

New “Hogs” in Hedgehog Transport and Signal Reception

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DOI 10.1016/j.cell.2006.04.016

A recent paper in *Cell* (Yao et al., 2006) and two papers in *Developmental Cell* (Tenzen et al., 2006; Zhang et al., 2006) identify a new receptor component for Hedgehog, a key morphogen in embryonic development. Many other proteins that bind to Hedgehog in the extracellular matrix or on the cell surface have been identified. In light of these recent discoveries, we discuss how these factors control the stability, transport, reception, and availability of Hedgehog in modulating Hedgehog-mediated responses.

Introduction

The Hedgehog (Hh) signaling pathway is vital for invertebrate and vertebrate embryonic development, and misregulation of the pathway is responsible for many human congenital defects and cancers (McMahon et al., 2003). Hh acts as a morphogen in diverse tissues and uses a largely conserved signaling apparatus at the cell surface to direct multiple cellular fate decisions. The genetic details of the Hh pathway have been characterized extensively, yet a precise mechanistic understanding of the numerous steps involved in the movement of Hh ligand and the transduction of its signal to the nucleus remains elusive. In addition, the means by which a gradient of lipid-modified Hh morphogen is shaped and translated into distinct transcriptional responses are poorly understood. Recently, several papers have described proteins that are able to bind to Hh either in the extracellular matrix (ECM) or on the cell surface and that play significant roles in transducing and shaping the Hh gradient. Three new papers in *Cell* and *Developmental Cell*

now report that Ihog (interference hedgehog) and Boi in the fruit fly *Drosophila* (Yao et al., 2006) and its mammalian homologs Boc and Cdo (Tenzen et al., 2006; Zhang et al., 2006) are transmembrane proteins that interact with Hh. These findings raise new questions about the physical composition of Hh receptors, and how distinct responses to a single signal could be achieved.

Patched Binds to Hh and Derepresses Smoothed

Patched (Ptc/Ptch) is a twelve-pass transmembrane sterol-sensing domain protein that binds Hh ligands with low nanomolar affinity (Lum and Beachy, 2004). In the absence of Hh, Ptc represses the activity of the seven-pass membrane protein Smoothed (Smo) and prevents activation of the Ci/Gli family of transcription factors (Figure 1). Binding of Hh to Ptc releases Smo from this inhibition. Through mechanisms that remain elusive, this event is communicated to the nucleus where Hh target genes (including *ptc*) are either derepressed or activated by Ci/Gli. After the initial binding event, the Hh-

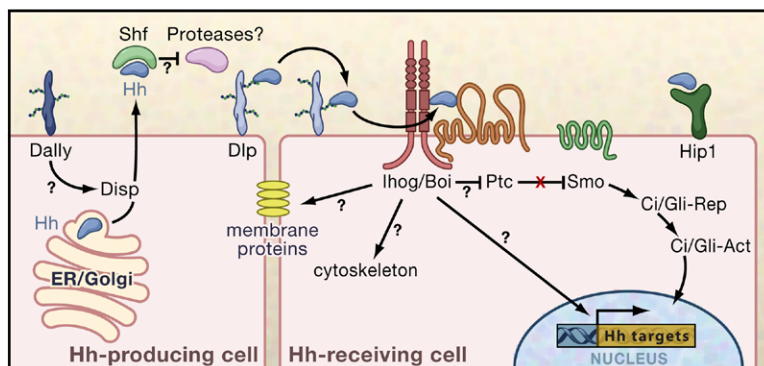


Figure 1. A Model of Hedgehog Release, Transport, and Reception

Release of Hh is mediated by Dispatched (Disp) and is assisted by HSPGs such as Dally and Dlp. After HSPG-facilitated release of Hh from its sites of synthesis, the stability and diffusion of Hh are controlled both by GAG chains of HSPGs and specificity factors such as Shifted (Shf), the fly ortholog of human Wnt Inhibitory Factor-1 (WIF-1) (Glise et al., 2005; Gorfinkiel et al., 2005). In receiving cells, Dlp directs Hh to membrane microdomains containing Hh receptors Ptc and Ihog/Boi. Synergistic binding of Hh to Ptc/Ihog/Boi complexes releases inhibition of Smo, resulting in conversion of Ci/Gli repressors to transcriptional activators and maximal expression of Hh target genes. Binding of Hh to Ptc alone still transduces the signal but at lower levels. The C-terminal tails

