Sonic hedgehog in the nervous system: functions, modifications and mechanisms

Karen S Ho and Matthew P Scott*

Signaling by Sonic hedgehog (Shh) controls important developmental processes, including dorsoventral neural tube patterning, neural stem cell proliferation, and neuronal and glial cell survival. Shh signaling involves lipid modifications to Shh itself, as well as changes in protein subcellular localization. Recent advances have revealed the importance of palmitoylation and acylation of Shh on its potency and migration capacity. Subsequent trafficking and organelle sorting in the Shh signaling pathway have been observed; these observations offer a new dimension to our understanding of downstream signal transduction events.

Addresses
Departments of Developmental Biology and Genetics, Howard Hughes Medical Institute, Beckman Center B300, 279 Campus Drive, Stanford University School of Medicine, Stanford, California 94305-5329, USA
*e-mail: scott@cmgm.stanford.edu

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Abbreviations

Disp	Dispatched
Dpp	Decapentaplegic
Hh	Hedgehog
NPC1	Niemann-Pick C1
opb	*open brain*
Ptc	Patched
Shh	Sonic hedgehog
ski	*skinny hedgehog*
sit	*sightless*
Smo	Smoothened
SSD	sterol-sensing domain
TGFβ	transforming growth factor β

Introduction

Sonic hedgehog (Shh), a member of the Hedgehog (Hh) family of secreted signaling proteins, carries out diverse functions during vertebrate development. Humans or mice lacking Shh develop holoprosencephaly and cyclopia due to a failure of separation of the lobes of the forebrain [1,2]. In the neural tube, Shh, secreted from the notochord and later from the floorplate, directs cell fate choices in a dose-dependent manner. Recent work from many groups has greatly expanded the depth and breadth of our understanding of Shh signaling in the nervous system. Here, we first review the advances that further characterize Shh's many roles in neural development, then we turn to a discussion of new insights gleaned from studies of the Shh and Hh signal transduction mechanisms.

The graded activity of Shh in patterning the neural tube was elegantly demonstrated using various concentrations of purified Shh to elicit dose-dependent gene activity in neural tube explants [3]. Shh organizes the developing neural tube by establishing distinct regions of homeodomain transcription factor production along the dorsoventral axis [4•]. These transcription factors, including Nkx, Pax, and Dbx family members, specify neuronal identity (reviewed in [5,6]; Figure 1). By ectopically activating Shh signaling in medial and dorsal cells of the neural tube, two groups [7,8] showed that Shh signaling acts directly on target cells, not through other secreted mediating factors, to specify neural cell fates. Different concentrations of Shh thus cause cells to choose appropriately among many potential cell fates.

Shh also plays important patterning roles elsewhere in the nervous system. In the ventral forebrain, Shh is necessary for the generation of cells of the medial and lateral ganglionic eminences [9]. In the midbrain and hindbrain, Shh is one of the signals necessary to generate dopaminergic and serotonergic neurons (reviewed in [6,10]). Shh and Hh proteins play important roles in retinal and eye development in vertebrates and invertebrates, as reviewed in this issue by Peters [11–16].

