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VISIONS AND REFLECTIONS

Hedgehog signaling pathway: a novel model and molecular mechanisms of signal transduction

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Abstract The Hedgehog (Hh) signaling pathway has numerous roles in the control of cell proliferation, tissue patterning and stem cell maintenance. In spite of intensive study, the mechanisms of Hh signal transduction are not completely understood. Here I review published data and present a novel model of vertebrate Hh signaling suggesting that Smoothened (Smo) functions as a G-protein-coupled receptor in cilia. This is the first model to propose molecular mechanisms for the major steps of Hh signaling, including inhibition of Smo by Patched, Smo activation, and signal transduction from active Smo to Gli transcription factors. It also suggests a novel role for the negative pathway regulators Sufu and PKA in these processes.

Keywords Smoothened · Patched · Gli · Signal transduction · PKA · Sufu · Crosstalk · GPCR · 7-DHC · Oxysterol · Primary cilium

Introduction: Hh pathway overview

The Hedgehog (Hh) signaling pathway is a key regulator of development, cell proliferation and stem cell maintenance [1–3]. Its activity is tightly regulated. Insufficiency of Hh signaling leads to developmental defects, such as cyclopia and holoprosencephaly, whereas its overactivation is involved in a various cancers including medulloblastoma and basal cell carcinoma [4, 5].

Hh signaling starts by the binding of the Hh ligand to its receptor Patched1 (Ptc). Ptc inactivation relieves repression of the receptor Smoothened (Smo), initiating the signaling cascade. This results in the activation of glioma-associated oncogene (Ci/Gli) transcription factors and the expression of Hh target genes.

Initially identified in *Drosophila* [6], the major Hh pathway components are evolutionally conserved. However, there are several important differences between flies and vertebrates. (1) Vertebrate homologues of the *Drosophila* transcription factor Cubitus interruptus (Ci), the Gli1, Gli2, and Gli3 proteins have more specialized functions. Mouse mutant studies indicate that Gli1 is exclusively a transcriptional activator and that its expression is induced by Hh signaling. Gli2 and Gli3 have dual activities, but although Gli3 functions essentially as a repressor (GliR), Gli2 is a major pathway activator (GliA) [7, 8]. (2) Drosophila has a single Hh ligand, whereas vertebrates encode three homologous proteins (Sonic Hh, Desert Hh and Indian Hh). (3) In vertebrates, Hh signaling critically depends on the primary cilium [9], a microtubule-based organelle, projecting from the cellular plasma membrane [10]. The selective import and export of proteins between the cytoplasm and cilia is mediated by intraflagellar transport (IFT) proteins and their associated kinesin II (Kif3 family) and dynein-2 (Dync2) motors [10]. Major Hh pathway components move through, and accumulate in cilia at different steps of the pathway activity; mutations in numerous ciliary genes impair Hh signaling. (4) Suppressor of Fused (Sufu) is a major Hh pathway inhibitor in vertebrates, but dispensable in flies, whereas in both vertebrates

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and flies, the twelve transmembrane receptor Patched (Ptc) and protein kinase A (PKA) negatively regulate Hh signaling. Hh ligand maturation and secretion which require lipid modifications are not discussed here, but the reader is referred to a recent review [2].

In the canonical vertebrate Hh pathway, Ptc and the G protein-coupled receptor (GPCR) Gpr161 are present on cilia in the absence of ligand. Ptc represses the activity of a seven transmembrane (7TM) receptor Smo [11, 12], while Gpr161 inhibits Hh signaling by activating PKA at the base of cilia [13] (Fig. 1). The Gli transcription factors are phosphorylated by PKA, Casein kinase 1 (CK1) and Glycogen synthase kinase 3β (GSK3β), processed by proteasomes into the C-terminally truncated repressor form GliR, and repress Hh target genes in the nucleus [14, 15]. Upon the binding of the processed and lipid modified Hh ligand by Ptc, Ptc and Gpr161 exit the cilia relieving Smo inhibition. Smo is phosphorylated by CK1 and G protein-coupled receptor kinase (GRK2), and is translocated into the cilia by the kinesin motor Kif3a [16], where it is anchored near the base of the cilia by Ellis-van Creveld syndrome proteins Evc1/Evc2 [17, 18]. Active Smo inhibits Gli processing. The full length Gli2 and Gli3 (GliFL) proteins, in a complex with Sufu, move into the cilia and accumulate at the cilia tip where the complex dissociates [19, 20]. Upon exiting the cilia, activated GliFL proteins act as transcriptional activators [2, 21, 22]. The transit of Gli2 and Gli3 proteins through the cilia is essential for GliR and GliA production and partially depends on kinesin-like protein Kif7, the *Drosophila* Cos2 vertebrate orthologue [2, 23–26].

In addition to Ptc, CAM-related/downregu-lated by oncogenes (CDO), brother of CDO (BOC), as well as growth arrest-specific 1 (GAS1) and hedgehog interacting protein (Hhip) receptors can also bind Hh with similar affinities, thus modulating Hh signaling [27]. Increased expression of the Hh targets Ptc and Hhip ensures feedback inhibition of the Hh pathway [28, 29]. The sum of all negative and positive signaling inputs, the GliR/GliA ratio, determines Hh pathway activity [30]. Hh generates numerous cellular responses. For example, Hh can behave as a morphogen by acting in a dose-dependent manner to specify distinct neuronal cell types [31]. Hh can also trigger several non-canonical signaling pathways involving Smo, but independent of Gli proteins, transcription and cilia [32].

