

# Hedgehog Signal Transduction: From Flies to Vertebrates

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**The patterning and morphogenesis of multicellular organisms require a complex interplay of inductive signals which control proliferation, growth arrest, and differentiation of different cell types. A number of such signaling molecules have been identified in vertebrates and invertebrates. The molecular dissection of these pathways demonstrated that in vertebrates, mutations or abnormal function of these signaling pathways were often associated with developmental disorders and cancer formation. The Hedgehog (Hh) family of secreted proteins provides a perfect example of such signaling proteins. In the following review, we will not discuss in detail the role of Hh as a morphogen, but rather focus on its signal transduction pathway and its role in various human disorders.** © 1999

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## HEDGEHOG

*Hedgehog (Hh)* was identified originally in *Drosophila melanogaster* in a genetic screen for mutations disrupting the polarity of the segments of the cuticle (segment-polarity genes) [2]. Although a single fly Hh regulates different aspects of *Drosophila* development, seven Hh homologues have been isolated in different vertebrates species [reviewed in 3]. In mammals three Hh homologues have been characterized and named Sonic Hh (Shh), Desert Hh (Dhh), and Indian Hh (Ihh). Shh, the best characterized mammalian Hh, controls patterning of the neural tube [4–10] limbs [11, reviewed in 12], somites [13, 14], gut [15, 16], lungs [17], and hair follicles [18; reviewed in 19]. Shh also participates in regulating left–right asymmetry [reviewed in 20]. Consistent with a pivotal role for Shh during vertebrate embryogenesis, mice lacking both alleles of Shh die at birth, are cyclops, and display severe defects in midline structures, limbs, brain, spinal chord, and axial skeleton [21].

Ihh was found to be expressed in the gut and in prehypertrophic chondrocytes of the cartilage [reviewed in 3], where it regulates bone morphogenesis

[22, 23]. Dhh is expressed mainly but not exclusively in the Sertoli cells of the testis, where its role appears to be critical for germ cell differentiation since Dhh<sup>-/-</sup> male mice are unable to produce mature sperm [24].

Complexity is present at every level of Hh signaling and starts with its biosynthesis. Hh is first synthesized as a precursor which undergoes an autoproteolytic cleavage catalyzed by the C-terminal moiety of the molecule, a domain sharing similarity with the splicing domain of inteins [reviewed in 25]. This cleavage liberates a 19-kDa N-terminal fragment (N-Hh) which possesses all the known signaling activities of Hh and a C-terminal peptide of about 26–28 kDa. During this reaction a cholesterol moiety is added to the C-terminal of the N-Shh fragment [26]. In addition, N-Shh is palmitoylated at its N-terminus. When tested in a cell-based assay for differentiation of fibroblasts into osteoblasts, the lipid-tethered forms of N-Shh were found to be 30-fold more potent than soluble N-Shh, suggesting a key role for these lipid modifications in Hh function [27]. The lipid-tethered N-Shh is thought to be limited in diffusion to a few cell diameter, thereby creating a localized source of inductive signal. In the ventral neural tube, different concentrations of N-Shh induce different cell fates. At high concentrations N-Shh acts as a short-range signal to induce floor plate differentiation, while at low concentrations N-Shh specify motor neurons differentiation, thereby acting as a long-range signal [reviewed in 28]. The mechanism by which N-Hh acts at long range is still unclear and might implicate in part the protein encoded by the *Drosophila tout-velu* (*ttv*) gene and its human homologue *EXT-1*, a gene associated with hereditary multiple exostosis [29]. Ttv is a type II transmembrane (TM) protein, and the genetic analysis of *ttv* in the wing imaginal disc elegantly demonstrated impaired Hh diffusion in the absence of Ttv activity. Nevertheless, the exact mechanism by which Ttv promotes Hh movement still needs to be defined. The analysis of *EXT-1* demonstrated that this latter locates in the endoplasmic reticulum and regulates synthesis of cell surface heparin sulfate glycosaminoglycans, suggesting an indirect mechanism of action for these molecules [30]. Long-range signaling by N-Hh might also depend on some recently described

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cell projections emanating from the receiving cells named cytonemes [31].

