

New players and puzzles in the Hedgehog signaling pathway

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Members of the Hedgehog (Hh) family of signaling proteins control cell fates and proliferation during animal development in part by regulating the transcription of specific genes. Depending on the tissue, Hh can act over long or short distances, to signal directly or by inducing secondary signals. Recent discoveries include new components of the pathway as well as novel regulatory mechanisms involving cholesterol, proteolysis, and the cytoskeleton. The role of Hh in carcinogenesis is underscored by the identification of mutations in several pathway components in skin and brain tumors.

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Abbreviations

BCC	basal cell carcinoma
BCNS	basal cell nevus syndrome
CBP	CREB-binding protein
Ci	Cubitus interruptus
Cos2	Costal 2
Fu	Fused
Hh	Hedgehog
NP-C	Niemann–Pick Type C disease
PKA	protein kinase A
Ptc	Patched
Shh	Sonic hedgehog
Smo	Smoothened

Introduction

In this commentary, we emphasize recent studies that have expanded our knowledge of the molecular mechanisms by which Hedgehog (Hh) signals regulate changes in gene expression. The relevance of this pathway to the study of human cancer has been highlighted by the involvement of some pathway components in several tumors and inherited syndromes (see Table 1). Developmental aspects of Hh signaling will be addressed with an emphasis on the mechanism of signal transduction.

In *Drosophila*, Hh regulates the transcription of specific genes such as members of the Wnt and TGF- β families of signaling proteins. Hh modulates gene expression by opposing the activity of Patched (Ptc), a multiple transmembrane protein [1,2]. In the absence of Hh, Ptc blocks gene expression but, when present, Hh relieves Ptc inhibition to activate transcription [3]. Biochemical studies

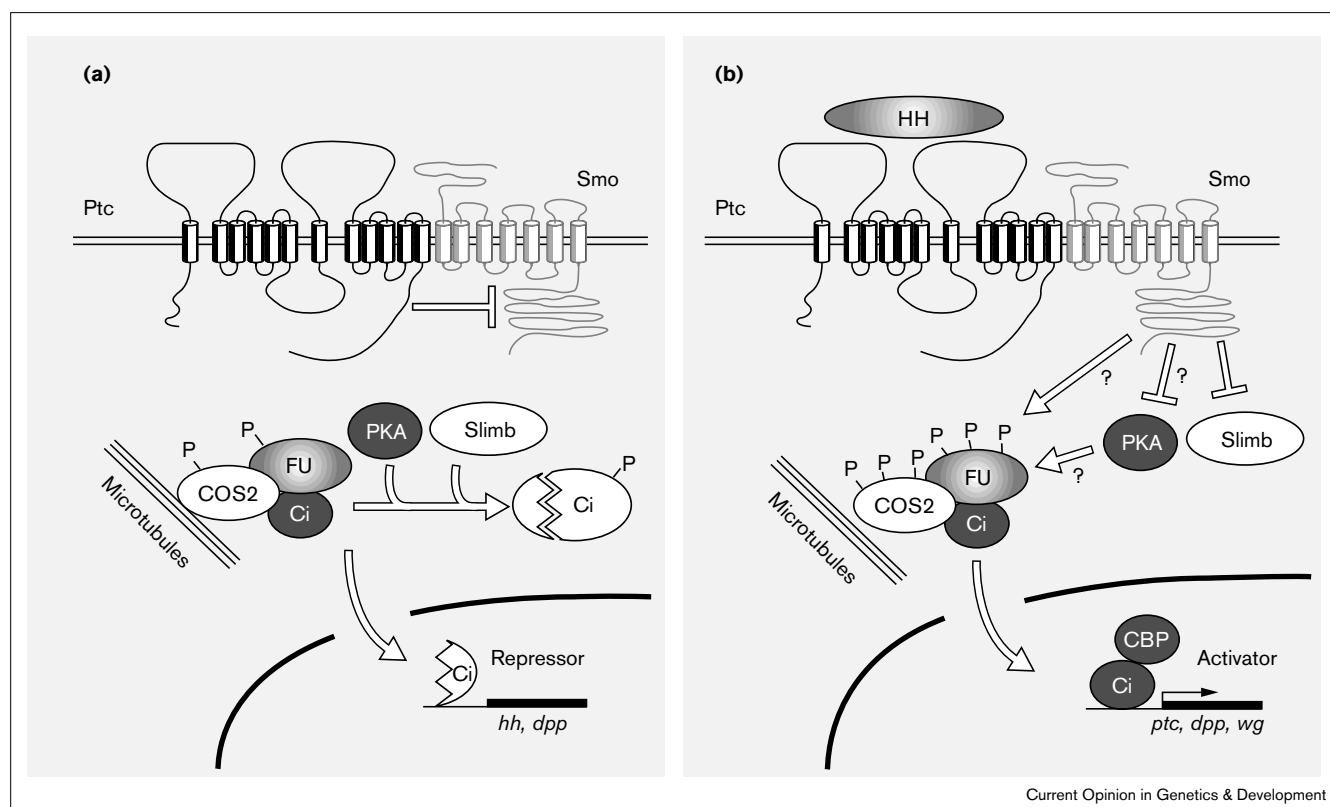
with vertebrate homologs show that Ptc can bind Hh proteins [4,5]. However, genetic data from *Drosophila* have revealed some effects of Hh that are independent of Ptc [6,7], suggesting that other Hh receptors exist. Ptc appears to inhibit signaling by associating with and inactivating Smoothened (Smo) [5], a seven transmembrane domain protein required to relay the Hh signal [8,9]. How signaling is transduced within the cell is not understood but pathway regulation converges on the control of Cubitus interruptus (Ci), a zinc finger transcription factor homologous to the vertebrate family of Gli transcriptional regulators [10]. Ci and Gli bind a similar consensus sequence and mutation of these sites results in loss of Hh activation *in vivo* [11–13]. Recent evidence has revealed a host of unusual molecules and mechanisms that intricately regulate this pathway.

