# Hedgehog Signaling Protocols

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METHODS IN MOLECULAR BIOLOGY™

# Hedgehog Signaling Protocols

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Cover illustration: Background and bottom left inset: Provided by Dr. Sudipto Roy: One day old transgenic zebrafish embryos expressing a GFP reporter in the midline (notochord, floor plate and ventral brain tissues) under the control of the regulatory elements of the *sonic hedgehog* gene. Transgenic strain generated by F. Muller and image captured by S. Roy.

Top left inset: *Shh* expression in the nervous system, limb bud and branchial arches in Stage 21 chick embryo. *See* Chapter 2 by Eva Tiecke and Cheryll Tickle.
Central image of inset: 10 day chick wing with a digit pattern of 432234 after implanting a Shh-soaked bead. *See* Chapter 2 by Eva Tiecke and Cheryll Tickle.
Central upper image of inset: Stage 48 wild type Xenopus embryo. *See* Chapter 3 by Thomas Hollemann, Emmanuel Tadjuidje, Katja Koebernick & Tomas Pieler.
Top right inset: Dorsal head view of rat telencephalon marked for neural explant assay. *See* Chapter 4 by Rina Mady and Jhumku Khotz.
Bottom right inset: is an unpublished one of mine: Drosophila third instar wing imaginal disc stained for Wingless (green) and Cubitus interruptus (red). Provided by Jamila I. Horabin.

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# Preface

When the *Hedgehog* gene was first described by Nusslein-Volhard and Wieschaus in their seminal paper of 1980 (*Nature* **287,** 795), it was one of many, identified because it affected patterning of the *Drosophila* embryo in a very specific way. Almost three decades and many experiments later, we have a good grasp of what their mutants were revealing. The story is not complete, but our understanding of developmental molecules and their mode of action is far beyond that of early embryologists who, with their insightful experiments told of morphogens (substances that regulate cell fates in relation to their concentration), but were without the technology and reagents to reveal them. From the gene collections of Nusslein-Volhard and Wieschaus as well as others who followed in their footsteps, we now know enough to appreciate the complexities of the Hedgehog (Hh)-signaling pathway and its importance in determining appendage and tissue types. Remarkably, the pathway is relatively well conserved across the animal kingdom, and utilized in very synonymous ways. We have also come to recognize that when functioning abnormally, the pathway can lead to various diseases and cancers, highlighting the importance of analyzing and understanding developmental signaling cascades.

This book is intended for Molecular Biologists, Geneticists, and Biochemists interested in manipulating and analyzing the Hh-signaling pathway. In the first half, it covers manipulating the Hh system in vertebrates, followed by a series of chapters describing various molecular and genetic tools available to the *Drosophila* experimentalist. The book winds down with chapters describing some biochemical approaches, done with *Drosophila* cells but the methods should be applicable to other cell types. Last, but not least, a chapter describing how to use sequence analyses to study the evolutionary history and determine functional conservation of Hh expression is included.

We begin with the chapter by Baker, Taylor, and Pepinsky, who describe how to purify the human and rat N-terminal signaling fragments of Sonic Hh (ShhN) from bacterial and insect cells. The ShhN protein is particularly sensitive to metal ion-induced oxidation, and the methods are devised to minimize this oxidation. As Hh is naturally modified in vivo, the authors also describe how to prepare *E. coli*-expressed human ShhN which has been modified at the N-terminus with various fatty acyl moieties.

Chapter 2 by Tiecke and Tickle discusses how to use purified Hh and apply it to chick wing buds to examine the resulting developmental effects. Varying the developmental timing and position of application uncovers different effects of Hh on tissues. To manipulate *Xenopus* development, Hollemann, Tadjuidje, Koebernick, and Pieler describe in Chapter 3 microinjection techniques for mRNA as well as inhibitors of Hh signaling. In Chapter 4, Mady and Kohtz describe a more accurate and reproducible use of rat neural explants to score the effects of Hh, a method which allowed them to demonstrate the induction and differentiation of unspecified neuronal progenitors.

These vertebrate systems provide good proof for the functional conservation of Hh and its homologs. With the Zebrafish genetic analyses can also be performed, and in Chapter 5 Roy describes a rapid and convenient assay that can be used to distinguish effects of loss of function or gain of function genes that affect Hh pathway activation during embryogenesis. To more rapidly analyze the effects of various alterations of the Hh system, cell culture experiments provide one of the best opportunities and in Chapter 6 Kasper, Regl, Eichberger, Frischauf, and Aberger describe how to use retroviral systems to introduce desired changes of Hh components in both dividing and quiescent mammalian cells. Detmer and Garner add to this with their use of flow cytometry in Chapter 7, which when combined with relevant signaling and differentiation markers can be powerful for isolating the population of Hh affected cells one wishes to analyze.