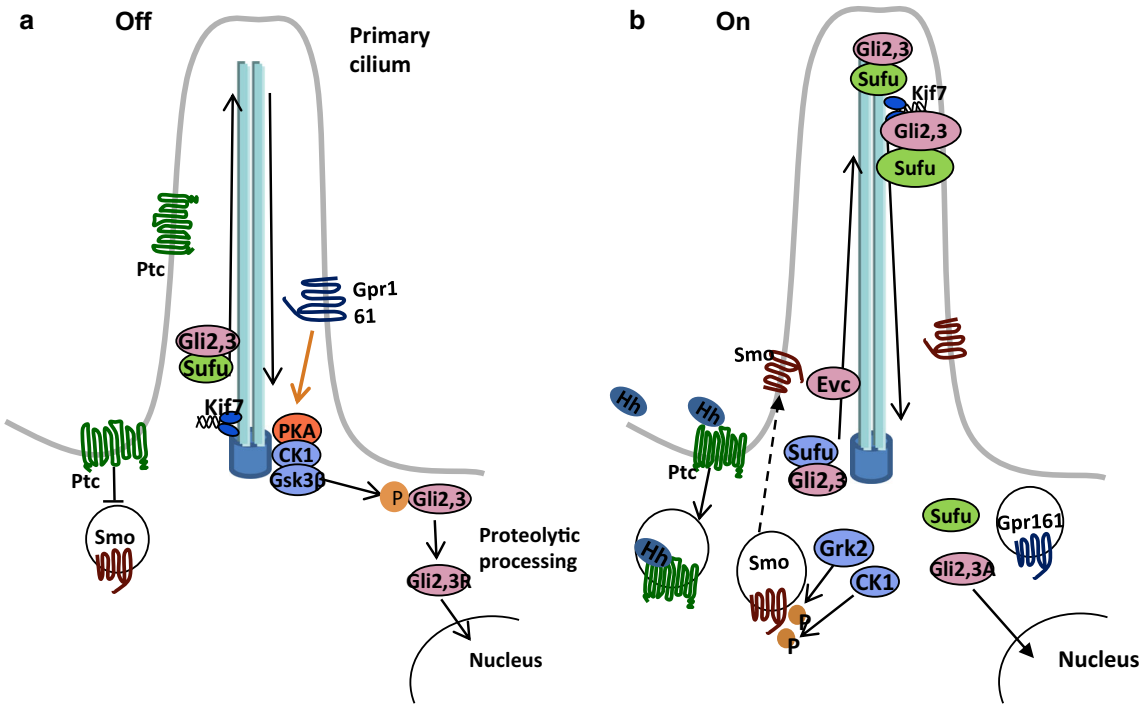

Fig. 1 Canonical Hh pathway (adapted from [2]). **a** In the absence of Hh, Ptc and Gpr161 are present in the cilium. Ptc inhibits Smo activity, preventing its accumulation in the cilium. Gpr161/Gs activates PKA, required for the production of the Gli repressor GliR. Sufu represses Gli2 and Gli3 activity by forming a complex with them. Gli2 and Gli3 proteins are phosphorylated at the base of the cilium by PKA, CK1 and GSK3β, and after passage through the cilium, are processed to the GliR form by the proteasome. **b** In the presence of Hh, Gpr161 and Ptc exit the cilium. Smo is phosphorylated by CK1 and GSK3β and transported to the cilium by the microtubule motor Kif3A, where the activated Smo is anchored near the base of the cilium by EVC (Ellis-van Creveled syndrome protein). The Sufu/Gli complex accumulates at the tip of the cilium, where the complex dissociates. Upon exiting the cilia, activated, full length Gli2 and Gli3 proteins enter the nucleus. The movement of the Sufu/Gli complex into the cilia is partially dependent on Kif7, the vertebrate Cos2 orthologue

Despite intensive studies, the mechanisms of Smo activation and the downstream signaling cascade remain poorly understood. Here is revised the existing model of Hh signaling in vertebrates and a novel interpretation of previously published data is provided. Based on the hypothesis that Smo functions as GPCR in the canonical Hh pathway, I propose molecular mechanisms for the major steps of Hh signaling: Smo inhibition by Ptch, Smo activation, and signal transduction from active Smo to Gli transcription factors. Finally, I suggest a novel role for the negative Hh pathway regulators Sufu and PKA and highlight the importance of cilia for all of these processes.

The structure of Smo

The crystal structure of the human Smo (hSMO) 7TM domain confirmed its canonical GPCR helical fold (Fig. 2), and its structural similarity to the Frizzled (Fz) receptor of the Wnt signaling pathway [33]. However, whether Smo functions as a GPCR remains a matter of debate. Smo constitutively dimerizes through its highly conserved extracellular N-terminal cysteine-rich domain (CRD) [34], containing ligand-binding pocket in many GPCRs, including Fz [33, 35]. The endogenous Smo ligand is still unknown, but the CRD seems to be dispensable for Hh-induced Smo activity [36, 37]. Recently, the Smo CRD was identified as a binding site for several natural activating oxysterols (see below) [36, 37]. Their role in Hh pathway regulation remains to be studied.

The Smo 7TM domain is a binding site for the Smo inhibitor cyclopamine, a plant steroidal alkaloid. Several small synthetic agonists and antagonists can compete with cyclopamine for binding to Smo [38–40]. Smo is believed to adopt multiple conformations, suggesting the existence of multiple binding sites on Smo for regulating its activity (Fig. 2). Indeed, structural studies have mapped distinct ligand-binding pockets to the 7TM domain associated with the extracellular loops [33, 41].

Smo can couple to the Gαi family of heterotrimeric proteins, inhibiting adenylyl cyclase (AC) and PKA activities, in various cell models. In *Xenopus* melanophores, hSMO is able to signal through the pertussis toxin (PTX)-sensitive Gi family of G-proteins [42]. Specific coupling of mouse Smo (mSmo) to G inhibitory protein family (Gi, Go, Gz) was shown using both SF9 insect cells and mammalian fibroblasts [43]. In HEK293 cells, mSmo spontaneously couples with endogenous Gi protein as does the canonical GPCR serotonin receptor 5HT1aR [44]. Smo-Gi coupling alone is sufficient for the activation of non-canonical Gli- and transcription-independent Hh signaling [32]. However, an additional signal from the Smo C-terminus is required for transcriptional activation of the canonical Hh pathway [43].

In the absence of signaling, small amounts of Smo protein are localized in cytoplasmic vesicles [45]. The Smo C-tail then adopts a closed conformation, masking the kinase-binding pockets. Hh activates Smo by inducing its conformational switch, phosphorylation and targeting to the ciliary membrane [34]. The ciliary localization of Smo

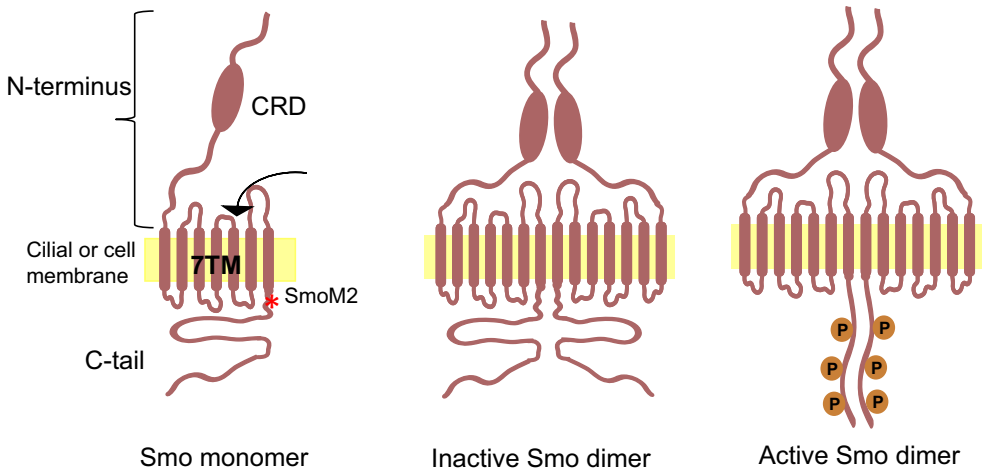

Fig. 2 The structure of Smo. Smo has a GPCR structure. It has a long extracellular N-terminus containing a cysteine-rich domain (CRD), a 7TM region and a long C-terminal cytoplasmic tail. CRD is a binding site for activating oxysterols [36, 37]. The Smo 7TM region and extracellular loop form binding pockets for cyclopamine and small synthetic Smo ligands [33]. Smo is constitutively dimerized through its CRD. In the inactive conformation, the C-terminus is in a closed configuration and the kinase-binding pocket is inaccessible [34]. Hh induces a conformational switch of Smo, phosphorylation of its C-termini and targeting of Smo to the cilia. The highly phosphorylated C-termini dimerize, giving rise to the active, open conformation of the Smo dimer [34]. The position of the activating SmoM2 mutation is indicated by the *red star*. The *black curved arrow* indicates a binding pocket for allosteric Smo ligands

is critical for mammalian Hh signaling [9, 46, 47]. The C-termini of Smo are then highly phosphorylated by CK1 and GRK2 at Ser/Thr clusters and dimerize giving rise to a Smo dimer in an open active conformation (Fig. 3). Moreover, Smo activation is proportional to the level of Smo phosphorylation [34, 48]. A similar conformational change is induced by the oncogenic Smo mutation W539L (or SmoM2) and the Smo agonist SAG [34].

Ptc catalytically inhibits Smo

The mechanism of Smo inhibition by Ptc is poorly understood. Ptc shares homology with proteins of the bacterial resistance-nodulation division (RND) transporter family [49], is present in and around the cilia, and inhibits otherwise constitutively active Smo in a cell-autonomous catalytic manner [50]. The leading hypothesis is that Ptc controls the transport of small endogenous Smo ligands (influx of inhibitor or efflux of activator) [39, 51, 52]. These molecules are supposed to be sterols or derivatives for several reasons: (1) Ptc contains a highly conserved sterol-sensing domain (SSD), required for sterol transport. Mutations in this region abolish Ptc repression of Smo [49, 53]; (2) the plant-derived Smo antagonist cyclopamine has a cholesterol-like structure, as do several synthetic Smo ligands [38]; (3) Perturbations in sterol synthesis abrogate Hh signaling [54, 55]. It is also possible that Ptc regulates Smo localization through lipid modification of Smo-containing endosomes [56, 57], or by binding to Smo at a site different from the potential ligand-binding pockets on CRD or 7TM [36] (Fig. 2).