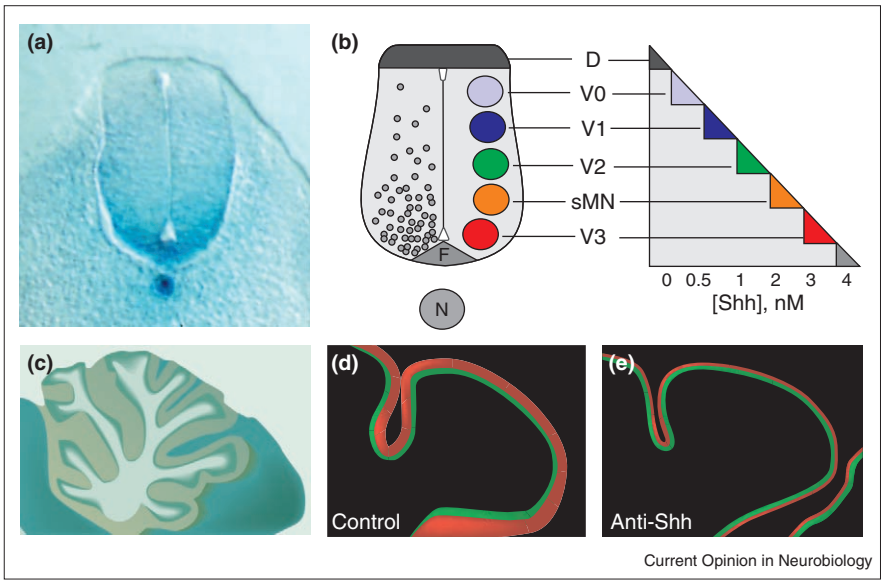
In addition to controlling cell fates, Shh promotes proliferation and inhibits differentiation of neuronal and non-neuronal cell types. One such example is the proliferative response of cerebellar granule cell neurons to Shh [17–19] (Figure 1). Shh also regulates the proliferation and survival of oligodendrocyte precursors [20], and of neural tube and neural crest cells [21,22] (reviewed in [23]). Hh, together with the transforming growth factor β (TGFβ)-family member Decapentaplegic (Dpp), promotes proliferation and motility of subretinal glia in the fly eye [24].

The myriad responses to Shh are achieved, in part, by controlling the production, amount, and biochemical nature of the signal itself. In addition, Hh signal transduction is regulated, so that cells respond appropriately to differing amounts of signal. Recent advances demonstrate how these strategies are employed to bring about proper developmental outcomes. Here, we discuss how covalent modifications of Shh alter its signaling activity, and how receptor trafficking may alter how cells interpret Shh signals.

A *skinny hedgehog* weighs in: the importance of cholesterol and palmitic acid modifications for Hedgehog signaling activity

The 45 kD Hh precursor protein is post-translationally processed. This 22 kD mature Hh signaling protein is formed by an autocatalytic cleavage that removes the carboxyl (C)-terminus. As part of this reaction, a cholesterol moiety is covalently attached to the C-terminal end of the

Figure 1



Two examples of the many functions of Shh in the nervous system. **(a,b)** Shh, secreted from the neural tube and later from the floorplate, patterns neurons along the dorsoventral axis of the neural tube. (a) The graded effect of Shh is illustrated by the gradient of *ptc-lacZ* activity in the neural tube (blue). This mouse embryo is a *ptc*$^{-}$/*ptc*$^{+}$ heterozygote, in which the *ptc*$^{-}$ chromosome was created by knock-in of the *lacZ* ORF, simultaneously disrupting the endogenous *ptc* gene and serving as a transcriptional reporter for Hh activity. (b) The gradient of Shh activity is interpreted by neural tube cells. Shh induces and represses homeobox-containing transcription factors, whose expressions are governed by the level of Shh protein seen by responding cells. These transcription factors go on to specify specific neural fates, such as motor neurons and interneurons, at appropriate positions along the dorsoventral axis. D: dorsal; F: floorplate; sMNs: sensory motor neurons; V0-V3: ventral neurons. **(c–e)** Shh plays a proliferation-inducing role in the cerebellum. (c) A spontaneous cerebellar tumor arising in a *ptc*$^{-}$/*ptc*$^{+}$ mouse embryo due to ectopic activation of Shh target genes. Abnormally high levels of *ptc* expression are shown in blue. (d) Granule cell precursors in the external granule cell layer (red) proliferate under the influence of Shh. (e) Anti-Shh treated cerebella show a reduced thickness of the external granule cell layer due to the ability of the antibodies to block Shh signaling.

22 kD protein [25]. Recent studies have shed light on the biological significance of this unique modification.