Chapters 8 to 15 describe some of the techniques used by those working with *Drosophila*. Callejo, Quijada, and Guerrero use green fluorescent protein tagged Hh in Chapter 8 to demonstrate in vivo immunocytochemistry techniques which analyze the extracellular distribution and intracellular trafficking of Hh. Chapter 9 by Gallet and Thérond gives an account of using the Confocal microscope to analyze fluorescent protein as well as *in situ* hybridization signals.

Although genetic analysis is the stronghold of *Drosophila*, the power of RNAi as a tool for regulating gene expression cannot be overlooked. Marois and Eaton in Chapter 10 describe a vector for temporally and spatially controlling expression of RNAi substrates in *Drosophila*. The mainstay in manipulating *Drosophila,* however, still relies on genetic analysis and with genes that are lethal, clonal analysis is indispensable. The generation of mutant clones to identify maternally acting genes in Hh signaling is described by Selva and Stronach in Chapter 11, while in Chapter 12 Bankers and Hooper walk you through the considerations for making somatic clones, how to induce them, and how to prepare and analyze the tissues, clones, and phenotypes. Chapter 13 by Busson and Pret is a tour de force compilation of the available GAL4/UAS reagents for the targeted expression of Hh pathway components and their variants. These

allow tests of signaling in different cell types with varied developmental timing, and continue to provide insights into mechanism and function.

The next two chapters go into the realm of Biochemistry, with Stegman and Robbins in Chapter 14 describing biochemical fractionations and how to begin characterizing the proteins in the resulting fractions. Tong and Jiang in Chapter 15 give a detailed account of how to perform immunoprecipitations from cultured cells, imaginal discs, and embryos. Both chapters also introduce culture and transfection procedures for two commonly used *Drosophila* cell lines.

Chapter 16, the closing chapter, by Müller and Borycki reminds us of evolutionary context. They describe the use of sequence alignment to build and analyze phylogenetic trees, and search tools for phylogenetic footprinting and transcription factor-binding sites to characterize *cis*-regulatory elements of developmental genes and *hh*.

Each of the chapters give fairly detailed descriptions, including internet resources where relevant, and pointers for success. For one inexperienced in manipulating the Hh system, they should offer a valuable resource. Additionally, because the techniques are generally utilized, we hope this book will serve the broader function of explaining techniques that, with small modifications, are applicable to other signaling systems.

***Jamila I. Horabin***

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# Overview

## Overview of Hedgehog Signaling

Hedgehog (Hh) is a secreted protein that patterns and specifies cell fate in several different tissues during the development of both vertebrate and invertebrate animals. It generally acts as a morphogen, patterning in a concentration dependent manner (reviewed in ***1–3***). Defects or misregulation of Hh signaling can lead to cancer, diseases and congenital defects such as basal cell carcinomas, holoprosencephaly, cyclopia and skeletal malformations to name a few (for a more complete description *see* ***4,5***).

The active Hh protein is synthesized as a precursor and undergoes autocatalytic cleavage. It is additionally modified at both its amino and carboxy termini by palmitoyl and cholesterol adducts, respectively (**Fig. 1**). These modifications not only alter the activity of Hh, but affect its properties, influencing signal strength and range of effect.

Many of the initial aspects of Hh signaling came to light from work on *Drosophila*, and analyses on vertebrate systems have shown that the pathway is relatively well conserved. Recent discoveries have also uncovered differences. For simplicity, we shall first discuss the system in *Drosophila* and use this framework to highlight some of the divergences in the pathway.

## The Hh Pathway in *Drosophila*

In *Drosophila,* response to Hh is mediated by Cubitus interruptus (Ci), a zinc finger transcription factor with both activator and repressor activities. Depending on the presence or absence of the Hh ligand, Ci is processed into either an activator or repressor. These fates of Ci are controlled by two membrane proteins, Patched (Ptc) a twelve-pass transmembrane protein, and Smoothened (Smo), a seven-pass transmembrane protein (*see* reviews ***2***,***3*** and references therein). In the absence of Hh, Ptc suppresses Smo and this triggers the events that lead to the proteolysis of Ci to its repressor form, a 75 kDa isoform. Hh relieves the Ptc-mediated suppression of Smo preventing the proteolysis of Ci to result in the activator form, the full length 155 kDa isoform.