The range of Hedgehog signaling and its control by Patched

In flies and vertebrates, Hh and Sonic hedgehog (Shh) can act either as long or short range signals. Recent experiments using membrane-bound forms suggest that the mechanism and range of signaling varies between tissues. In the developing wing of *Drosophila*, membrane-tethered Hh constitutes just part of the patterning activity of the normal protein, suggesting that Hh diffuses over several cell widths to pattern the wing [14*]. In the chick limb bud, however, membrane-tethered Shh is highly localized and yet has long-distance effects similar to those of a freely diffusible form. In this instance, Shh may be inducing other long-range signals [15*]. The complexity of Hh patterning is revealed in studies of the developing *Drosophila* abdomen, where Hh appears to act directly to specify different cell fates but indirectly to establish polarity by inducing secondary signals [16,17].

A reliable indicator of Hh signaling (Figure 1) is thought to be high-level *ptc* transcription as *ptc* is induced in most tissues in response to Hh proteins [18,19]. The increased Ptc protein made in response to Hh constitutes an important feedback mechanism that limits the range of Hh signaling. In cells where Ptc is absent, Hh protein diffuses further [20] and induces target gene expression at a longer distance [21]. In the mouse, Hh signaling is widely operative throughout early development because, in embryos lacking Ptc1 function, *ptc1* transcription is derepressed in all tissues except the endoderm [22**]. A second *ptc* gene, *ptc2*, has been identified in mice and, in the epidermis, it is regulated differently than *ptc1* [23,24*]. In the developing tooth and follicles, *ptc2* is expressed not in cells that receive the Shh signal, but in cells which produce Shh. Although Ptc1 and Ptc2 share ~60% amino acid identity, they are not well conserved in some regions, such as the carboxy-terminal