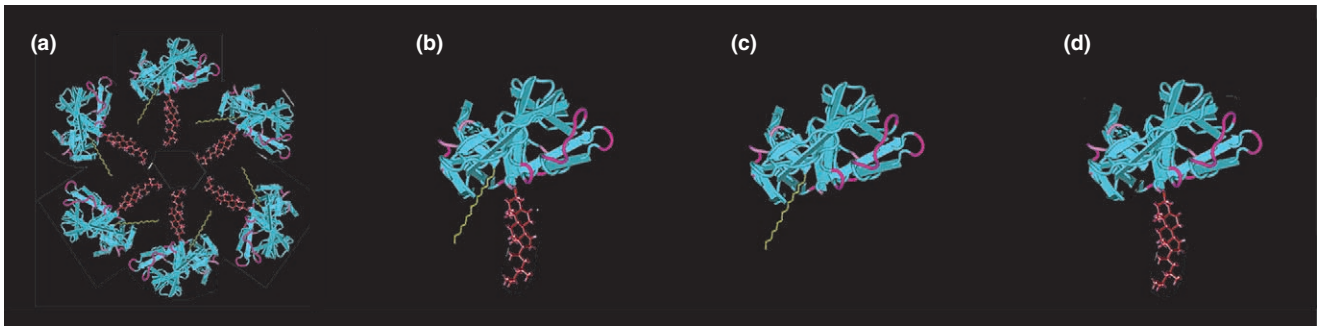
In *Drosophila* and perhaps other animals, cholesterol-modified Hh requires a transmembrane protein, called Dispatched (Disp), for secretion. The sequence of Disp [26] is similar to that of the Hh-binding protein Patched (Ptc), the apparent Hh receptor. Disp is required in Hh-secreting cells, in contrast to Ptc, which is active in receiving cells. Both Disp and Ptc have sequences related to the 'sterol-sensing domain' (SSD) of HMG CoA (3-Hydroxy-3-methylgluatryl coenzyme A) reductase, Niemann-Pick C1 protein (NPC1), and SCAP (sterol regulatory element binding protein), which are all proteins involved in cholesterol metabolism. SSDs are thought to influence the activity or stability of the proteins that contain them, in response to local sterol concentrations. Sterol levels could therefore modulate Disp activity, or Disp may require its SSD in order to recognize and secrete cholesterol-modified Hh.

The significance of cholesterol modification for Hh signaling was, for a time, a mystery, because unmodified Hh produced from an engineered construct was also able to induce known target genes [25]. New studies have shed light on the importance of cholesterol for Hh signaling.

Once Hh is secreted, the shape of the concentration gradient formed in various tissues may depend on the cholesterol moiety. In *Drosophila*, cholesterol-modified Hh travels less distance through the anterior wing disc than does its unmodified, engineered counterpart [25,26]. However, cholesterol-modified Shh in the chick limb bud moves further from its source, the zone of polarizing activity at the posterior end of the bud, than an engineered form of secreted Shh without the cholesterol modification [27]. A new biochemical study shows that a potent, cholesterol-modified form of Shh can diffuse through limb bud tissue [28•]. This soluble form of Shh is in a multimeric complex, as shown by gel filtration and immunoprecipitation of tagged Shh [28•] (Figure 2). Thus, cholesterol modification of Shh may modulate the normal range of its movement through responsive tissues. In certain tissues, cholesterol may prevent the spread of Hh, as in the *Drosophila* wing, whereas in other tissues, such as the chick limb, cholesterol modification of Hh allows it to travel greater distances. The difference may lie in the interaction of Shh or Hh with different extracellular matrix proteins in different tissues [29–34].

Shh is acylated on its amino (N)-terminus, in addition to its C-terminal cholesterol modification, meaning that both ends are covalently linked to strongly hydrophobic groups (Figure 2). The N-terminal lipid is most likely palmitate, but other lipids with different chain lengths have been detected [35]. Two recent studies of a new *Drosophila* mutant underscore the importance of acylation for Hh function. *skinny hedgehog (ski)* [36•] and *sightless (sit)* [37•] are the same *Drosophila* gene. The gene encodes an acyltransferase that is required in Hh-emitting cells. The apparent mass of Hh produced in embryos lacking this acyltransferase comigrates with an engineered form of Hh that cannot be acylated. This 'Hh-light' form has a mass that corresponds to the mass of Hh protein without a palmitoyl group. In *sit/ski* embryos, Hh is produced and secreted normally, and