The processing of Ci is achieved through a complex of Ci with the cytoplasmic components of the pathway, known members of which are Costal-2 (Cos2), Fused (Fu) and Suppressor of Fused [Su(fu)]. Cos2 has sequence similarity to the motor domain of kinesin, Fu appears to be a serine threonine kinase, while Su(fu) shows no homology to any known protein. The Hh cytoplamsic complex is tethered to the Smo cytoplasmic tail by Cos2 ***(6)***.

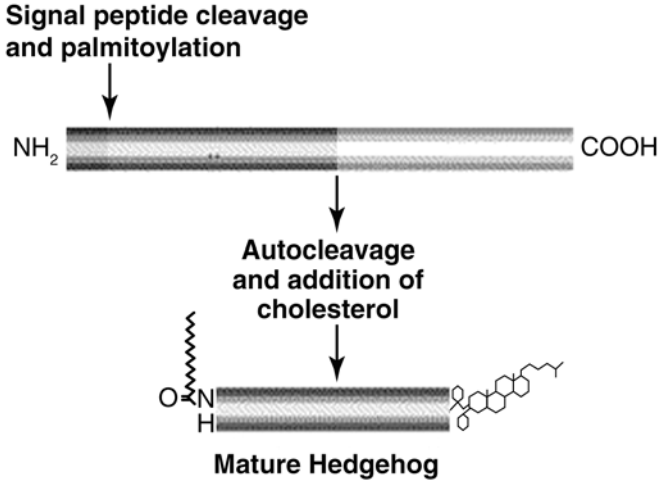


**Fig. 1.** Processing and modification of the Hh ligand. SP at the amino end of the protein represents the Signal Peptide which is removed.

Integral to regulating the processing of Ci is its phosphorylation by Protein kinase A (PKA), as well as Glycogen synthase kinase-3β and Casein kinase I (CKI). These kinases appear to use Cos2 as a scaffold ***(7)*** and promote the processing of Ci to the 75 kDa repressor, with the activity of the F-box protein, Slimb, a component of the SCF ubiquitin ligase complex. Hh reduces the phosphorylation of Ci by PKA which prevents its proteolysis, releases and activates it to result in full length Ci in the nucleus. Hh also promotes phosphorylation of Smo by PKA and CKI, activating it and increasing its levels at the plasma membrane **(Fig. 2)**.

Depending on the level of Ci activation, different downstream Hh targets are turned on. These include *wingless, decapentaplegic* and *ptc,* potent molecules which themselves direct cell fates and developmental processes. The upregulation of Ptc is one of many intriguing aspects of the effects of Hh, because Ptc binds to and limits the spreading of Hh. Elevating Ptc levels results in Hh shaping its own gradient and activity.

Other proteins also modulate the stability and spreading of Hh. Dispatched, a protein with homology to Ptc, appears to be dedicated to the release of Hh from secreting cells ***(8)***. The heparin sulfate proteoglycans (HSPGs), molecules which cover the cell surface, affect the spreading of Hh from producing cells as well as its receptor binding in receiving cells (reviewed in ***9***). The Wnt inhibitory factor-1 protein (WIF-1) also appears to regulate the specificity of Hh binding ***(10)***.

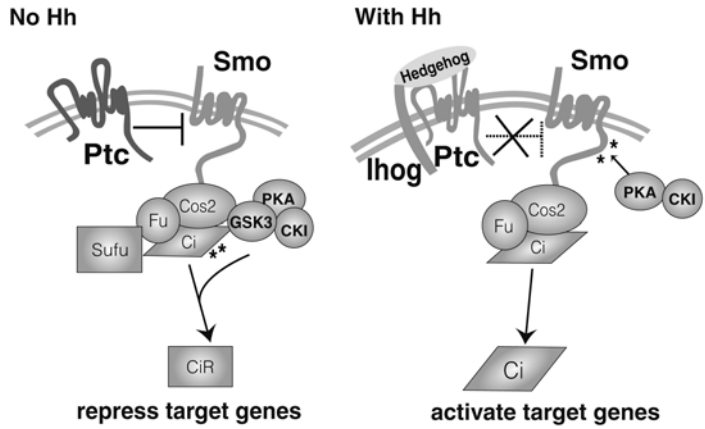


**Fig. 2.** Simplified depiction of Hh signaling. Without Hh, Ptc represses Smo and the Hh cytoplasmic complex in conjunction with the kinases, PKA, CKI and GSK3 which phosphorylate Ci (asterisks), lead to formation of the repressor form (CiR). Hh binds to Ptc and its co-receptor Ihog (and Boi, not shown) to relieve the inhibition of Smo by Ptc. This prevents the proteolysis of Ci which translocates to the nucleus and, with the transcriptional co-activator CREB, activates transcription. Smo is phosphorylated (asterisks) in the process. See text for additional details and abbreviations.