### THE Hh RECEPTOR COMPLEX

During this past decade, several components of the Hh pathway have been isolated through genetic screens in *Drosophila*. Two TM proteins candidate receptors for Hh, Patched (Ptc) [32, 33] and Smoothened (Smo) [34, 35], were implicated in reception and transduction of the Hh signal respectively [36].

Ptc is a 12-TM protein with a structure reminiscent of an ion channel. Genetic analysis in *Drosophila* indicates that Ptc is a negative regulator of the pathway required to limit Hh diffusion and to repress induction of Hh target genes [36; reviewed in 37]. Two vertebrate Ptc homologues, Ptc1 and Ptc2, have been identified so far. Both *Drosophila ptc* and vertebrate *Ptc1* transcripts are upregulated in response to Hh, as part of a negative feedback loop aimed at restricting Hh signaling; therefore, *ptc* can be considered a universal marker for activation of the Hh pathway [reviewed in 38]. Biochemical assays in vertebrate systems demonstrated similar high-affinity binding ( $K_d = 0.5\text{--}1\text{ nM}$ ) of all three mammalian Hh to both Ptc1 and Ptc2 [39–41]. However, the more restricted expression pattern of Ptc2 which is present at high levels in spermatocytes raises the possibility that it is more specifically involved in mediating the Dhh signal [41].

Interestingly, Ptc contains a sterol sensing domain (SSD) similar to the one found in the Niemann Pick type C syndrome protein and other cholesterol metabolism enzymes [reviewed in 38]. Several groups have reported that the steroidal alkaloid cyclopamine induces severe holoprosencephaly (HPE) by interfering with Shh signaling. Apparently, this teratogen does not affect the sterol modification that occurs during Shh biosynthesis, but rather seems to prevent Shh signaling in the recipient cell, potentially by interfering with the SSD of Ptc1 [42, 43]. However, direct interaction between cyclopamine and Ptc1 has yet to be demonstrated.

The seven-TM protein Smo was genetically identified as a positive regulator of the pathway. Smo has an overall structure similar to G-protein-coupled receptors (GPCRs) and contains putative protein kinase A (PKA) phosphorylation sites in its C-terminus [34, 35]. Smo does not bind Shh [40], neither does it participate in the binding of Hh to Ptc, as this latter can occur in the absence of Smo [44]. Smo as been proposed to act as the signaling component of the receptor complex and is constitutively active in absence of Ptc [34, 36].

Overexpression of vertebrate Smo in a Shh-responsive cell line which expresses low levels of endogenous Ptc1 (C3H10T1/2; [45]) is sufficient to activate Gli, a transcription factor downstream effector of the pathway (see below). Cotransfection of Ptc1 in this system

represses Smo activity, while addition of recombinant N-Shh abrogates Ptc1-mediated repression of Smo [46]. Ptc1 and Smo form a physical complex when transfected in mammalian cells, suggesting that Ptc represses Smo through direct interaction between these two proteins [40]. It remains to be established whether endogenous Ptc and Smo can form a similar complex *in vivo*. The current model inferred from these data suggests that, upon binding of Hh to Ptc, the repressive effect of Ptc on Smo activity is relieved, allowing Smo to transduce the Hh signal [36, 39, 40, 46]. However, there is high homology between Smo and the Frizzled family of Wnt/Wingless (Wg) receptors, specially in the ligand binding N-terminal extracellular domain (also referred to as cysteine-rich domain) [reviewed in 47]. Therefore, one cannot exclude the existence of a ligand able to directly bind Smo and modulate its activity.

Another cell surface protein able to bind all mammalian Hhs with affinities similar to Ptc1 has been characterized recently in vertebrates and named Hh-interacting protein (Hip) [48]. Hip is a type I TM protein which is expressed in all Hh target tissues, and similarly to *Ptc1*, the *Hip* transcript is upregulated in response to Hh. Hip lacks an intracellular domain and therefore is not capable of transmitting a signal directly. Ectopic expression of Hip in the cartilage of transgenic mice antagonized the action of Ihh, demonstrating that Hip attenuates Hh signaling and like Ptc1 is a negative regulator of the pathway [48].