Figure 2



The engineered and naturally occurring forms of Shh: a comparison of their signaling potencies and range of activity in tissues.
(a–d) Hypothetical models of the forms of Shh and Hh. Palmitic acid (yellow) and cholesterol moieties (red) have been superimposed on the crystal structure of *Drosophila* Hh (blue). Note that the palmitoyl and cholesterol moieties are predicted to be located on the same face of the molecule, despite being attached to opposite termini of the protein. The crystal structure of *Drosophila* Hh was obtained from Cn3D, the 3D structure viewer of NCBI's website. (a) One possible conformation of the soluble hexamer detected by gel filtration. It is potent in the limb bud and can travel several cell diameters from its source. (b) A model of palmitoylated and cholesterol-modified Hh. This form is potent and can be found several cell diameters from its source. In *Drosophila*, however, this form of Hh appears to be tethered to cells closer to its source as compared to Hh without the cholesterol modification. (c) An engineered form of Shh, in which the C-terminal catalytic domain has been deleted, creating a secreted Shh molecule that lacks the cholesterol modification. In comparison with wild-type Shh, this form appears to be less potent in patterning the chick limb because more protein has to be expressed to achieve the same or similar effects. This form is not found as far from its source as cholesterol-modified Shh; in contrast, this form of Hh does spread greater distances than its modified counterpart in the *Drosophila* wing disc. (d) A mutant form of Shh in which the N-terminal Cys is mutated to Ser blocking acylation. Although this form has some activity in patterning the chick limb, it has very little activity in patterning ventral forebrain neurons. In *Drosophila*, this form is devoid of activity and can act as a dominant negative when over-expressed. The lack of acylation does not affect the spread of this protein through tissue.

is detected by antibodies at normal distances from its source [36•]. Nevertheless, loss-of-function *ski/sit* embryos die during late embryogenesis with a phenotype identical to that of *hh* null mutants, indicating the importance of the modification for effective Hh function, and the specificity of the acyltransferase activity to Hh signaling.

To assess the importance of acylation of Shh in signaling in vertebrates and in *Drosophila*, the N-terminus cysteine of Shh and Hh was mutated to serine (C24S) to prevent the addition of palmitate [37•]. Vertebrate or invertebrate mutant proteins were tested for function in chick limbs and *Drosophila* wing imaginal discs. Acylation was necessary for Hh function in fly discs, but not for Shh action in chick limb buds [38]. The limb experiments employed over-expression of Shh, so dose-dependent activity differences might not have been detected. In contrast, palmitoylation does appear to be essential in vertebrate neural tissue. The C24S mutant is severely deficient in ventral forebrain neuron patterning [39•]. Acylation of Hh protein may therefore be important for patterning in some tissues, but less so in others.