of Ihog/Boi convey other signals to cytoskeletal or integral membrane proteins, induce morphological changes in the cell, and signal to the nucleus through unknown mechanisms. Species-specific components such as Shf and Hip1 are utilized to shape the Hh gradient. This series of increasingly specific interactions between Hh and its environment could effectively funnel the ligand to its final destination and prevent inappropriate movement of the ligand outside of the morphogenetic field.

Ptc complex is internalized and targeted to lysosomes, presumably for degradation (Torroja et al., 2005). This, in combination with upregulation of the *ptc* gene, ensures tight control of Hh signaling and likely contributes to shaping the morphogen gradient.

Ptc does not bind directly to Smo, and the mechanism by which Ptc inhibits Smo activity remains unclear. The observation that substoichiometric amounts of Ptc are able to inhibit Smo led to the hypothesis that Ptc may have a catalytic function (Taipale et al., 2002). Experiments examining different ratios of Ptc Δ L2 (which lacks a loop region thought to be involved in Hh binding) and chimeric, constitutively active Hh-Ptc demonstrated that the presence of Hh-Ptc inhibited the activity of unbound Ptc Δ L2 (Casali and Struhl, 2004). These results predict that the ratio of Hh bound to unbound Ptc is a critical determinant of pathway activation and supports a model where inhibition of a multimeric Ptc receptor could be achieved by binding of Hh to only one subunit. Whether a ratiometric mechanism for Ptc function is physiologically relevant requires further investigation.

HSPGs Control Movement, Retention, and Reception of Hh

Heparan sulfate proteoglycans (HSPGs) consist of several families of core proteins covalently linked to large glycosaminoglycan (GAG) chains and are found on the cell surface and in the ECM (Lin, 2004). The enzymes encoded by *tout-velu* (*ttv/Ext1*), *sister-of-tout-velu* (*sotv/Ext2*), and *brother-of-tout-velu* (*botv/Ext3*) are responsible for the addition of alternating glucuronic acid and N-acetylglucosamine residues after initiation of the heparan chain (Lin, 2004). Flies deficient in *ttv*, *sotv*, or *botv* display embryonic phenotypes indicative of perturbed Hh, Wingless (Wg/Wnt), Bone morphogenetic protein (Bmp), and Fibroblast growth factor (Fgf) signaling. In the wing imaginal disc, Hh protein is not able to completely diffuse through *ttv*, *botv*, or *sotv* mutant clones; this is exacerbated in *ttv-ptc* or *ttv-botv* double mutants (Lin, 2004). Loss of Hh protein from the ECM in *ttv*, *sotv*, and *botv* clones may indicate that HSPGs protect Hh from degradative processes. Alternatively, HSPGs may participate in releasing Hh ligand from producing cells (Figure 1).

To date, two core HSPG proteins, the glycosylphosphatidylinositol (GPI)-linked glypicans Dally and Dally-like protein (Dlp), have been shown to function in the movement and reception of the Hh signal in *Drosophila*. Dally and Dlp play redundant roles in facilitating Hh movement from its sites of synthesis (Lin, 2004). In contrast, RNAi of *dlp*, but not *dally*, in clone-8 cells results in a loss of responsiveness to exogenous Hh, but this defect can be rescued by cell-autonomous expression of Hh (Lum et al., 2003). Epistasis tests in vitro and in vivo show that Dlp acts upstream or at the level of Ptc. Conflicting data exist concerning the role of GAG chains in achieving maximal Hh signal transduction (Desbordes and Sanson, 2003; Lum et al., 2003; Callejo et al., 2006).