The search for endogenous Smo ligands identified several activating oxysterols, hydroxylated cholesterol derivatives [55, 58]. They bind to the Smo CRD and act synergistically with Hh. However, they are unlikely to be transported by Ptc molecules because mutants of Smo, in which the CRD has been deleted, still respond to Hh [36, 37]. The sterol vitamin D3 and its derivatives were described as Smo inhibitors, competing with cyclopamine

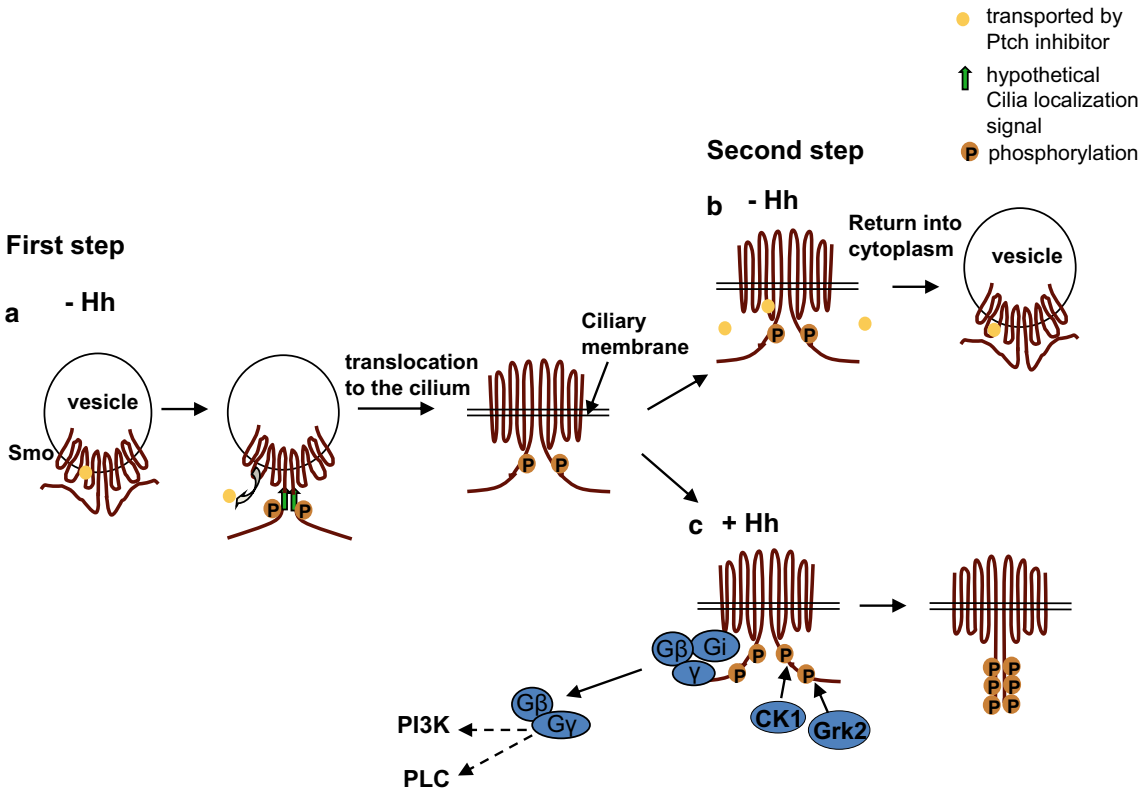

Fig. 3 Model of Smo activation as a GPCR. **a** In the absence of Hh: small lipidic molecules are transported by Ptc, bind to Smo, and inhibit its constitutive activity. Inactive Smo is localized to cytoplasmic vesicles. Dilution of the inhibitor in the cytoplasm would lead to the release of bound inhibitor by Smo and its conformational change. This allows Smo transport into the cilia by the kinesin protein Kif3A and Smo localization to the ciliary membrane. In cilia, two scenarios are possible. **b** In the absence of Hh: a high concentration of the Smo inhibitor is present in cilia. Binding of the inhibitor to the internal part of the Smo 7TM region induces a closed conformation and Smo returns to the cytoplasm. **c** In the presence of Hh: Ptc binds Hh and exits the cilia [121]. The concentration of Smo inhibitor decreases. This permits the phosphorylation of the Smo C-terminus by CK1 and GRK2 [48], the coupling of Smo with trimeric G protein, and Smo activation as a GPCR. The level of C-tail phosphorylation regulates the accessibility of the Gαi protein to the third intracellular loop of Smo and, consequently, Smo activity. Dissociated Gβγ subunits could activate PI3K or PKC signaling pathways

[59]. However, biosynthesis of vitamin D3 requires ultraviolet (UV) irradiation of its precursor, 7-dehydroxycholesterol (7-DHC) in the skin [60]. The lack of UV in the mammalian embryo environment argues against vitamin D3 to be the physiological Smo inhibitor. Endogenous Smo ligand that is transported by Ptc remains to be discovered.

Proposed model of Smo regulation

Based on presented above data, it is suggested that Smo functions as a GPCR and proposed a model for Smo regulation in vertebrates. This model includes three cases of Smo activation: by Hh, by the oncogenic SmoM2 mutation, and by synthetic ligands. An important point of this model is the constitutive dimerization of Smo [34].

(a) Smo activation by Hh

Smo activation requires ciliary localization and phosphorylation of the C-terminus, two processes that can be pharmacologically separated. Thus cyclopamine targets Smo to the cilia, but blocks Smo C-tail in the closed, inactive conformation inaccessible to phosphorylation [34]. These results have served as the basis for a two-step model of Smo activation [61]. Molecular mechanisms underlying these two steps are proposed here. They take place in two cell compartments—the cytoplasm and the cilia.

In the absence of Hh: Ptc is present in and around the cilium and controls the influx of small molecules inhibitors (sterols or derivatives). Binding of these inhibitors to the Smo 7TM domain induces an inactive, closed conformation, retaining Smo inside cytoplasmic vesicles (Figs. 3, 4a, b). At the base of the cilium, the Smo inhibitor is rapidly diluted in the cytoplasm and its concentration is low. Release of bound inhibitor from Smo induces a conformational switch from the closed to open form. The open conformation allows Smo to interact with the kinesin protein Kif3, and Smo is transported into the cilium, where it localizes to the membrane near the base of the cilium. This is the first necessary, but insufficient, step for Smo activation. Indeed, genetic studies of IFT proteins indicate, that Smo transits through the cilia even in the absence of ligand [62, 63]. The second step of Smo activation or inhibition takes place in the cilium (see below).

It could be proposed, that the open Smo conformation allows its coupling with the trimeric Gαi protein, leading to the activation of Smo as a GPCR in cilia. The level of C-tail phosphorylation could regulate the accessibility of Gi proteins for coupling with Smo and consequently its activity (Fig. 3). As a result, the inhibition of AC and PKA activity is limited to the ciliary space. Indeed, three AC (AC3, AC5 and AC6) have been detected in cilia [64]. I hypothesize, that Smo functions as a GPCR, not only in non-canonical, but also in canonical Hh signaling.

In support of this model, the deletion of the Smo C-tail significantly increases Gi-coupled Smo activity in HEK293 cells [43]. Functional analysis of eight point mutations, critical for mSmo activity, revealed that two are localized to the third intracellular loop and six to the C-terminus [65], regions that are important for interaction with G proteins in many GPCRs. Presumably, a ciliary localization sequence also resides in this region because, in mouse fibroblasts, C-tail-deleted Smo is not targeted to the cilia [17, 66, 67], and is transcriptionally inactive [43]. By contrast, fusion of the mSmo C-terminus to *Drosophila* Smo is sufficient for its transport into the cilia [66]. Altogether, the C-terminus is neither required for Smo expression on the cell membrane nor activation of Gi in HEK293 cells. However, a ciliary localization sequence present in the Smo C-tail renders it indispensable for signaling to Gli in the cilia of mouse fibroblasts [43].