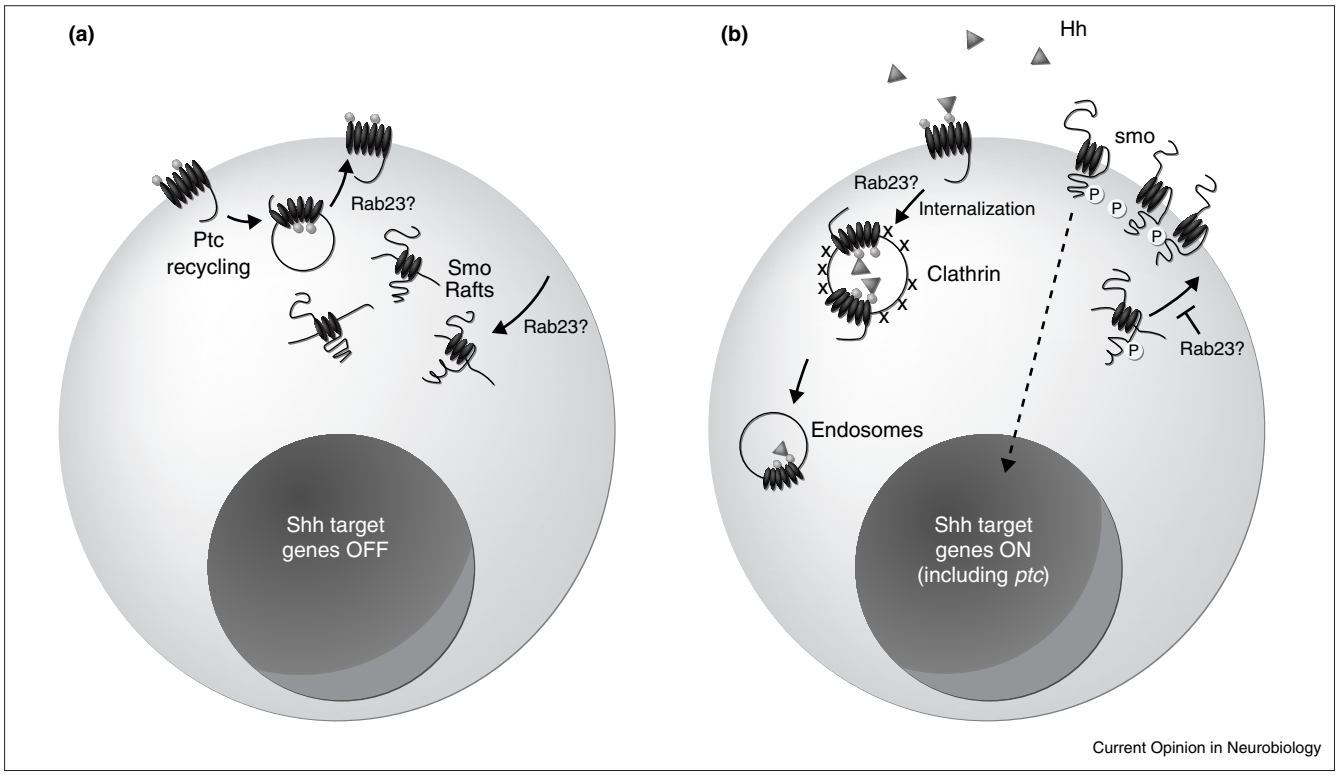
One can imagine how different forms of Shh, such as Shh modified by different fatty acids, could be used by organisms to elicit specific responses from particular tissues. As we have discussed, the presence or absence of a fatty acid modification changes the ability of Shh to signal. Whereas lipid modifications of Shh do not affect the affinity of Shh for Ptc, they do affect the ability of Shh to activate reporter constructs in cultured cells and target genes *in vivo* [27,35,36•,38,39•,40•]. In mammalian cells, dedicated acyltransferases, orthologs of Ski/Sit, could modify Hh in different ways to perform different functions, though this remains to be seen. Cholesterol modification of Shh affects its distribution in neural and other tissues [25,27], and may shape the Shh gradient. Thus, the hydrophobic modifications of Shh have important and complex effects on Shh function.

Intracellular organelle and protein movements in Hedgehog signaling

New and intriguing observations show that subcellular protein movements occur in response to Hh signals. Several lines of evidence indicate that subcellular movements of downstream components of the Hh signaling system are important for signal transduction, both in the developing nervous system and elsewhere. We focus here on new developments involving three components of the Hh signal transduction system: the 12-transmembrane domain Ptc receptor, the seven-pass transmembrane protein Smoothened (Smo), and the small GTPase and organelle trafficking regulator Rab23.

Ptc, a protein that binds Hh with nanomolar affinity, is required for repression of target genes in the absence of Hh. The Hh signal induces target genes by binding and

Figure 3



A highly speculative model depicting the trafficking of Hh, Ptc and Smo, and possible roles for Rab23. Models representing protein localization in **(a)** the absence and **(b)** the presence of Hh. (a) Ptc is located on the plasma membrane and in internal compartments of the cell. Smo is associated with rafts. Hh target genes are OFF in the absence of Hh. (b) In the presence of Hh, Ptc and Hh are rapidly internalized into endosomes. Smo appears at the cell surface. Smo also becomes phosphorylated in response to Hh. Target genes are ON. Rab23 may exert its negative regulatory activity on Hh target genes by: promoting Ptc-containing vesicle trafficking in the absence of Hh; preventing Smo from going to the cell surface in the absence of Hh; aiding in Ptc-dependent internalization, and possible break-down, of Hh.

inactivating Ptc. Inactivation of Ptc function allows Smo to become active, which leads to transcription of downstream genes. When Ptc is inactivated by mutation, inappropriate transcription of Hh target genes results. In humans and in mice, loss of *ptc* function causes medulloblastomas, tumors of the cerebellum, as well as spina bifida, large body size, and other developmental abnormalities, all of which result from inappropriate expression of Shh target genes [41,42] (reviewed in [43]).