Although it is apparent that the choice of experimental system (animal versus cell culture) and the method of achieving gene loss of function influence many of the conclusions reached in these studies, the data suggest that both the core protein and GAG chains contribute to reception and transduction of the Hh signal. HSPGs could facilitate Hh ligand presentation to responding cells, concentrate Hh ligand in membrane microdomains proximal to Ptc, or participate as part of a larger receptor complex. Although loss of HSPG core proteins in vertebrates has not been linked with defective Hh signaling, it is unclear if this is a result of functional redundancy resulting from gene duplication or a true divergence in protein function.

Ihog Proteins and the Hh Receptor Complex

The RNAi-based screen in which the requirement of Dlp for cell-autonomous Hh reception was elucidated also identified three other components (Lum et al., 2003). The role of one new component, named *interference hedgehog* (*ihog*), has been recently characterized in depth in fruit fly (Yao et al., 2006). *ihog* encodes a protein with four extracellular Ig-like domains, two extracellular fibronectin type III (FNIII) domains, a transmembrane domain, and a C-terminal tail with no significant homology to other proteins. The *Drosophila* genome contains a related gene, named *brother of ihog* (*boi*). *Ihog* localizes to the cell surface, and both *Ihog* and *Boi* bind Hh through the first FNIII domain. In vivo analysis of *ihog* mutants supports a positive role in Hh signal transduction because *Drosophila* embryos lacking *ihog* maternal germline clones produce embryos that phenocopy hypomorphic *hh* alleles. One reason that the *ihog* mutants do not mimic complete *hh* loss of function may be redundancy with *boi*; testing this hypothesis will require generation of *boi* null alleles. RNAi epistasis tests confirm that *Ihog* functions at the level of Ptc, and on the basis of these studies it appears that *Ihog* is a component of the Hh reception machinery. This is supported by the striking observation that coexpression of Ptc and *Ihog* results in synergistic binding of a HhN-Renilla luciferase fusion protein to the cell surface. Coexpression of *Boi* with Ptc yielded similar results. Thus, it was proposed that *Ihog* family members are new components of the Hh pathway involved in signal reception. Interestingly, although the second FNIII domain does not participate in Hh binding, it is essential for *Ihog* function, as truncated *Ihog* constructs lacking this domain do not restore the loss of Hh response resulting from *ihog* knockdown.

Another unexpected function of *Ihog* is that its overexpression is able to complement loss of Dlp in a dose-dependent fashion. However, the converse is not true. Overexpression of Dlp does not rescue *ihog* knockdown in vitro. Perhaps more surprisingly, coexpression of Ptc and Dlp does not result in a synergistic increase in Hh binding to the cell surface. Therefore, despite the fact that both *Ihog* and Dlp appear to function in receiving the Hh signal, their biochemical functions are unlikely to be

equivalent. The synergism observed between Ihog and Ptc argues in favor of a physical association between these two membrane proteins, but this awaits definitive biochemical proof.

The Ihog family plays a conserved role in Hh signaling across species, as vertebrates have orthologs of Ihog and Boi named Cdo and Boc. Mice lacking *Cdo* exhibit holoprosencephaly (often associated with loss of Shh signaling), with the severity dependent on the genetic background (Cole and Krauss, 2003; Zhang et al., 2006). Direct Hh target genes such as *Ptch1* and *Gli1* are downregulated in mice lacking Cdo, and additional gain- and loss-of function studies in vitro and in vivo support positive roles of Cdo and Boc in Hh-mediated patterning of the spinal cord and forebrain (Tenzen et al., 2006; Zhang et al., 2006). Close examination of neural tube markers, however, showed that overexpression of Cdo or Boc also resulted in a cell-non-autonomous expansion of cells of the dorsal neural tube into ventral regions (Tenzen et al., 2006). This is consistent with idea that Cdo and Boc bind Shh and that they would positively transduce the signal while preventing Shh ligand from traveling further through the morphogenetic field. Indeed, biochemical studies demonstrate that one of the three FNIII repeats of Cdo and Boc is able to bind Shh, in this instance the third domain. This region is distinct from the first FNIII and cytoplasmic domains of Cdo/Boc, which positively promote myogenesis but do not appear essential for Shh signaling (Zhang et al., 2006). It is unknown if the region which binds Shh also participates in myogenesis, and it will be interesting to see if Cdo and Boc provide a direct, physical link between Hh signaling and muscle development.