Figure 1



A model of Hedgehog signaling. **(a)** In the absence of Hh, Ptc inhibits Smo activity to keep the pathway quiescent. In the cytosol, a protein complex containing Ci, Fu, and Cos2 is attached to microtubules and the negative regulators, Cos2 and PKA, inhibit Fu and Ci. Ci is proteolytically cleaved into a transcriptional repressor presumably through PKA and Slimb activity. **(b)** In the presence of Hh, Ptc is inhibited, allowing Smo activation. Smo presumably causes Cos2 and Fu hyperphosphorylation and dissociation of the complex from microtubules. Ci proteolysis is prevented and an activator form of Ci moves to the nucleus and, in association with CBP, induces gene expression.

domain [24•]. Whether these proteins function differently in regulating Hh signaling is not yet known.

Control of Hedgehog-regulated transcription by cytoplasmic and nuclear proteins

Recent work has shown that Ci can repress and activate target gene expression. Ci has at least three domains of function: an amino-terminal region involved in transcriptional repression [11,25], a zinc finger domain that binds DNA [26]; and a carboxy-terminal region that mediates transcriptional activation [11,25]. In Gli1, the activator domain has been resolved to 18 amino acids and is proposed to form a negatively charged helix similar to that of viral protein 16 [27].

How can Ci behave both as an activator and repressor? Initial studies in *Drosophila* suggested that Hh activates gene expression by elevating Ci protein levels post-transcriptionally [28] but recent work suggests that Hh stabilizes full-length Ci by preventing its cleavage into a repressor form [29••]. In the absence of Hh, full-length Ci (155 kDa) is proteolytically cleaved into a 75 kDa fragment containing the amino-terminal half including the zinc finger domain.

The fate of the carboxy-terminal half of Ci is not known. The 75 kDa cleavage product is found in the nucleus and can repress target gene expression. In the presence of Hh, proteolysis is blocked and the full-length form is stabilized [29••]. The activator form of Ci has not been identified but it may be the full-length protein or an alternatively processed product.

Ci cleavage may be linked to the ubiquitin/proteasome pathway. In cells mutant for *slimb*, Ci cleavage is blocked and Hh-mediated gene expression is induced [30••]. *Slimb* is related to Cdc4p, a component of the yeast ubiquitination pathway, and appears to oppose Hh action by facilitating Ci degradation [30••]. It is not known whether the vertebrate Gli family is also regulated by proteolysis but some evidence suggests that the activator and repressor functions have been delegated to different Gli members [12,27,31,32].

In the cytosol, Hh signaling appears to regulate a multi-component complex that controls Ci localization and/or processing. Biochemical studies in *Drosophila* have identified two proteins associated with Ci: the serine/threonine kinase

Table 1

Components of the Hedgehog signaling pathway.

<i>Drosophila</i> gene	Vertebrate homologs	Function of product	Effect on Hh Target genes	Human disease
<i>hedgehog</i>	<i>Sonic hedgehog</i>	Secreted protein	+	Holoprosencephaly
	<i>Desert hedgehog</i>	Secreted protein	+	
	<i>Indian hedgehog</i>	Secreted protein	+	
<i>patched</i>	<i>patched1</i>	Hh reception	-	Basal cell nevus syndrome, basal cell carcinoma, medulloblastoma, trichoepitheloma [72], meningioma [70], breast carcinoma [70].
	<i>patched2</i>	Hh reception?	?	
<i>smoothened</i>	<i>smoothened</i>	Hh reception	+	Basal cell carcinoma
<i>fused</i>		Serine/threonine kinase	+	
<i>Suppressor of fused</i>		Unknown, PEST motif [73]	-	
<i>Protein kinase A</i>	<i>PKA</i>	Serine/threonine kinase	-/+	
<i>costal 2</i>		Kinesin related protein	-	
<i>cubitus interruptus</i>	<i>Gli1</i>	Transcription factor	+	Glioma [74] Greig cephalopolysyndactyly [75,76], Pallister-Hall syndrome [77]
	<i>Gli2</i>	Transcription factor	+	
	<i>Gli3</i>	Transcription factor	-/+	
<i>CREB-binding protein</i>	<i>CBP</i>	Transcriptional coactivator	+	
<i>slimb</i>		Ubiquitination facilitator	-	

Note: references are added only to subjects which are not referred to in the text.