In addition to repressing target gene transcription, Ptc regulates the movement of Hh through tissues. Binding of Hh to Ptc limits the spread of Hh from its source. The removal of Ptc genetically from *Drosophila* cells allows Hh to act at a greater than normal distance, implying that Ptc restricts Hh movement [44]. Ptc protein is predicted to have 12 transmembrane domains and two large extracellular loops. A form of Ptc lacking the second extracellular loop is incapable of binding Shh. When the mutant Ptc is produced in the neural tube, an abnormal spread of Shh to more dorsal cells is observed [7]. In addition, ventral neural tube cells that contain the mutant protein take on inappropriate dorsal cell fates, indicating that mutant Ptc can still block Shh signaling, and its signaling function is thus separable from its Hh-sequestering function.

The binding of Shh to Ptc triggers rapid, dynamin-mediated internalization of Shh into endosomes [45•]. Whether Shh is then directed to lysosomes for degradation, or moved to other parts of the cell, is unclear. One intriguing possibility is that Ptc controls the destinations of vesicles that contain Ptc and Hh. Ptc has sequence similarity to the lipid trafficking mediator, NPC1 protein. NPC1 is capable of transporting fatty acids [46], and is required for the normal movements of organelles, such as that of late endosomes [47,48]. Mutations of conserved residues in the Ptc SSD cause mutant Ptc protein, designated Ptc^{SSD}, to accumulate in late endosomes, implying that the SSD is necessary for normal intracellular movements of Ptc [49]. Ptc and NPC1 have been reported to colocalize extensively in Cos1 cultured cells, implying that the trafficking pathway through which Ptc moves may converge to some extent with the pathway traversed by NPC1 [50].

How might the movements of Ptc and Hh be important for Hh signaling? In the case of both the TGFβ family member Dpp and the Wnt family member Wingless, internalization,

controlled movement and degradation of signaling molecules, coupled with their regulated transcytosis and exocytosis, is important in establishing and maintaining morphogen gradients [51•,52•,53•]. During *Drosophila* eye development, Hh protein is transported down photoreceptor axons to cause lamina precursor cells in the brain to divide once [11,13]. Vertebrate neural cells and other tissues may also have mechanisms in place for moving Hh towards target cells. Hh bound to Ptc may move to specific subcellular locations, and this could have an impact on the distribution of Hh in tissues.

In addition to directing the proper intracellular movements of Ptc, the SSD of Ptc is necessary for Ptc to repress Hh target genes. In the presence of Ptc^{SSD}, Smo becomes active, leading to inappropriate activation of target genes. The Ptc^{SSD} protein can still bind Hh, so its sequestration of Hh is intact, despite its loss of ability to regulate downstream events. Mutation of the SSD of Ptc therefore affects two aspects of its function: its ability to traffic appropriately and its ability to regulate Smo activity [49,54]. The next challenge will be to determine how the movement of Ptc is related to the regulation of the activity and localization of Smo.

The seven-transmembrane protein Smo, a relative of the Frizzled proteins that act as Wnt receptors, is required in both vertebrates and invertebrates for Hh signal transduction. Genetic manipulations have shown that Smo acts downstream of Ptc and is a positive regulator of Hh target gene transcription. New findings suggest that Smo activity may be regulated by a Hh-dependent change in its subcellular localization. In an elegant series of experiments, Denef *et al.* [55••] showed that Smo becomes hyperphosphorylated and accumulates rapidly at the cell surface in response to Hh, while Ptc is simultaneously removed from the cell surface.