Surprisingly, Cdo is also able to influence Hh pathway activation downstream of Hh binding, as RNAi of *Cdo* or *Boc* reduces the ability of overexpressed Gli1 and Gli2 to activate the pathway (Zhang et al., 2006). These effects on Gli activity may be indirectly mediated through unknown factors not dedicated to the Hh pathway, as Cdo does not appear to directly modulate the function of the Gli2 repression or transactivation domains. Therefore, in addition to its function at the cell surface, Cdo might affect Gli activity through parallel pathways that regulate transcriptional cofactors that are required for maximal activation of Hh target genes. It is unclear at this point if Ihog or Boi influence Ci activity in a similar fashion. Further dissection of Cdo and Boc function will undoubtedly provide greater insight into the different levels at which they control the Hh response.

Regulation of Cdo and Boc expression would be important in modulating Hh signaling, and it is interesting to note that transcriptional profiling of embryos that are either wild-type, *Smo* deficient, or *Ptch1*-deficient reveals that *Cdo* and *Boc* are downregulated in response to Hh signaling (Tenzen et al., 2006). *Cdo* is transiently expressed in the notochord and floor plate and is strongly expressed in the somites and anterior limb bud, whereas *Boc* is expressed in the dorsal neural tube and limb bud. Loss of *Shh* confirms the dependence of floor

plate *Cdo* expression on Hh signaling, as *Cdo* message is lost. In contrast, the domains of *Cdo* and *Boc* expression in the limb, somites, and dorsal neural tube expand. This complicated control of the expression of Ihog family members is postulated to play key roles in shaping and transducing Shh gradients, with Cdo initially amplifying Shh signaling near the notochord and floor plate despite its subsequent downregulation (Tenzen et al., 2006). In addition, low levels of Cdo and Boc in combination with Ptc may enhance signal transduction at regions distal from Shh sources (Tenzen et al., 2006).

Outlook

Although Ptc is essential for inhibition of Smo activity and binding of Hh, the Hh receptor has not been biochemically defined in vitro, and additional components may participate in the initial step of Hh ligand binding. Ihog and Boi are to date the strongest candidates for coreceptors, based on their synergistic binding of Hh with Ptc and positive transduction of the signal. It has also been hypothesized that Dlp may function as a Hh coreceptor, but the fact that Dlp and Ptc do not synergistically bind Hh argues against this. Furthermore, the FNIII domains of Ihog and Boi are critical both for Hh binding and influencing signal transduction; corresponding domains of similar importance have not been identified in Dlp. Utilization of either HSPGs or other membrane cofactors for signal reception is not unprecedented for major signaling pathways. HSPGs are thought to stabilize dimerization of FGF receptors in response to ligand binding, and the LDL-related protein Arrow may function as a coreceptor with Frizzled proteins to bind Wnt ligands (Dailey et al., 2005). The embryonic phenotypes of *ihog* and *Cdo* mutants resemble partial loss of *hh/Shh*, which would indicate that Ihog/Cdo does not function with Ptc to inhibit Smo activity but is instead involved in counteracting the repressive function of Ptc. Hypothetically, this could be achieved by promoting binding of Hh and inactivating Ptc function. It will be interesting to discover if Ihog/Cdo and Ptc physically interact, to investigate whether Ihog could interact productively or destructively with a Ptc oligomer and to further define the Hh receptor biochemically.