### FROM THE RECEPTOR TO DOWNSTREAM EFFECTORS: A LESSON FROM THE FLY

The mechanisms by which the Hh signal is transduced from its receptor to the transcriptional activation of Hh-target genes are still poorly characterized. Smo activity requires the third intracellular loop of the protein, a domain typically involved in the coupling of seven-TM receptors to G protein effectors. However, so far there is no evidence that second messengers implicated in GPCRs signaling take part in the Shh response [46].

Again, most of the molecules involved in transmitting the Hh signal downstream of its receptor have been identified by *Drosophila* genetics. Several segment-polarity genes have been found to modulate Hh signaling downstream of Smo by regulating the activity of the Cubitus interruptus (Ci)/Gli family of zinc-finger transcription factors [reviewed in 38, 49]. Ci/Gli family members bind to a 9-bp consensus motif [50] located in the promoter region of Hh-target genes, such as *ptc*, *wg*, and *decapentaplegic (dpp)*, and directly regulate their transcription [51–53]. In the absence of Hh, full-length Ci (Ci-155), which is localized in the cytoplasm, is proteolytically processed into an N-terminal nuclear repressor form (Ci-75) [54]. The cleavage

of Ci is triggered in part by PKA-mediated phosphorylation of the C-terminus of Ci, which contains four PKA phosphorylation sites [55, 56], and potentially by the F-box/WD40 repeat ubiquitin targeting protein Slimb [57]. Consistent with these data, proteasome inhibitors were shown to prevent accumulation of Ci-75, but so far, no Hh-regulated ubiquitination of Ci could be detected [58]. In response to Hh stimulation, the cleavage of Ci is blocked and Ci-155 is activated into a labile and still uncharacterized form [59, 60]. This allows a combinatorial regulation mechanism by which activation of Hh-target genes requires either the elimination of the repressor form, Ci-75 (i.e., *hh*), or the presence of activated Ci-155 (i.e., *ptc*) or both (i.e., *dpp*) (Fig. 1) [60]. Preventing cleavage of Ci-155 by the use of an uncleavable mutant form of the protein is not sufficient to activate Hh-target genes such as *ptc* [60]. The maturation of Ci-155 into a transcriptional activator requires Hh-dependent modifications, and this step is positively regulated by a putative serine threonine/kinase designated Fused (Fu) [61–63], and inhibited by a protein named Suppressor of Fused (Su(*fu*)) [59, 64–66].

Mutant embryos for *fu* or *ci* display a phenotype reminiscent of *hh* loss of function, consistent with positive roles for these two genes in regulating Hh signaling. In contrast, mutant embryos for negative regulators of the Hh pathway, such as *ptc* or *pka*, induce ectopic expression of Hh-target genes [reviewed in 49]. Molecular analysis of Fu revealed that Fu can be subdivided into two domains, an N-terminal serine/threonine kinase domain of about 292 amino acids, and a long C-terminal regulatory region (~580 residues). But kinase activity has yet to be detected *in vitro*, and no substrates for Fu have been identified to date [63, 67]. Mutations affecting the N-terminal catalytic domain of Fu have been classified as type I mutant alleles, mutations disrupting the C-terminus of the protein as class II mutant alleles, and amorphic alleles devoid of Fu protein as class 0. Genetic data demonstrated that although both regions are required for Fu function, they play different roles as revealed by the different phenotypes generated in a *Su(fu)* mutant background [62].