Smo is associated with lipid rafts, which are implicated in trafficking events [56]. Lipid rafts are biochemically defined fractions of membranes enriched in cholesterol, sphingolipids, and certain proteins such as caveolin. The relationship between Smo localization and Smo activity has yet to be elucidated, but the internalization of Ptc–Hh complexes could permit, or even coordinate, the emergence of Smo at the surface, perhaps borne upon rafts (Figure 3). Movement of Smo to the surface could place it in contact with proteins, lipids, or other molecules that serve to activate it and the signal transduction cascade. In addition, Smo movement may protect it from the activity of a currently unidentified type 2A protein phosphatase. In the absence of Hh, the phosphatase may cause Smo destabilization [55••].

Keeping an *open brain* about Hedgehog

The recent report of a new gene involved in Shh signaling, *open brain* (*opb*), further underscores the importance of lipid trafficking for Hh signaling. The *opb* mutant mouse strikingly resembles a partial loss-of-function *ptc1* mutant mouse, with a ventralized neural tube that fails to close, abnormal cranial and eye development, and polydactyly [42,57••]. The *opb* gene acts cell-autonomously in the dorsal and lateral neural tube, locations where Ptc actively represses Shh target genes [58•]. Chimeric neural tubes composed of both wild-type and *opb* cells were used to show that Shh target genes are ectopically activated in *opb* cells in dorsal and lateral neurons, far from the Shh source.

Because of similarities between the *opb* and *ptc* loss-of-function phenotypes, Eggenschwiler *et al.* [57••,58•] asked whether *opb* acts in the Shh signaling system. Because Shh activates target genes, and *opb* represses them, one way to examine the functional relationship between *shh* and *opb* is to make the double mutant. In *opb*|*shh* double mutants, the downstream targets of Hh signaling are derepressed, as they are in the *opb* single mutant. As target genes are activated in the *opb shh* double mutant, we can conclude that activation of target genes in this genetic background does not depend on functional Shh. Therefore, *opb* is not likely to control the activity, secretion, or transport of Shh. Because *opb* acts cell-autonomously in responding cells, it is most likely to be involved in controlling components of the Shh signal transduction system [57••].

The *opb* gene encodes Rab23, a member of a family of small GTPases that mediate organelle trafficking and fusion events (reviewed in [59]). Because the subcellular location of Smo may be important for its function, a model, in which Rab23 negatively regulates Smo movement to the membrane, has been proposed [60]. An alternative, consistent with the genetics, is that Rab23 positively regulates Ptc activity, perhaps by aiding its movement to the surface, or by blocking its movement to lysosomes for degradation (Figure 3). The formal possibility still exists that Rab23 is not directly involved in Shh signal transduction but, instead, plays a role in a dorsalizing pathway that is antagonized by the Shh signal. Further investigation of these possibilities will be important to our understanding of Shh signaling. Eggenschwiler *et al.* [57••] favor a model in which Rab23 interacts with components of the Shh signal transduction system because the simplest interpretation of the genetic experiments places its activity downstream of *shh* and upstream of Shh target genes. The involvement of a Rab in regulation of Shh target genes further emphasizes how important it will be to track intracellular movements of the members of the Shh signaling components.

Conclusions

Neural cells respond in many ways to Shh signaling, sometimes taking on a specific cell fate and other times dividing. A goal for the future will be to better understand the mechanisms that allow distinctive responses to a rather generic signal. The potency of the signal requires that it be restrained and the inhibition by Rab23, the binding by Ptc, the opposing signal transduction effects of Ptc, and the tethering by lipophilic modifications all contribute to measured and buffered Hh activities. The involvement of *ptc* mutations in at least a substantial fraction of medulloblastoma

cases, an often fatal tumor that usually affects children, provides additional impetus toward obtaining a full understanding of this pathway. The paths to discovery in Hh signaling have benefited from connecting fly development, human disease, mouse genetics, and sterol metabolism. The many lines of evidence for the relevance of protein and perhaps organelle movements, and the links to sterol metabolism, suggest the ancient origins of the Hh signaling pathway in fundamental cell processes. From these origins a new pathway emerged, and it evolved to play a fundamental role in the developing nervous system.

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- • of special interest
- •• of outstanding interest

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