The second step of Smo regulation takes place in the cilium, separated from the cytoplasm by a diffusion barrier (Fig. 3b, c). In the cilium, the concentration of Smo inhibitor, transported by Ptc, depends on the extracellular level of Hh: it is maximal in the absence of Hh but decreases, as a result of Ptc inactivation, in the presence of Hh. In the absence of Hh, the inhibitor rapidly binds to the intracellular side of the Smo 7TM domain inducing the closed, inactive conformation. Inactivated Smo returns to the cytoplasm (Fig. 3b). In the presence of Hh, the inhibitor concentration decreases. This increases the probability of phosphorylation of the Smo C-terminus, coupling with Gi protein, and Smo activation in cilia (Fig. 3c). Therefore, competition for binding to Smo between Smo inhibitor and Gi protein may regulate the initiation of signaling. By transiting through the cilium Smo could act as a sensor for the presence of Hh.

(b) Smo activation by the SmoM2 mutation

A similar conformational change is induced by the oncogenic Smo mutation W539L (SmoM2) [34] (Fig. 4c). In contrast to WT Smo, overexpressed SmoM2 does not localize to the cell membrane in HEK293 cells. However, a significant increase of Gi-coupled activity in membrane extracts from these cells indicates that SmoM2 can signal through Gi protein [43]. Notably, SmoM2 has strongly reduced sensitivity to inhibition by Ptc and some small molecule antagonists such as cyclopamine, GDC-0499 (Vismodegib), Cur14614, ALLO2, and SANT-3 [39, 51, 68–70]. It is possible that inhibitor transported by Ptc partially shares a binding site with these ligands on the Smo 7TM region, or at least, that W539 is a critical residue for maintaining Smo in a conformation, allowing their

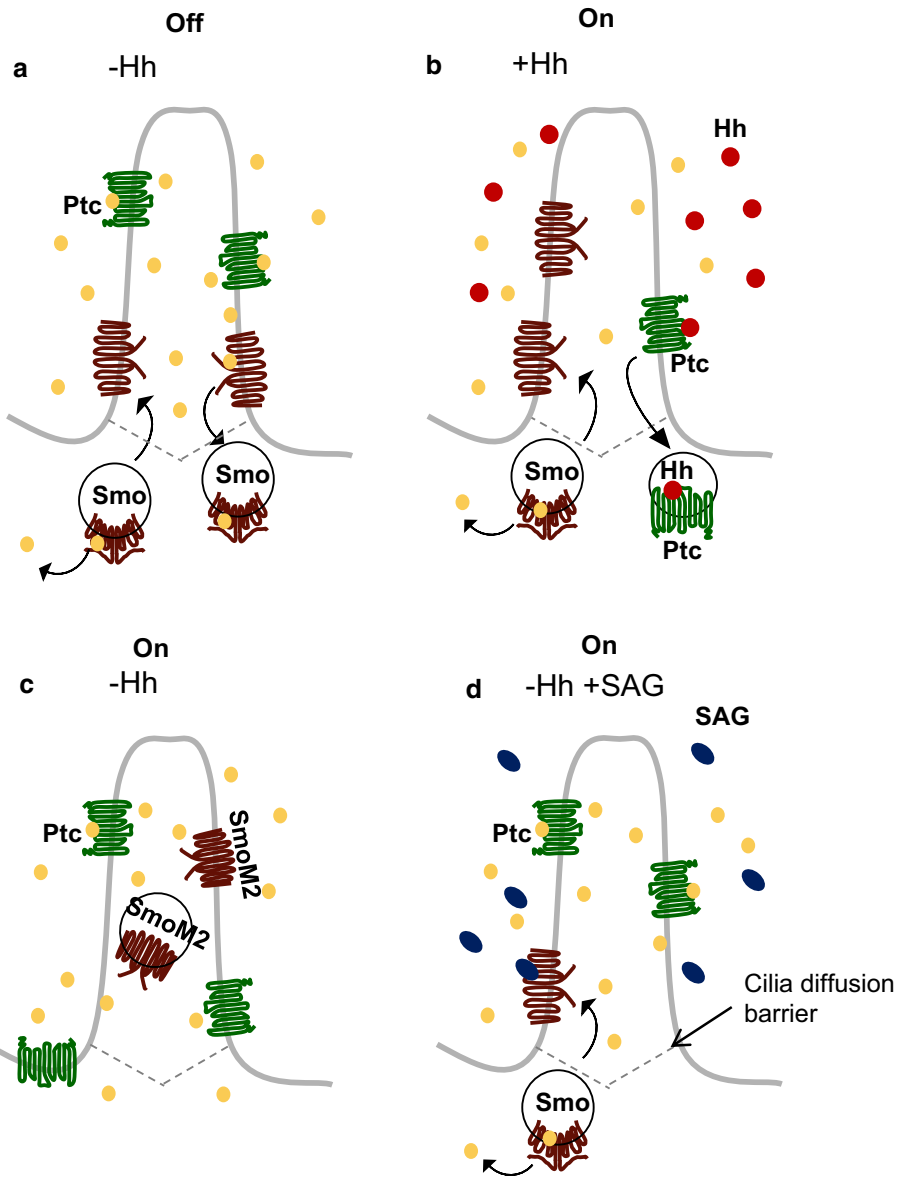

Fig. 4 Three cases of Smo activation. **a** In the absence of Hh, the concentration of the inhibitor transported by Ptc in the cilium is maximal. The inhibitor binds to the intracellular side of the Smo 7TM and induces the inactive Smo conformation. Smo returns to the cytoplasm in intracellular vesicles; **b** Binding of Hh ligand to Ptc and its internalization stops the transport of the Smo inhibitor and its ciliary concentration decreases. This increases the probability of Smo phosphorylation, coupling with Gi protein, and signaling; **c** The oncogenic SmoM2 mutation induces open Smo conformation. SmoM2 is constitutively present in the cilium (probably within vesicles) and active, but is not phosphorylated. Ptc remains on the cilium, but endogenic antagonists have a low affinity for SmoM2. Presumably, SmoM2 is transiently localized to the ciliary membrane, but is rapidly internalized, explaining the capacity of some synthetic antagonists to inhibit SmoM2 activity; **d** The Smo agonist SAG traps Smo in the ciliary membrane in the presence of Ptc

binding. Indeed, all of these molecules lose their affinity to SmoM2. By contrast, a number of antagonists with different chemical structures (ALLO1, SANT-1, SANT2) efficiently reduce SmoM2 activity [51, 70], presumably acting at a site allosteric to the cyclopamine-binding pocket or interacting with different Smo residues.

The open conformation of SmoM2 and WT Smo may be different. Although SmoM2 is constitutively active and present on cilia [9, 43, 69], it is only weakly phosphorylated or not at all [51, 65]. Moreover, sterol depletion using cyclodextrins decreases WT Smo but not SmoM2 activity in *Ptc−/−* mouse fibroblasts, indicating that sterols are required for WT Smo, but not SmoM2, activity [71]. Another interpretation of these results could be that cyclodextrins, which complex with hydrophobic compounds, not only deplete cells of cholesterol, but also of active, highly phosphorylated, and hence, hydrophobic WT Smo. Non-phosphorylated SmoM2 is not a target of cyclodextrins and thus remains active. In the SmoM2 conformation, the C tail is probably inaccessible for phosphorylation, thus preventing β-arrestin anchoring and SmoM2 inactivation. These features, along with the reduced sensitivity of SmoM2 to Ptc inhibition, could explain its constitutive activity. However, genetic sterol depletion inactivates both WT Smo and SmoM2 activity [36, 71]. Probably, sterol deficiency could affect the ciliary membrane properties and/or cilia or vesicular transport, thus preventing Smo targeting to the cilia. In agreement, sterol depletion blocks Hh-induced ciliary Smo accumulation [36].