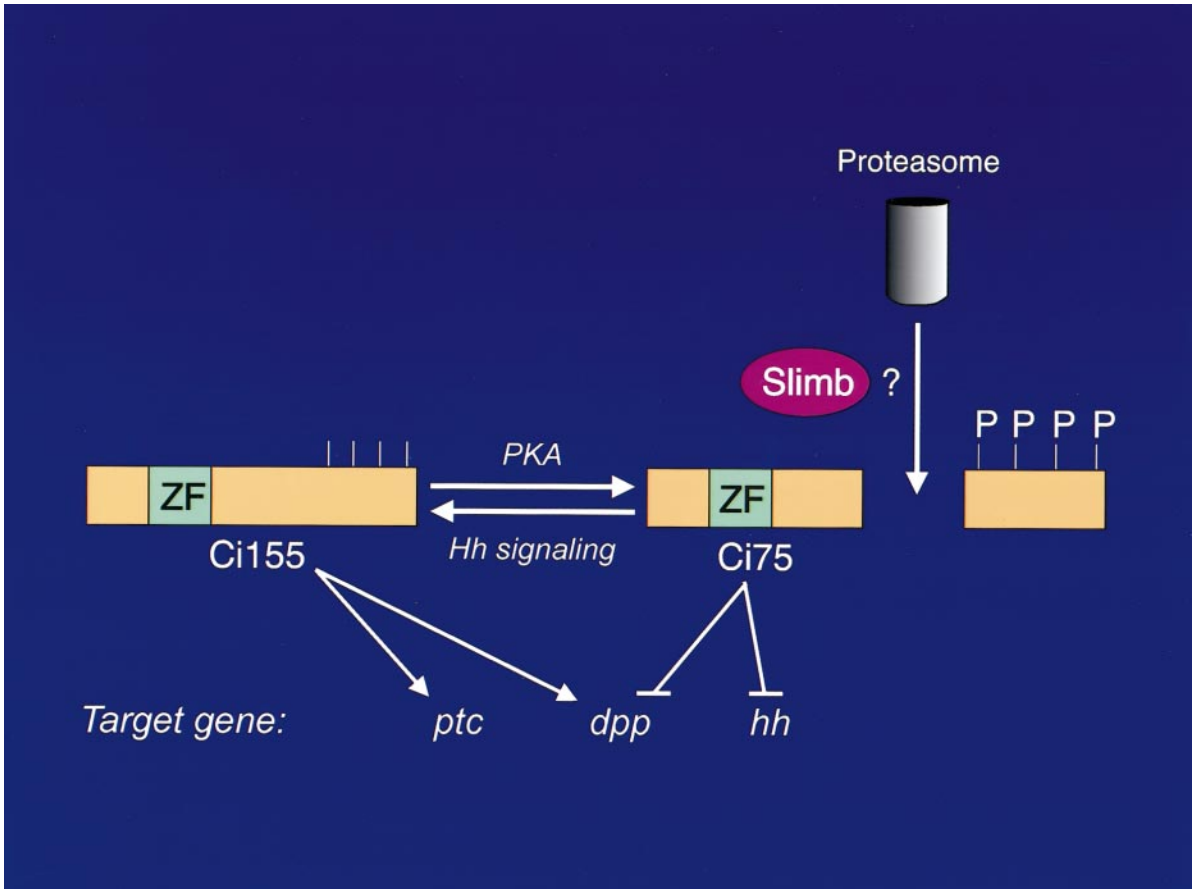
Su(*fu*) appears to negatively regulate the Hh pathway by antagonizing the role of Fu [59, 64, 66, 68]. *Su(fu)* encodes a protein with no homology to any other known proteins [65] and contains a putative PEST sequence, a motif implicated in rapid protein degradation [69]. *Su(fu)* was originally identified as a gene which, when deleted, was able to suppress the embryonic and adult phenotypes of all *fu* mutant alleles. However, combination of type II mutant alleles of *fu* in a *Su(fu)* null background resulted in a *hh* gain of function phenotype similar to mutations in the *costal-2* (*cos-2*) gene (see below), while the combination of type

I or 0 alleles with *Su(fu)* results in a wild-type phenotype [62].

