drosophila* and mammalian Smo proteins undergo a conformational change in response to Hh [104]. In response to Hh stimulation or Ptc inhibition, *Drosophila* Smo is phosphorylated by protein kinase A (PKA) and casein kinase I (CK1) at its C-terminal tail (C-tail) [4, 22, 39, 99], which triggers a conformational switch and increased proximity of two Smo C-tails within a Smo dimer [104]. Mechanistically, these phosphorylation events activate Smo by counteracting multiple Arg clusters that maintain Smo in a closed inactive conformation [104]. Mammalian Smo (mSmo) C-tail does not harbor PKA/CK1 sites, but does contain a long stretch of basic residues that inhibits its activity; and mSmo undergoes a similar conformational change upon Shh stimulation [104]. mSmo is phosphorylated either directly or indirectly by the G protein-coupled receptor kinase GRK2, which positively regulates Hh signaling [14, 59, 69], raising the possibility that GRK2 and related kinases may substitute for PKA and CK1 to regulate Smo conformation and trafficking in vertebrates.

Downstream of Smo: G Protein and Cos2/Kif7-Ci/Gli Signaling Complex

G protein $G\alpha_i$ is activated by Smo in both *Drosophila* and mammalian cultured cells [66, 71], and $G\alpha_i$ is required for the expression of Hh target gene *decapentaplegic* (*dpp*) in *Drosophila* wing imaginal discs [66]. However, whether $G\alpha_i$ plays a physiological role in Shh signaling is not clear, as inhibition of $G\alpha_i$ activity had minimal effects on Hh-dependent ventral neural tube patterning in chick embryos [53].

Smo likely signals through both $G\alpha_i$-dependent and -independent mechanisms. In *Drosophila*, Smo directly interacts with a multi-protein signaling complex containing Ci, the kinesin-like protein Costal 2 (Cos2), and the Ser/Thr protein kinase Fused (Fu) [38, 54, 65, 75]. Cos2 serves as a molecular scaffold to bring Ci and Fu together with PKA, GSK3, and CK1, leading to efficient phosphorylation and proteolytic processing of Ci [103]. Activated Smo attenuates Cos2-Ci-kinase complex formation, thus inhibiting Ci phosphorylation and processing [74, 103].

mSmo does not interact directly with the vertebrate Cos2 homologs Kif7 and Kif27 [83]. However, recent studies demonstrated that Kif7 is a functional homolog of Cos2. Kif7 forms complexes with Gli proteins and its deletion or mutation leads to aberrant regulation of Hh signaling [16, 26, 51]. Cos2 can move along the microtubules and its motor activity appears to be required for Ci processing [28]. Similarly, Kif7 function is dependent on intact IFT machinery and Hh signaling promotes ciliary localization of Kif7 [26]. Furthermore, Gli3 processing is compromised in *Kif7* null embryos [16, 26, 51]. In *Drosophila*, $G\alpha_i$ is associated with Cos2 upon Hh stimulation [66]. It remains to be determined whether $G\alpha_i$ or related proteins serve as a link between Smo and Kif7.

Control of Gli Protein Degradation and Processing

Ci/Gli activity is regulated by multiple mechanisms, including phosphorylation, proteolysis, and cytoplasmic/nuclear shuttling. In the absence of Hh, full-length Ci/Gli protein can be proteolytically processed into a truncated repressor (Ci, Gli3 and, to a lesser extent, Gli2) or degraded (Gli1 and Gli2). Hh signaling blocks the production of the truncated repressor, and stimulates nuclear translocation and activation of accumulated full-length Ci/Gli. Ci/Gli processing requires the activities of PKA, GSK3, and CK1 as well as the F-box protein Slimb/β-TRCP of the SCF ubiquitin ligase complex [42]. PKA, GSK3, and CK1 sequentially phosphorylate multiple sites in the C-terminal region of Ci/Gli, resulting in the recruitment of Slimb/β-TRCP [40, 76, 80, 87]. A *p*rocessing *d*eterminant *d*omain (PDD) located between the Zn-finger DNA-binding and Slimb/β-TRCP-binding domains of Ci/Gli appears to be critical for proteasome-mediated degradation that selectively removes its C-terminal half. Deletion of this domain from Ci blocks the production of Ci^R [61] and renders complete degradation of Ci [77]. Gli3 is processed more efficiently than Gli2 into a truncated repressor form probably due to a more potent PDD, and Gli1 lacks a PDD and does not exhibit repressor activity [68].

In mammalian cells, Gli2 and Gli3 are localized to the tip of primary cilia in an Hh-dependent manner (Fig. 1.2; [13, 33, 46, 90]). Upon Hh stimulation, Gli2 shifts from a predominantly cytoplasmic localization to the distal tip of the cilium and within the nucleus [51]. While $Gli3^R$ is predominantly nuclear and not found at the ciliary tip [33, 90], Hh stimulation leads to its disappearance and accumulation of full-length Gli3 ($Gli3^{FL}$) at the tip of the cilium as well as in the nucleus [103]. Importantly, Hh signaling also promotes degradation of full-length Ci/Gli2/Gli3 through an ubiquitin ligase containing HIB/SPOP [13, 45, 88, 90, 100, 101], and this mechanism serves as a negative feedback loop to tune down Hh signaling

activity in *Drosophila* [45, 100]. Gli1 is not a strong substrate for SPOP [13, 101] and its degradation involves Numb, which acts in conjunction with the E3 ubiquitin ligase Itch [23]. $Gli3^R$ is also degraded by the proteasome but this likely utilizes a different ubiquitin ligase system [90]. Control of Gli protein degradation might play a central role in preventing tumorigenesis [23, 35].