(c) Smo regulation by small molecules

A conformational switch and Smo activation can also be driven by allosteric agonists [34]. In this case, agonists bind to the extracellular side of Smo 7TM competing with the endogenous inhibitor and trapping Smo in the cilia, even in the presence of active Ptc (Fig. 4d), as has been observed for SAG and activating oxysterols [36, 72]. As opposed to activation by Hh, where Smo is anchored near the base of the cilium, Smo is found throughout the entire organelle [73]. Little, if any, retro-inhibition of signaling by Ptc would then be expected. Similarly, Smo synthetic antagonists compete with endogenous inhibitor and stabilize the inactive Smo conformation, inducing Smo internalization, and preventing its activation by Hh and transport to the cilia. Oxysterols potentiate Hh and SAG-induced Smo activity, presumably favoring the open Smo conformation. That oxysterols do not compete with cyclopamine for binding to Smo [36, 37] suggests that the binding of these allosteric ligands to the 7TM region or CRD induces mutually exclusive Smo conformations: oxysterol binding to CRD renders the 7TM cyclopamine binding pocket inaccessible and vice versa.

If sterols are endogenous Smo inhibitors, the intriguing possibility arises that Hh pathway is regulated by sterol metabolism. According to the "Oxysterol hypothesis", oxysterol production is related to increased cholesterol synthesis. Oxysterols can decrease it in a feedback loop by inhibiting the key cholesterol synthesis enzyme HMG-CoA reductase [74]. Potentiation of Hh-induced Smo activity by oxysterols could thus be a mechanism to assure Hh signaling when Smo inhibitor is in excess.

The activation of Gα protein by GPCRs is accompanied by the release of Gβγ subunits that can activate phosphotidylinositol 3-kinase (PI3K) or protein kinase C (PKC) (Fig. 3). The involvement of these kinases in Hh signaling has been described in LIGHT2 cells [43, 75]. Moreover, PI3K up-regulation was observed in Hh-related cancers, acquired resistance to Smo antagonists [68, 76]. Alternatively, PI3K or PKC could be activated after Smo internalization. Similar to other GPCRs, active Smo is phosphorylated by GRK2, promoting β-arrestin (βarr) recruitment and clathrin-dependent Smo internalization [77]. βarr can also serve as a scaffold for different signaling pathway components, including ERK1/2, PKA and PI3K [78]. Smo can likely switch to G-protein independent and βarr-dependent signaling after internalization, as described for some GPCRs [78]. Further studies are needed to clarify this question.

Thus, Smo activation results from Smo de-repression. Hh, the SmoM2 mutation, or synthetic agonists all induce an open, active Smo conformation, permitting its coupling with Gi protein. Although experimental confirmation that Smo-Gi coupling is required for Hh signaling in vivo is still lacking, the data presented here are in accordance with the idea that Smo functions as a GPCR.

The bell-shaped activity curve

An indirect argument in favor of the hypothesis proposed above comes from the well-known phenomenon that synthetic Smo agonists, such as SAG or purmorphamine, induce a biphasic bell-shaped activity curve: weak agonist concentrations increase Smo activity, but strong concentrations inhibit it [39, 40, 51]. A similar dose–response curve was observed for the human olfactory receptor OR1740 (a canonical GPCR) expressed in cultured cells and its ligand Helional [79]. Olfactory receptor ligands bind to the 7TM region, and according to BRET data, OR1740 is constitutively homodimerized in cells. Authors suggested, that binding of one odorant ligand to the receptor dimer activates it, but two bound odorants (one on each protomer) induce inappropriate dimer conformation and inhibits signaling [79].

It is tempting to apply a similar model to Smo (Fig. 5), taking into account its GPCR structure, constitutive dimerization [34] and binding of synthetic agonists to the 7TM region [33, 38]. Although experimental confirmation remains to be provided, the finding that Smo agonists of different chemical structures produce a bell-shaped curve [39, 40, 51], and that a similar curve is induced by SAG in cultured cells, expressing mouse Smo and Ptc [80], argues in favor of the proposed model. In this context, the mechanism of Smo activation, resulting from the release of

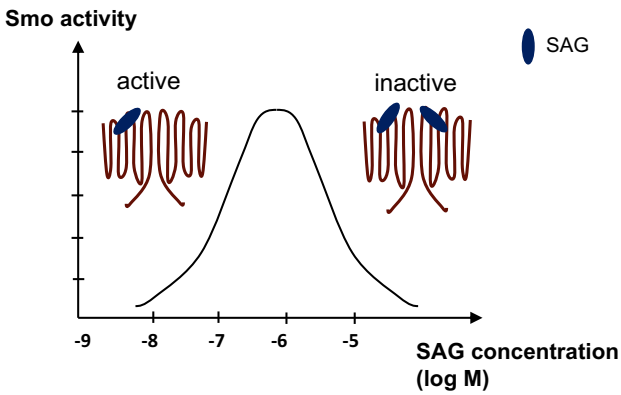

Fig. 5 The *bell-shaped* activity curve. In the presence of small synthetic agonists, the Smo activity curve is *bell-shaped*. We hypothesize that the binding of one agonist molecule to the Smo dimer activates it; but the binding of two agonist molecules to the Smo dimer further changes Smo conformation, blocking either its interaction with Gi protein or its activation and thus blocking Smo activity at high agonist concentrations

its ligand after Ptc inhibition, excludes the possibility of a bell-shaped curve at any Hh concentrations.

Model of Hh pathway regulation by PKA

If Smo acts as a canonical GPCR by inhibiting AC and PKA activity, how does it transduce a signal regulating GliR or GliA production? Formation of GliR and GliA depends on the passage of the Sufu/GliFL complex through the cilium [46, 47, 81]. Interestingly, the stability of both Sufu and Gli is regulated by phosphorylation and proteasomal degradation, but in an opposite manner. In the absence of Hh, PKA and GSK3β phosphorylate Sufu leading to its stabilization [82], whereas phosphorylation of Gli2 and Gli3 by PKA, GSK3β and CK1 lead to their partial proteolysis to GliR by the proteasome [14, 15]. GSK3β absolutely requires priming phosphorylation of its substrates [83]. CK1 preferentially phosphorylates primed substrates, but can also phosphorylate non-primed sites or act as a priming kinase. Thus, CK1 can phosphorylate Gli2 and Gli3 in vitro [14, 15]. PKA, localized to the base of the cilium, primes substrates and regulates GliR/GliA formation [22, 84]. Hh blocks Sufu and Gli phosphorylation and induces their accumulation at the tips of the cilia [14, 15, 82], where GliFL is modified to become GliA and the Sufu/Gli complex dissociates [19, 20]. GliA then enters the nucleus and activates the transcription of its targets genes.

A simple mechanism could be proposed for the regulation of Gli activity by PKA (Fig. 6). In the absence of Hh, both Sufu and Gli in the Sufu/Gli complex are primed by PKA at the base of the cilia. The complex then enters the cilium, where Sufu is further phosphorylated by GSK3β and stabilized. Gli is phosphorylated by CK1 and GSK3β and after the exit of cilium, is converted to GliR by the proteasome. A possible function for phosphorylated Sufu is dicussed below.