*Su(fu)* mutant flies have a wing phenotype similar to but not as strong as *ptc* or *pka* mutants [59]. Crossing *ptc* or *pka* mutations in a *Su(fu)* mutant background enhances the *ptc* and *pka* mutant phenotype, supporting the idea that these genes cooperate in negatively regulating Hh signaling. PKA negatively regulates the Hh pathway, probably by acting on targets other than Ci-155 as well. For instance, PKA-mediated phosphorylation of the C-terminus of Smo might control its turnover or cellular distribution. In addition, PKA might also impinge on the CREB-binding protein (CBP), which has been identified by yeast two hybrid as a cofactor for Ci. Indeed, CBP binds Ci in a phosphorylation independent manner, and CREB in a phosphorylation-dependent manner, suggesting that binding of CBP to Ci could be competed indirectly by PKA through CREB phosphorylation [70].

Several indications tend to suggest that PKA activity in the Hh pathway might be regulated independently of cAMP. It has been shown in *Drosophila* embryos that cAMP-dependent regulation of PKA activity is not required for Hh signaling [71], and stimulation of C3H10T1/2 with N-Shh does not trigger variations in the level of cAMP [46]. Interestingly, this mechanism is reminiscent of the mechanism through which NF $\kappa$ B-associated PKA catalytic subunit (PKAc) regulates NF $\kappa$ B activation. Recent data have shown that PKAc is in a complex with I $\kappa$ B- $\alpha$  or I $\kappa$ B- $\beta$ , and this interaction maintains PKAc in an inactive state. Signals that trigger degradation of I $\kappa$ B lead to the activation of PKAc in a cAMP-independent manner and subsequent phosphorylation and activation of NF $\kappa$ B p65 [72]. It would therefore be tempting to speculate that, similarly to NF $\kappa$ B, PKA might be part of a multiprotein complex. This complex contains Ci and regulates its activity independently of the concentration of cAMP, but through its interaction with one or multiple components of the complex.

Several additional levels of regulation appear to be involved in modulating Ci activity. These include the control of Ci nuclear shuttling [58], as well as its association with microtubules. The latter is mediated by the kinesin-like molecule Cos-2, which, in absence of Hh signaling, tethers Fu, Ci, and potentially other regulators of the pathway to microtubules (Fig. 2). *Cos-2* is a negative regulator of the pathway as mutations in *cos-2* produce a phenotype similar to constitutive activation of the Hh pathway [73]. Stimulation of insect S2 cells with Hh leads to the dissociation of the Ci/Fu/Cos-2 multiprotein complex from microtubules, to the appearance of complexes heterogeneous in size, and to the phosphorylation of both Fu and Cos-2 (Fig. 2) [67, 73]. The estimated molecular weight of the complex is greater than the arithmetic sum of the molecular weight of the known components (Ci, Fu,



**FIG. 1.** Model of combinatorial gene activation by Ci (adapted from Ref. [60]). Bars on Ci represent the four PKA phosphorylation sites present in the C-terminus moiety of the protein; ZF indicates the region of Ci containing five zinc fingers.