Fused (Fu) and the kinesin-related protein, Costal2 (Cos2) [33^{**},34^{**}]. Fu is required for the transmission of Hh signaling whereas Cos2 opposes Hh function [35]. Ci, Fu, and Cos2 are associated in a soluble complex, or set of complexes, of ~400–800 kDa in size. Cos2 binds microtubules and appears to tether the complex to the cytoskeleton [33^{**},34^{**}]. Hh signaling apparently releases the complex as in cultured cells, Fused and Cos2 dissociate from microtubules after Hh stimulation. This dissociation may be mediated in part by phosphorylation as both Fu and Cos2 phosphorylation levels increase in the presence of Hh [33^{**},36]. The kinase(s) involved has not been identified but do not appear to be either Fu or PKA. How these findings apply to vertebrates is not yet known but Shh has been found to regulate a serine/threonine phosphatase to induce Gli-dependent and independent gene expression [37^{*}].

In *Drosophila*, protein kinase A (PKA) acts at multiple points to either oppose or augment Hh signaling. PKA appears to oppose Hh in part by regulating Ci cleavage. In cultured cells, Ci proteolysis is blocked and transcriptional activation is increased when the PKA consensus sites in Ci are mutated [38], suggesting that PKA phosphorylation makes Ci susceptible to a protease that converts it into a repressor form. Paradoxically, the activity of this mutated

Ci is further increased by PKA [38]. PKA facilitates Hh signaling in *Drosophila* embryos too because a constitutively active PKA induces Hh target genes [39^{*}]. In this activating role, PKA might be targeting Smo, Ci [39^{*}], or the CREB-binding protein (CBP), a coactivator protein that interacts with the carboxy-terminal region of Ci [40^{*}]. CBP is required for Hh signaling and contains several consensus PKA sites [38,40^{*}].

The convergence of cholesterol metabolism and Hedgehog signaling

The involvement of cholesterol in Hh signaling was first noted in studies of Hh biosynthesis. Within the secreting cell, Hh undergoes an autocatalytic cleavage to attach a cholesterol moiety to the amino-terminal half which anchors it to the cell membrane [41]. Signaling is mediated by the amino-terminal half alone and does not require the cholesterol attachment. The carboxy-terminal half catalyses the cleavage reaction [42] and its structure is similar to self-splicing proteins [43^{*}]. Mutations in the human *hh* gene, *Shh* are responsible for some cases of holoprosencephaly, a developmental disorder that results in craniofacial defects [44–46]. Severe holoprosencephaly resembles the phenotype of *Shh* null mice which have cyclopia and a rudimentary nasal structure [47].

Cholesterol may also regulate Ptc function. Ptc contains domains similar to those of NPC1, a candidate gene for Niemann-Pick Type C disease (NP-C) [48*,49*]. NP-C is a neurovisceral degenerative disease caused by a defect in cholesterol homeostasis (reviewed in [50]). Cultured NP-C fibroblasts are defective in cholesterol trafficking as seen by the accumulation of exogenously provided cholesterol in lysosomes and the delay in movement to the endoplasmic reticulum [51,52]. NPC1 and Ptc are similar in their hydrophobic regions, including a putative sterol-sensing domain, a motif found in several sterol-regulated proteins such as HMG CoA reductase, a key enzyme in cholesterol biosynthesis [48*,49*]. Although NPC1 and Ptc have not been shown to be modulated by sterols, the NPC1 homology implies that Ptc may respond to sterols and be involved in intracellular trafficking. Whether kinesin-related proteins like Cos2 have a role in vesicle trafficking to regulate Hh signaling or cholesterol movement is unknown. Investigation of the cellular localization and molecular function of both NPC1 and Ptc may yield additional insights.