In the presence of Hh, active Smo inhibits AC, decreasing PKA activity at the cilium base. The Sufu/Gli complex, not having been phosphorylated by PKA, enters the cilium, where Sufu is not recognised by Gsk3β as its substrate (no priming phosphorylation) and is not phosphorylated. Gli is phosphorylated at several sites by CK1, but not by GSK3β (because there are no CK1-primed sites for GSK3β) and the Sufu/Gli complex dissociates. After exit from the cilium, GliA activates the transcription of target genes in the nucleus. Unphosphorylated Sufu is ubiquitinated and degraded. In summary, the decrease of PKA activity increases the probability that the Sufu/Gli complex enters the cilium, bypassing PKA phosphorylation, leading to the production of GliA, supposed to be CK1-phosphorylated GliFL. This mechanism allows for adjusting the GliR/GliA ratio in a rheostat manner, depending on the level of Smo-regulated PKA activity (Fig. 6). The basal level of GliA is maintained due to the low probability to bypass PKA in the absence of Hh.

In support of this model, a Gli2 protein lacking PKA phosphorylation sites is more stable than WT Gli2, and is constitutively active in mouse embryos [85–87]. Phosphorylated Gli3FL protein was detected in the nucleus of Hh-treated NIH3T3 cells [20]. Finally, CK1 inactivation results in the loss of Gli2A showing that CK1 is required for Gli2A stabilization [88].

In contrast to previous models [2, 22], we suggest that CK1 and Gsk3β phosphorylate Gli in the cilium. Indeed, Hh induces ciliary CK1α accumulation and subsequent phosphorylation of Smo [48]. Sufu recruits Gsk3β for the phosphorylation of Gli3 [89], indicating that Gsk3β is present in cilia. The homology between the Gli2 and Gli3 proteins suggests a similar mechanism for the phosphorylation of Gli2. Thus, cilia are absolutely required for both GliR and GliA formation. Gli accumulation on the tips of the cilia suggests that the phosphorylation of non-primed sites by CK1 is a rate-limiting step in GliA production. In addition, CK1 phosphorylates Smo targeted to the cilium in response to Hh. The increase of Sufu in the cilium would be a consequence of its translocation in the Sufu/Gli complex. Indeed, Sufu is not detected in cilia of Gli-deficient mutants [90].

In the absence of Hh, Sufu/Gli proteins transit through the cilium for GliR formation, but do not accumulate there [46, 47]. Presumably, phosphorylation of substrates primed by PKA is more efficient, and Smo is therefore absent from the cilium. It is also possible, that the passage of the Gli proteins into the cilium is partially restricted by Kif7, a functional vertebrate homolog of *Drosophila* Cos2, kinesin-like protein. Kif7, localized at the base of the cilium, can bind to all three Gli proteins [23, 24, 26] and probably retains them in the absence of signaling. In response to Hh, Kif7 transports the Gli/Sufu complex to the cilium tip and also accumulates there [20]. Kif7, similar to Cos2, has a double role in the Hh pathway. *Kif7*-null mutants display increased basal Hh signaling, but reduced ciliary Gli2 and Gli3 accumulation in response to Hh [24, 25]. Because phosphorylation can regulate kinesins function, it is speculated that Kif7 is also a PKA substrate, and that PKA regulates Kif7 activity or its interaction with IFT proteins and its movement into the cilium. Indeed, the ciliary accumulation of Kif7 depends of active Smo [24] and hence PKA inhibition. In contrast, the PKA activator forskolin (Fsk) blocks the entrance of Gli and Sufu into the cilium [19, 90, 91].

This model suggests a dual role for CK1, required both for GliR and GliA production, but a negative role for PKA in accordance with its inhibitory function in the Hh pathway [92–95]. Interestingly, another GPCR, Gpr161, has the

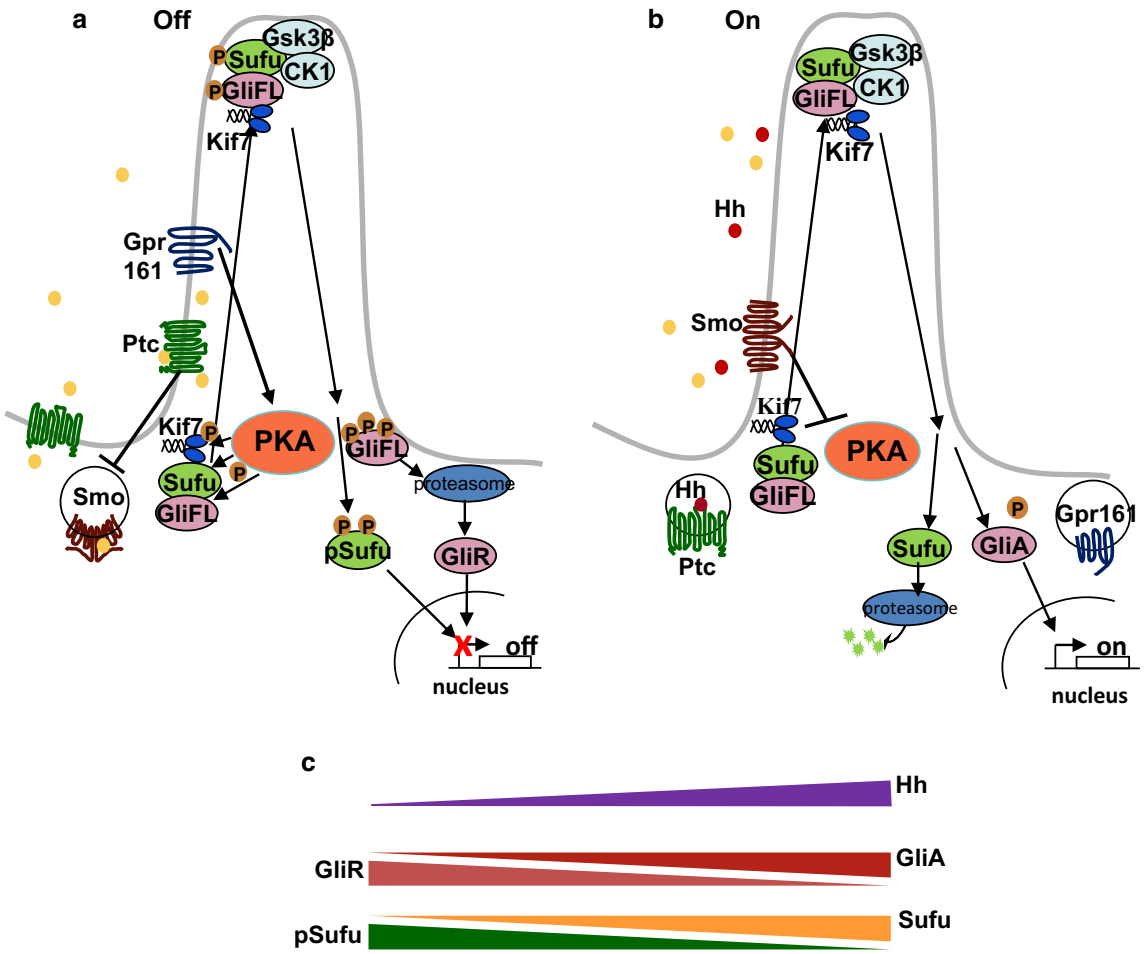

Fig. 6 Hh pathway regulation by PKA. **a** Ptc and GPR161 are present in the cilium. Ptc transports the Smo inhibitor (*yellow circles*) and represses Smo/Gi activity. Gpr161/Gs activates PKA. This double action retains the Hh pathway in the "off" state. Primed by PKA, the Sufu/Gli complex is further phosphorylated by CK1 and GSK3b in the cilium, where the complex dissociates. After the exit of cilium, Gli is partially processed by the proteasome to GliR and both, GliR and pSufu, repress Hh target genes in the nucleus (see text for details). **b** In the presence of Hh (*red circles*), Ptc is internalized, thus relieving Smo inhibition. Gpr161 then exits the cilium, reducing PKA activity. Active Smo enters the cilium and further inhibits PKA through Gi protein. Not having been primed by PKA, the Sufu/Gli complex enters the cilium, where Sufu is not phosphorylated by Gsk3β. GliFL, phosphorylated by CK1, becomes GliA and the complex dissociates. GliA exits the cilium and activates target gene transcription in the nucleus, and Sufu is degraded by the proteasome. The passage of the Sufu/Gli complex into the cilium is partially dependent on Kif7. Phosphorylated by PKA, Kif7 is retained at the ciliary base with the Sufu/Gli complex, but some probability to bypass PKA phosphorylation exists in the absence of ligand (**a**). Non-phosphorylated Kif7 transports the Sufu/Gli complex to the cilium (**b**). In cilia-deficient mutants, Sufu can be phosphorylated at the base of cilia (not shown). **c** Regulation of GliR/GliA and pSufu/Sufu production by increasing Hh concentration