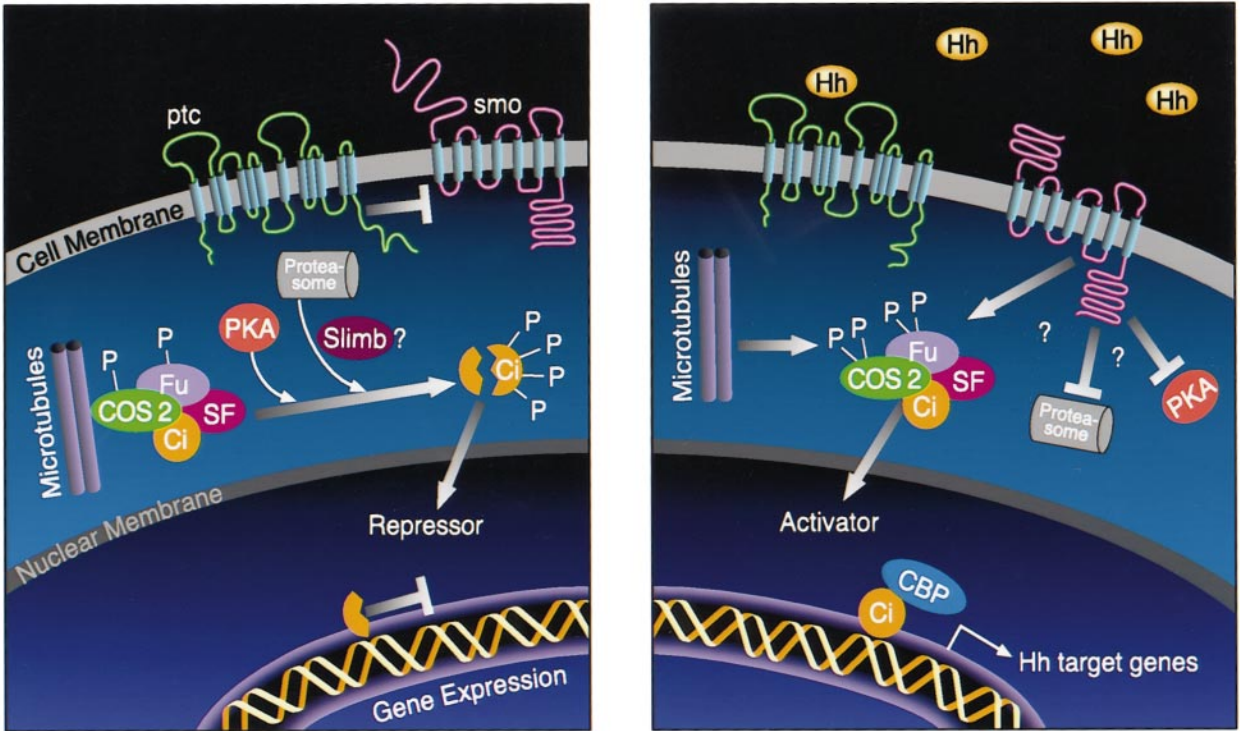
Cos-2), suggesting the presence of additional proteins and/or that some of the known components might be present more than once per complex. Two hybrid and biochemical data have identified Su(fu) as being potentially part of this complex, binding to both Fu and Ci [74]. This complex may also contain a phosphatase whose activity is required for Ci activation [58]. Indeed, it has been shown that a phosphatase pharmacologically related to protein phosphatase 2A (PP2A) is required to prevent processing of Ci-155 in response to Hh [58] and to activate Gli in mammalian cells [75]. Interestingly, immunolocalization data have demonstrated association of pools of PP2A with microtubules [76]. In addition to the exact physical composition of this signaling complex, an important question which remains to be elucidated is how the signal is transmitted from the receptor to the complex upon Hh stimulation and whether this complex can directly interact with components of the receptor.

#### THE ROLE OF VERTEBRATE Glis IN TRANSDUCING THE Hh SIGNAL

Many aspects of the Hh pathway seem to be evolutionarily conserved between flies and vertebrates.

Therefore, the activity of vertebrate Glis is likely to be regulated by a multiprotein complex and other regulatory proteins similar to those identified in *Drosophila*. Consistent with this idea, PKA has long been known as a common negative regulator of the pathway in both flies and vertebrates [77]. Moreover, vertebrate homologues of Su(fu) [78] (Stone *et al.*, in press), Fu (Murone *et al.*, submitted for publication), and Cos-2 (D. Stone, personal communication) were recently identified. Chick Su(fu) was shown to bind to human Gli1 and Gli3 in GST pull-down assays and to *Drosophila* Fu in yeast two hybrid [78]. Although similarities exist between flies and vertebrates, some important differences also exist, as exemplified by the characterization of the three vertebrate Glis identified so far (Gli1, 2, 3). Vertebrate *Glis*, in contrast to *Drosophila ci*, are regulated at the transcriptional level. *Gli1* expression is upregulated by Shh while *Gli3* is repressed [79–82].