How does the discovery of a potential Hh coreceptor impact our understanding of the pathway? Ihog may alter the affinity of a cell for Hh, change the specificity of signaling, and provide a mechanism for diverse responses to the signal. Clearly, a cell expressing both Ptc and Ihog binds Hh with greater affinity, and this may provide a mechanism for a cell to sense lower ambient concentrations of Hh. Additionally, different receptor combinations could affect interactions with Hh in higher molecular weight complexes, although the in vivo relevance of these complexes remains controversial (Hooper and Scott, 2005; Jia and Jiang, 2006). Participation of Ihog family members in Hh signaling may also influence the types of response generated in a cell. Yao et al. (2006) propose that the C-terminal tails of Ihog and Boi may

integrate the Hh pathway with other signaling cascades. Interestingly, these cytoplasmic domains are the least similar regions of Ihog and Boi and also show little similarity to the corresponding domains of Cdo and Boc. Both the extracellular and intracellular domains of Cdo and Boc mediate their heterodimerization and are able to associate with cadherins and netrins (Zhang et al., 2006, and references therein). The C-terminal domains of Ihog and Boi could act in similar fashion or could possibly influence the activity of transcription factors which cooperate with Ci/Gli. Coupling of Ihog family members to Ptc could therefore provide an elegant method for Hh to execute both short-term morphological changes and longer-term transcriptional programs as well as integrate responses with those of other signaling cascades.

The fact that overexpression of Cdo or Boc results in both a cell-autonomous increase in Shh signaling and a cell-non-autonomous reduction in signaling implies that in addition to positively transducing the Hh signal, Cdo and Boc may also function to restrict movement of Hh ligand. In this regard, Ihog family members appear to share characteristics of both Ptch and negative regulators of ligand movement such as Hip1 (Hooper and Scott, 2005) in the sequestration of Hh ligand. Thus, the function of Ihog proteins is likely to be heavily context dependent and perhaps even tissue specific but could profoundly shape the number of Hh-responding cells and the magnitude of the response. Further confirmation and complete understanding of the dual roles of Ihog proteins are hindered by genetic redundancy, exemplified in mice lacking *Cdo*, which display neural defects but lack overt limb phenotypes (Tenzen et al., 2006; Zhang et al., 2006). This is also apparent in *Drosophila*, where no role for Hh sequestration by Ihog or Boi has yet been shown. Future study of *Ihog-Boi* and *Cdo-Boc* compound mutants will undoubtedly shed light on any potential synergistic and distinct contributions of Ihog family members to Hh reception and/or transport in vivo.

Recent studies of Hh signaling in vertebrates have led to speculation that aspects of the cytoplasmic signaling apparatus may utilize different components or function in a manner distinct from *Drosophila* (Hooper and Scott, 2005; Jia and Jiang, 2006). It is notable that the general function of Ihog family members appears to be conserved across species, in that they promote Hh signaling and appear to function at the cell surface. However, two key differences exist. First, the Hh binding FNIII domains in fly and mouse are not conserved; the significance of this difference in domain architecture remains to be determined. Second, Cdo may exert influence on the Gli proteins through a mechanism distinct from Hh binding and release of Smo inhibition. As mentioned above, it remains to be seen if Ihog/Boi function in a similar fashion, or if this represents another vertebrate innovation in the cytoplasmic signal transduction apparatus. Fur-

ther functional comparison of the Ihog family members from different species, as well as analysis of the different classes of Hh binding proteins, will be essential to a complete understanding of the shaping and reception of the Hh morphogen gradient.

The involvement of HSPGs and the Ihog family in Hh signal transduction raises many more questions than answers. Additional genetic, cell biological, and biochemical studies are needed to tease out the fascinating, intricate, and often unexpected mechanistic details of the powerful Hh signaling pathway.

ACKNOWLEDGMENTS

We thank members of the Chuang laboratory for helpful discussion. We sincerely apologize to authors whose works were not directly cited because of space restrictions. This work was supported by NIH grant HL67822 and NHLBI-funded Program for Genomics Applications ("BayGenomics") HL66600.

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