opposite effect on PKA and Hh signaling [21]. In the absence of Hh, Gpr161 is localized to cilia and activates PKA through Gs protein, thus inhibiting the Hh pathway. *Gpr161−/−* mutants display mildly elevated Hh signaling compared to *PKA−/−* mutants [13, 21]. Hh leads to the exit of Ptc and Gpr161 from the cilium, reducing PKA activity. Smo further inhibits PKA activity upon entering the cilium, and the pathway is on. The anchoring of active Smo near the base of the cilium by the Evc/Evc2 proteins [17, 18] could lead to more efficient PKA inhibition.

PKA appears to be the major Hh signaling target (Fig. 6). PKA activity is tightly regulated in an opposite manner by two GPCRs, Gpr161/Gs and Smo/Gi. Phosphorylation by PKA in turn determines activation or degradation of Hh pathway components (Gli2, Gli3, Sufu) and their ciliary transport by Kif7. Moreover, PKA may be a point of cross-talk between Hh and other PKA regulating signaling pathways, as has been described for TGF-β [96] and pituitary adenylate cyclase polypeptide (PACAP) [97]. The central role of PKA in Hh signaling provides a key for understanding why this pathway is dependent on cilia. The cilium is a local compartment. Small changes of PKA activity can be detected in the cilium space and transduced into an adequate GliR/GliA ratio for adjusting a global

cellular response. This makes the cilium a very sensitive and economic organelle. The cilium is also a scaffold to assemble different pathway components at different times. In Drosophila, this role is played by Cos2 and the end of the Smo C-terminus that is absent from the Smo C-terminus of vertebrates [98].

According to the model proposed here, the inhibition of PKA activity by Smo allows for GliA production in cilia. An increase in GliA would be inevitably accompanied by a decrease in GliR and vice versa. The same would be true for the phosphorylated/non-phosphorylated Sufu (pSufu/Sufu) ratio (Fig. 6). Together, GliR/GliA and pSufu/Sufu balances determine Hh pathway activation.

Sufu as a Hh pathway inhibitor

Genetic studies point to Sufu as one of the major negative Hh pathway regulators in vertebrates [54, 99]. Mouse *Sufu−/−* embryos are embryonic lethal by E9,5 exhibiting an open neural tube, similar to *Ptc−/−* and *PKA−/−* mutants [22, 54, 99]. However, the proposed mechanisms for the inhibitory function of Sufu—i.e. the cytoplasmic sequestration of Gli1 and Gli2A and suppression of nuclear GliA activity by recruitment of the SAP18-mSin3 corepressor complex [100–103] seems counterintuitive. They suggest that Sufu inhibits Gli1 and Gli2A, produced in response to Hh, while the inhibition of target genes is required in the absence of ligand.

Importantly, Sufu retains its inhibitory function in cilia-deficient *Kif3−/−* and *Ift88−/−* mutants with strongly reduced GliR and GliA levels [104, 105]. In contrast, Sufu inactivation in these mutants results in high Hh pathway activity [104, 105], which is no further modified by the deletion of *Gli3* [22]. Hence, in cilia-deficient mutants with low GliR and GliA levels, Sufu controls inhibition of the Hh pathway, suggesting that some inhibitory Sufu functions are neither dependent on cilia, nor Gli proteins.

The inhibition of the Hh pathway in *Kif3−/−* mutants correlates with the increased accumulation of the stable, phosphorylated form of Sufu (pSufu) in these cells [104] that probably acts as a repressor. Production of pSufu, as well as Gli3R, requires PKA activity, and mutation of all PKA sites completely abolishes Sufu phosphorylation in vitro [82]. The absence of pSufu in both *Sufu−/−* and *PKA−/−* mutants could explain the similarity of their phenotypes, which is stronger than the *Gli3−/−* mutant phenotype. Accordingly, PKA activation with its agonist Fsk does not repress Hh signaling in *Sufu−/−* mouse embryonic fibroblasts (MEFs) [104], though it efficiently inhibits the Hh pathway in *Ptc−/−* and SmoM2 mutants [69]. Smo antagonists have no effect in *Sufu−/−* MEFs [99], placing Sufu downstream of Smo and PKA in Hh signaling.

Proposed mechanism for the inhibitory function of Sufu

How can Sufu inhibit the Hh pathway? Sufu is a nuclear-cytoplasm shuttling protein [103]. Sufu increases binding of Gli1 and Gli3 proteins to Gli binding sites (GBS) in vitro [100, 101, 106]. It was proposed that simultaneous pSufu and Gli3R binding have a cooperative repressor effect [100]. Moreover, overexpressed Sufu can inhibit Gli1 and Gli2 transcriptional activity independently of Gli sequestration [107]. But, can Sufu act as a repressor without Gli? Recent studies of cis-regulatory modules (CRM) in genes responding to the Hh morphogen (Fig. 7) in the neural tube identified GBSs of different affinity [108, 109]. Thus, the highest affinity GBS was detected in CRM of the most ventral genes *FoxA2*, *Nkx2.2* and *Nkx2.9* [108]. It could be predicted that pSufu recognizes high affinity GBSs in ventral gene promotors and represses them (presumably by recruiting a repressor complex). This prediction is based on a number of studies suggesting the inability of Gli3R to inhibit the transcription of all Hh target genes, and the possible existence of an additional repressor. Indeed, Gli3R overexpression mostly rescued neural tube patterning in a *Sufu−/−* mutant, except for residual *Foxa2* and *Nkx2.2* expression [110]. This situation is mirrored in the *Gli3−/−Smo−/−* mutant where the most ventral progenitors are not specified [111]. But, in *Sufu−/−* embryos, *FoxA2* and *Nkx2.2* are expressed throughout the neural tube [54, 99]. *FoxA2* and *Nkx2.2* expression thus correlates with the absence of Sufu, suggesting that these genes may be specifically inhibited by pSufu.

This also indicates that Gli3R and pSufu have partially overlapping inhibitory functions. Indeed, pSufu represses Hh targets in cilia-deficient cells compensating for a low level of Gli3R [104, 105], while Gli3R overexpression partly rescues the *Sufu−/−* phenotype [110]. In the *Gli3−/−* mutant V0, V1 and V2 dorsal progenitor domains are mixed [111]. It could be suggested, that this phenotype reflects the capacity of pSufu to bind to low affinity GBSs, normally occupied by Gli3R. This binding is unstable, resulting in chaotic repression of target genes and mixed progenitor marker expression. A similar mixture of progenitors is observed in other mutants with reduced Gli3R levels [13, 47]. A comparison of mildly dorsally shifted *Gli3−/−* [111, 112] and completely ventralized *Sufu−/−* mutant phenotypes [54, 99] evidences, that the major Hh pathway repressor is pSufu. I suggest that pSufu, similar to Gli3R, directly inhibits Hh target genes in the nucleus.