The exact locations for phosphorylation and proteasomal degradation/processing of Ci/Gli proteins are not known. As the proteasome is enriched at centrosomes that give rise to the basal body underneath the primary cilia [91], Gli proteins might be phosphorylated at primary cilia and then targeted to the centrosome-associated proteasomes for proteolysis. A recent study showed that, in the presence of Shh, the inactive catalytic subunit of PKA is enriched in the cilium base of proliferative cerebellar granular neuronal precursors and that this localization of PKA is essential for Shh-induced proliferation [5]. These observations raise an intriguing possibility that the cilium base might serve as the prime site for phosphorylation and degradation/processing of Gli proteins. In the absence of Hh, the primary cilium may act as a "cAMP gun" to locally activate PKA. Smo might activate $G\alpha_i$ in the ciliary membrane, which in turn represses the adenyl cyclase in the cilium, leading to a local drop of cAMP level and PKA activity. This model is consistent with the genetic data that Gli^A and Gli^R levels are affected in various mutant backgrounds with defective IFT and/or ciliogenesis. How Gli proteins in the cilium are linked to the transcriptional activation of Hh target genes in the nucleus remains unknown. A recent study has highlighted the involvement of cytoplasmic microtubules in ciliary entry of Gli2, but not of Smo [46]. Full-length Gli proteins may need to be "activated" at the cilia before they translocate to the nucleus to activate Hh target genes.

Sufu: A Key Regulator of Mammalian Hh Signaling

A striking difference between *Drosophila* and mammalian Hh signal transduction is the divergent roles of Fu and Sufu [41]. In *Drosophila*, *fu* is a positive regulator essential for Hh signaling, whereas *Sufu* is a genetic suppressor of the *fu* mutation, but its loss does not elicit ectopic Hh signaling and has minimal effects on development. However, in mice, Fu is not involved in Hh signaling [12, 60] and loss of Sufu has profound effects on Hh signaling with ectopic pathway activation [19, 78, 83]. Sufu may have assumed a major inhibitory function in the mammalian Hh pathway due to the existence of multiple Gli proteins. To inhibit Gli^A function, Sufu could impede Gli nuclear localization [24] or suppress Gli activity by recruiting a corepressor complex [15]. Recent studies indicate that Sufu plays a major role in Gli3 processing [13, 34, 37, 47]. Furthermore, Sufu also plays a positive role in mammalian Hh signaling through stabilization of Gli2, in part through counteracting the activity of SPOP [13, 101]. Why Fu kinase is not involved in mammalian Hh signaling? One possibility is that the role of Fu kinase in *Drosophila* Hh signaling is replaced by other protein kinases in mammals. Indeed, multiple protein kinases, including DYRK1a, DYRK2, MAP3K10, ULK3 and Cdc2l1, have been identified to influence Gli activity in mammalian cultured cells [27, 56, 57, 82].

Unresolved Questions in Mammalian Hh Signaling

Numerous studies have revealed the differential utilization of Gli^A and Gli^R in various developmental systems during mammalian embryogenesis [41]. While Gli^A levels are central to cancer formation [35], the involvement of Gli^R has been implicated by several recent reports linking primary cilia to Hh pathway-dependent tumorigenesis [32, 93]. Though deletion of primary cilia blocks the ability of an oncogenic form of Smo (SmoM2) to induce tumorigenesis, it promotes tumorigenesis induced by activated Gli2, Gli2ΔN. Since primary cilia are essential for Gli3 processing, these results suggest that reduction of Gli^R levels may accelerate Gli2ΔN-induced tumorigenesis. Sufu and Kif7 have different functional requirements for IFT or primary cilia [13, 26, 37]. It is possible that they function in separate processes downstream of Smo and deletion of primary cilia may disrupt Kif 7 function, leading to increased tumor incidence in the above studies. Further studies will be needed to decipher the distinct as well as potentially overlapping functions of Sufu and Kif 7 in Hh signaling during development and tumorigenesis.

Several genomic scale studies on Gli target genes revealed that though many target promoters contain a consensus related to the sequence TGGGTGGTC, other target genes may not require this consensus sequence for Gli-dependent transcriptional regulation [31, 85]. Whether Gli^A or Gli^R regulates these genes through interactions with other transcription factors or cofactors remains to be determined. Furthermore, there is increasing evidence that Hh exerts its effects through Gli-independent non-transcriptional mechanisms [52, 67, 94]. However, the involvement of this Gli-independent Hh signaling in development and tumorigenesis has not been studied. As detailed in the rest of this book, Hh signaling plays major roles in a wide variety of tumors and it can act via both autocrine and paracrine mechanisms. Importantly, the requirement of Hh pathway activity in tumor formation and growth seems to differ largely in a context-dependent manner. Further understanding of Hh signal transduction mechanisms at different levels along the pathway will certainly be rewarding to current efforts in targeting the pathway for cancer therapy.

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