The pattern of expression of the three *Gli* genes in the mouse indicates that *Gli1* is expressed close to Shh-secreting cells, while *Gli2* and *Gli3* appear to be more broadly distributed [reviewed in 83], suggesting that they may play a role in other pathways [84]. Functional data suggest that Gli1 and Gli2 might act



**FIG. 2.** Model of Hh signal transduction in *Drosophila*. (Left) In absence of Hh stimulation, Ptc represses the latent activity of Smo and therefore prevents activation of Hh target genes. Ci, in a microtubules-bound complex with Cos-2, Fu, and potentially Su(fu), is processed into a repressor form (Ci-75) by PKA and the proteasome. (Right) When Hh binds to Ptc, it probably induces a change in conformation which allows signaling by Smo. Hh signaling inhibits proteolysis of Ci and triggers dissociation of the Cos-2/Fu/Su(fu)/Ci-155 complex from microtubules. Ci-155 is then converted in a transcriptional activator by Fu, which antagonizes Su(fu) and by association with CBP. The activity of an as yet unidentified phosphatase is also implicated in activating Ci.

as positive mediators, while Gli3 could act, similarly to Ci-75, as a repressor and support the idea that, in vertebrates, the activator and repressor functions of Ci have evolved into different *Gli* genes [53, 80, 81, 85, 86].

Knockout mice carrying disrupted alleles of *Gli1* are viable and have no obvious defects, suggesting overlapping roles for the three mammalian *Gli* genes [85]. Mice lacking both alleles of *Gli2* die at birth and fail to develop a floor plate [85, 86], but develop motoneurons, suggesting that Gli2 might be implicated in transducing the Shh signal where Shh acts at high concentrations. *Gli2*<sup>-/-</sup> embryos also display severe skeletal defects as well as lung, trachea, and esophagus abnormalities [87, 88]. Interestingly, the extra toes mutant mice (*Xt*<sup>l</sup>), which have a large 3' deletion in the *Gli3* gene, display a different subset of skeletal abnormalities and, at the homozygous state, die at birth with severe craniofacial defects [87; reviewed in 83]. The phenotype of *Gli2*<sup>-/-</sup>, *Gli3*<sup>-/-</sup> (*Xt*<sup>l</sup>) double mutant mice demonstrates the synergistic action of these two genes in patterning the embryonic skeleton [87] and the foregut derivatives [88] and indicates overlapping and distinct functions for Gli2 and Gli3.

More recent data now suggest that, similarly to Ci,

Gli family members undergo proteolytic processing. Indeed, C-terminal truncation forms of Gli3 but not Gli1 were detected in transfected 293T cells and mouse embryos, raising the possibility that Gli3 may act both as an activator and as a repressor [89]. In different systems (frog embryos and COS-7 cells) the three Glis produced C-terminally truncated forms, supporting a model where the three Gli proteins may have context-dependent activating and repressing functions [90]. Dissection of the different domains of Glis revealed that the N-terminal region of the protein has dominant negative/repressor activity, while the C-terminal is an activation and cytoplasmic tethering domain, which contains a candidate TAF<sub>II</sub>31-interaction motif in Gli1 and Gli2 and a candidate CBP-interacting domain in Gli2 and Gli3 [90, 91]. In reporter assays, Gli2 is a weak activator when compared to Gli1, while Gli3 is a repressor, but when various constructs carrying truncated N-terminal repression domain were tested, Gli2 and Gli3 are converted in activators; in contrast, little variations of Gli1 transactivation activity are observed. These data suggest that in this system, the N-terminal region of Gli1 somehow lacks repressive activity and that Gli1 is a constitutive activator [91]. Many questions remain to be addressed to further un-

derstand Gli regulation. Do these various cleavage forms of Gli play a relevant role *in vivo*, and does Shh signaling (or another pathway) modulate the switch of Gli2 from a weak to a putative strong activator? Furthermore, the role of Gli may not be limited to transcriptional regulation. It has been shown recently that the *Caenorhabditis elegans* Gli homologue TRA-1 also operates posttranscriptionally by regulating nuclear export of *tra-2* mRNA, suggesting potentially additional roles for Gli family members in controlling gene expression [92].