Roles of Hedgehog signaling in carcinogenesis

The Hh signaling pathway figures prominently in the skin tumor, basal cell carcinoma (BCC). Loss-of-function mutations have been identified in a human homolog of *ptc1* in sporadic BCCs and in the basal cell nevus syndrome (BCNS) [53–55]. BCNS (also known as Gorlin's syndrome) is an inherited disorder characterized by large numbers of BCCs and a variety of developmental defects (reviewed in [56]). The mutations identified in BCC and BCNS do not localize to specific regions of the Ptc1 coding sequence but instead are rather dispersed. About three quarters of *ptc1* mutations are predicted to prematurely truncate the protein. In addition, there does not appear to be a correlation between phenotypes of BCNS with specific types of *ptc1* mutations [57,58*].

In *Drosophila*, Hh-regulated genes are derepressed in *ptc* mutant cells [18]. Similarly, in BCCs, genes controlled by Shh (such as *ptc1* and *Gli1*) are transcriptionally derepressed [59*,60,61]. Overexpression of Shh would be expected to overwhelm Ptc activity and mimic a *ptc* mutant phenotype. Consistent with this notion, immune-deficient mice with skin grafts of human or mouse keratinocytes overproducing Shh develop a condition that resembles human BCC [62*,63*]. Mutations in a human homolog of *smo* have been found in several sporadic BCCs and these mutant forms are capable of cellular transformation in conjunction with E1A [64**]. Presumably, mutated Smo is constitutively active and is free from Ptc inhibition. Collectively, these studies suggest that mutations which cause unregulated activation of Hh signaling in the skin can give rise to BCCs.

Mutations in *ptc1* have also been detected in the brain tumor, medulloblastoma. Although most medulloblastomas occur sporadically, ~3% are found in BCNS individuals [65].

ptc1 mutations have been found in sporadic medulloblastomas and, in most cases, both alleles of *ptc1* appear to be mutated [66–70]. In one study, ~20% of 37 independent medulloblastomas contained *ptc1* mutations or were missing one *ptc1* allele [68]. Mice that are heterozygous for *ptc1* have a 30% incidence of medulloblastoma after six months of age, providing a promising mouse model of the disease. In these tumors, *ptc1* transcription is derepressed, suggesting that Ptc1 function is compromised [22**].

How do mutations in the Hh signaling pathway lead to skin and brain tumors? At present, there is little information to address this critical question. In the skin, the cells which are the target of Shh signaling and which give rise to BCC are not known. Whereas little *Shh* or *ptc1* expression is detected in normal human skin [53], high levels of expression are seen in the presumptive follicles of the developing mouse [63*]. Perhaps BCCs arise from cell types involved in follicular growth or regeneration. Medulloblastomas have been proposed to arise from precursor granule cells in the cerebellum [71]. In *ptc1* heterozygous mice, cells that express high *ptc1* levels are frequently detected in the cerebellar region where granule cells arise during development [22**]. It is possible that these cell populations later become medulloblastomas. Studies of BCC and medulloblastoma have been hampered by the inability to culture these tumors *in vitro* and to identify the stem cell population. The involvement of Hh signaling in the formation of these tumors now provides a molecular means for tackling these problems.

Conclusions

Recent investigations of Hh signaling have revealed a complexity and novelty in how these signals are transmitted. The identification of additional components and regulatory mechanisms provides a better understanding of how Hh is transduced. Future work will be directed at identifying and linking the components of this pathway and in discovering how Hh signaling is integrated with other signaling cascades. A particularly fruitful area will be understanding the cell biology of Hh signal transduction; where do the proteins reside in the cell and where does signal transmission occur? Resolving the many perplexing and fascinating aspects of Hh signaling may bring new ideas for cancer therapy and prevention. The continuing rapid progress in this field has bright prospects. If present trends continue, many more surprises await us.

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