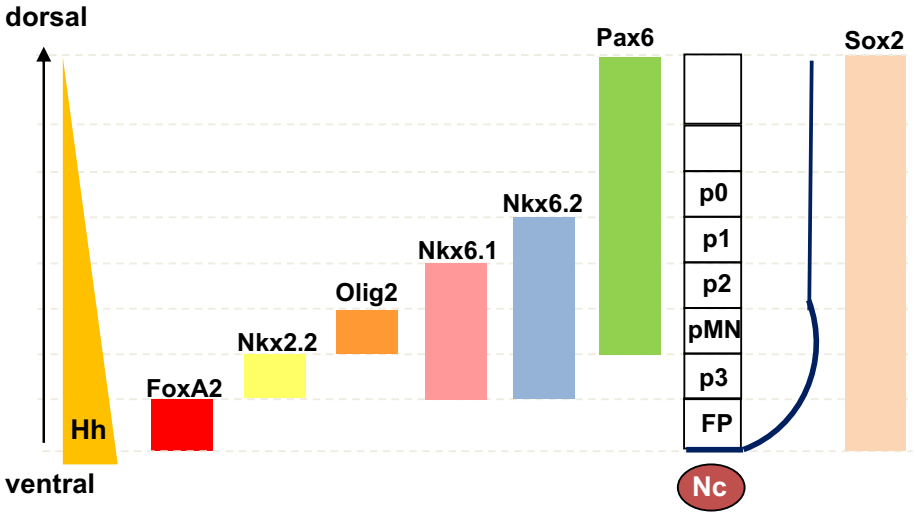

Fig. 7 Neural tube patterning in response to Hh morphogen. In vertebrate development, Hh secreted from the notochord (Nc) and floor plate (FP), forms a ventral to dorsal gradient and functions as a morphogen [31]. Increasing concentrations of Hh induce the expression of different transcription factors (TFs). These include (from ventral to dorsal) FoxA2, Nkx2.2, Olig2, Nkx6.1, Nkx6.2 and Pax6. The combinatorial expression of these TFs defines distinct progenitor domains (floor plate, p3, pMN, p2, p1 and p0 coordinately) and generates different neuronal subtypes. In *Hh−/−* and *Gli2−/−* mutants, ventral neural cell fates are lost. Pan-neural determinant TF Sox2 is expressed throughout the neural tube

Sufu and neural tube patterning

The role of Sufu, that is proposed here, predicts its implication in the regulatory network of neural tube patterning in response to the Hh morphogen (Fig. 7). Sufu contains a PEST domain and is believed to be a protein with a high turnover rate [113]. Expression of *FoxA2* and *Nkx2.2*, the most ventral progenitor markers, requires highest Hh concentrations and sustained pathway activity, during which the GliA level remains constant [114]. It could be suggested, that this time is required for the release of the *FoxA2* and *Nkx2.2* promotors by pSufu and pSufu degradation in conditions where its production is strongly reduced (low PKA activity). Once transcription starts, expression of these genes becomes less sensitive to decreases in the Hh level, due to the presence of excess GliA in the ventral neural tube and the time required to renew the pSufu pool. Feedback regulation of the Hh pathway could also decrease the sensitivity to Hh levels [114, 115].

Presumably, there are three types of genes which respond positively to the Hh morphogen in the ventral neural tube. In the most dorsal part, where the level of GliR is maximal and GliA minimal, only GliR function is required, while the activation of Hh target genes is provided by Sox2, a member of the SoxB1 transcription factors family [108, 109] (Fig. 7). A decrease in the level of GliR would liberate the promotors of these genes, permitting their transcription. In the middle part of the neural tube, transcription is regulated by a competition between GliR and GliA and is sensitive to the GliR/GliA ratio. Multiple GBS's are present in promotors of these genes [108]. Finally, in the most ventral part of the neural tube, the Hh concentration is maximal and the GliR level is low or absent. So inhibition of ventral genes is assured by pSufu, but their expression depends on Gli2A. In accordance, in *Gli2−/−* mutants, *Nkx2* and *FoxA2* genes are not expressed [7, 8], while in *Sufu−/−* embryos they are expressed throughout the neural tube [54, 99]. Further studies are needed to define all pSufu-regulated genes in this complex network.

Regulation of Sufu activity

The phosphorylation status of Sufu seems to determine its interactions with Gli proteins. Co-immunoprecipitation experiments showed that non-phosphorylated Sufu binds to Gli3FL, but not to Gli3R [20, 110] or GliA [19]. Formation of the Sufu/Gli complex stabilises Gli [101, 106, 113] and passage through the cilia would determine the phosphorylation state of both proteins and the production of GliR/pSufu or GliA/Sufu. In both cases Sufu loses its affinity for Gli and the complex dissociates, allowing GliR or GliA to enter the nucleus and act on target gene promotors.

Structural studies have revealed that Sufu adapts a "closed" conformation in complex with Gli and an "opened" conformation in response to Hh [116, 117]. The authors suggested that phosphorylation of Sufu or Gli proteins in cilia could provoke a conformational change,

leading to Sufu/GliA dissociation [117]. The model proposed here is in accordance with these data. Presumably, this is pSufu, which sequesters Gli1 and Gli2A in the cytoplasm, or in the nucleus. This function of pSufu could be interpreted to be the retro-inhibitory mechanism of Hh signaling.

The model presented here suggests that in WT embryos, primed by PKA, Sufu is phosphorylated by GSK3β in cilia; whereas in cilia-deficient mutants, Sufu is phosphorylated at the base of cilia, where GSK3β is also present [2]. Why Gli protein phosphorylation is absolutely cilia-dependent [85] is not clear. Probably, GliFLs are attached to IFT proteins or cytoplasmic microtubules, thus restricting their movement and interactions within the cell. Indeed, overexpressed Gli2 and Gli3 continue to move through the cilia in the absence of Sufu [104], while Sufu is not targeted to the cilia in *Gli−/−* mutants [90]. Specific disruption of cytoplasmic, but not ciliary microtubules in mouse fibroblasts blocks Hh-induced Gli2 accumulation on the cilia. This treatment does not block the ciliary accumulation of Smo indicating that the Gli proteins, but not Smo, require cytoplasmic microtubules to move into cilia [62]. Further studies are needed to clarify this question.

Conclusions

The model presented here opens the way for further understanding of the regulation of the Hh pathway, important in development and diseases. It provides a novel interpretation of published data and, for the first time, proposes molecular mechanisms for the major steps of Hh signal transduction. The importance of the Hh pathway is emphasized by a double security mechanism at different signal transduction levels. In the absence of Hh, inhibition of target genes is assured by Gli3R and pSufu and pathway inactivation—by Gpr161 and Ptc (both favour PKA activation). In the presence of Hh, inactivation of both Gpr161 and Ptc is required for maximal pathway activity.

Mutations in Ptc, Sufu and Smo are associated with Hh-driven cancers [5, 118, 4]. Smo, as many other GPCRs, is a pharmacological target for small molecules, a number of them being in clinical trials [52]. Sufu, as the most downstream Hh pathway inhibitor, may be a valuable target for pharmacological regulation. As such, the model proposed here has implications for new therapeutic strategies. It will be useful for understanding the mechanisms of cross-talk between Hh and other signaling pathways and for the identification of the endogenous Smo inhibitor. One potential candidate is 7-dehydrocholesterol (7-DHC), a cholesterol and vitamin D3 precursor. Mutations in 7-DHC reductase, converting 7-DHC into cholesterol, lead to the human disease Smith-Lemli-Opitz-Syndrome (SLOS) associated with aberrant Hh signaling [119]. This effect may be related to the accumulation of 7-DHC rather than to a decrease in the level of cholesterol. This idea has been previously discussed [120] and is worth revisiting in light of recent data and the model proposed here.

Although Gli-independent repression of Hh target genes by Sufu remains to be demonstrated, its critical nuclear inhibitory role was recently highlighted [122]. The existence of two Hh pathway repressors (Gli3R and pSufu) substantially simplifies the explanation of the action of the Hh morphogen in neural tube patterning and necessitates a revision of the model of graded Hh interpretation. The model presented here thus provides a new framework for understanding Hh target gene regulation in response to the Hh morphogen.

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Compliance with ethical standards

Conflict of interest The author declares no competing or financial interests.

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