Not all the vertebrate Hh responses appear to be mediated by the different Gli proteins. A novel Shh-responsive element was identified in the promoter of the *COUPTFII* gene and shown to be upregulated in response to Shh independently of Gli [75]. Very little is known on the signal transduction pathway leading to induction of *COUPTFII*. Similarly to the activation of Ci/Gli pathway [58], *COUPTFII* stimulation requires a phosphatase with pharmacologic properties similar to PP2A [75]. These data suggest that Gli activation may not be the only output of the Shh pathway. Analysis of the chick *talpid*<sup>3</sup> mutation supports a model where the Shh pathway bifurcates for Glis-mediated activation of Shh-target genes and *COUPTFII* [93].

#### HEDGEHOG SIGNALING AND HUMAN DISORDERS

A number of developmental disorders and cancers are associated with mutations in the human counterparts of the Hh pathway, supporting a key role for vertebrate Hh signaling in development and tissue homeostasis. Mutations in one allele of the human *SHH* gene lead to HPE which, in its most severe form, results in cyclopia [94]. This phenotype is reminiscent of Shh-deficient mice where homozygous knockouts develop cyclopia as well as a large number of patterning defects leading to death at birth [21].

The human *Ptc1* gene (*PTCH1*) was first identified as the gene responsible for the basal cell Nevus syndrome (BCNS) or Gorlin's syndrome, an autosomal dominant disorder characterized by a number of developmental defects and by a high incidence of medulloblastomas and basal cell carcinomas (BCCs) of the skin. In the BCCs of BCNS patients the remaining allele of *PTCH1* is lost or mutated, suggesting that *PTCH1* acts as a tumor suppressor. Both copies of the *PTCH1* gene were also found to be mutated in a large portion of sporadic BCCs and other types of sporadic tumors [reviewed in 95]. *Ptc1*<sup>-/-</sup> mice are embryonic lethal [96, 97] and, consistent with its role as a tumor suppressor, *Ptc1*<sup>-/+</sup> mice develop medulloblastomas and rhabdomyosarcomas as well as developmental disorders similar to those observed in BCNS patients. It would be interesting to determine whether *Ptc1*<sup>-/+</sup> mice develop BCC-like lesion upon loss of the remaining allele of *Ptc1* in the skin.

Oncogenic mutations in human *Smo* (*SMO*) were identified in sporadic BCCs and primitive neuroectodermal tumors [98, 99]. Characterization of some of these SMO mutants in tissue culture systems indicated that they are capable of cellular transformation [98] and that their signaling activity is not repressed by coexpression of PTCH1 [46]. Overexpression of one of these SMO mutants in the skin of transgenic mice leads to a phenotype similar to transgenic mice overexpressing Shh in the skin [18], reminiscent of human BCCs [98]. Together these findings support the Ptc-Smo receptor complex model described above, where Smo functions as a Ptc-regulated signaling component, and Ptc as a ligand-regulated repressor of Smo. Mutations leading to a loss of PTCH1 function or gain of SMO function cause unregulated Shh signaling in the skin and hyperproliferation of basal cells.

Mutations in Gli family members also lead to developmental abnormalities or cancer. Example of this is provided by *Gli1* which was first discovered as a gene amplified in glioblastomas [100] and subsequently found to be overexpressed in a large number of BCCs [101]. *Gli1* has also transforming potential when overexpressed in fibroblasts [102]. On the other hand, haploinsufficiency of *Gli3* results in the Greig cephalopolysyndactyly syndrome, while frameshift mutations in *Gli3* which generate truncated version of the protein that still have their zinc-finger region cause the Pallister-Hall syndrome and postaxial polydactyly type A [reviewed in 83]. Some recent work demonstrated that the different mutations in *Gli3* translate into different phenotypes in part by creating repressor forms of Gli3 similar to Ci-75 [103]. Finally, mutations in the gene encoding the Gli-related zinc finger protein ZIC2 were also identified as responsible for HPE [104]. It is likely that additional disorders will be caused by mutations in the Hh pathway and that other tumor suppressors and oncogenes will be uncovered as more components of the human Hh signaling pathway are being discovered.

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