Completing the complex network of proteins that support both generation of the Hh ligand and its reception in receiving cells, are the recently described Ptc co-receptors, Ihog (interference hedgehog) and Boi (brother of Ihog). These transmembrane proteins bind to Hh and influence the ability of receiving cells to transduce the signal ***(11)***.

## Conservation and Divergences of the Hedgehog Pathway

Vertebrate orthologs of almost all the *Drosophila* Hh signaling proteins have been identified, including those that mature the ligand. In many cases they function similarly, but there frequently are multiple forms with each contributing differentially in their respective roles e.g. mammals have three Hh forms: Sonic (Shh), Indian and Desert, but Shh has the greatest scope of activity. The Ci counterpart is represented by three forms of Gli protein, Gli1, Gli2 and Gli3. The latter two are the primary targets of Hh signaling. In the absence of Hh, Gli3 is processed and appears to provide most of the repressor activity, the counterpart of Ci75 in *Drosophila*. The presence of Hh prevents the processing of Gli3 to the repressor form and activates Gli2 to provide the transcription activator function reminiscent of *Drosophila* Ci155 (*see* reviews ***2,12,13*** and references therein).

Molecules such as Dispatched, the Ptc co-receptors, Ihog and Boi (Boc and Cdo; ***14***,***15***), and WIF-1 (Shifted) also have their vertebrate counterparts suggesting that many of the steps up to the binding of Hh to its receptor, including the effects of the HSPGs, are conserved. Mammals also have additional Hh binding proteins, such as Hip1 (Hh interacting protein), that bind to secreted Hh and shape the gradient, for which a *Drosophila* counterpart is not known.

The major difference, however, appears to involve Smo and the events downstream of the membrane components, with respect to the role of Cos2, the Hh cytoplasmic complex and the activation of Gli. The closest mammalian orthologs to *Drosophila* Cos2, Kif27 and Kif7 (kinesin family), do not affect Shh signaling ***(16)***. Rather, it is Su(fu) that plays a role more akin to Cos2, unlike in *Drosophila* where Su(fu) is essentially dispensable ***(17)***. Consistent with this functional difference, the tail of *Drosophila* Smo, where Cos2 binds, is much longer and only the residues near the last membrane spanning region are conserved between the vertebrate and fly counterparts. Note that in the Zebrafish, Kif7 a Cos2 like protein, is functionally similar to the *Drosophila* protein ***(18)***.

This difference in Smo and the utilization of Cos2 may be due to the reliance on cilia and their intraflagellar transport (IFT) proteins for mammalian Hh signaling (***19*** and references cited). Recent data suggest that in the presence of Hh, Smo is transported to the tip of the cilium where it activates Gli2 and prevents the processing of Gli3 to its repressor form. Gli2 is then subsequently transported down the cilium and then to the nucleus where it activates Hh targets. In the absence of Hh, Ptc appears to inhibit Smo from entering the cilium so inhibiting the activation process; an effect that is in principle similar to *Drosophila* but mechanistically different. Many vertebrate cells that respond to Hh signaling have cilia, suggesting this may be the normal setting.

Mammals also use the G-protein-coupled receptor kinase 2 to phosphorylate the Smo tail on activation of the pathway and β-arrestin 2, which binds to phosphorylated Smo, to activate the Hh signal. Intriguingly, Zebrafish with a Cos2-like counterpart and reliance on a β-arrestin 2, would appear to have a Hh signaling system that is intermediate between flies and mammals. There are no data indicating whether or not Zebrafish require IFT proteins and cilia for Hh signaling.

The conservation and differences in Hh signaling highlight the need for further analyses. We hope the following chapters facilitate the process and bring out more of the fascinating twists and turns, still to be appreciated.

## References

1. McMahon, A. P., Ingham, P. W., and Tabin, C. J. (2003) Developmental roles and clinical significance of hedgehog signaling. *Curr. Topics Dev. Biol.* **53,** 1–114.
2. Hooper, J. E. and Scott, M. P. (2005) Communicating with Hedgehogs. *Nature Rev. Mol. Cell Biol.* **6,** 306–317.

3. Torroja, C., Gorfinkiel, N., and Guerrero, I. (2005) Mechanisms of Hedgehog gradient formation and interpretation. *J. Neurobiol*. **64,** 334–356.
4. Ingham, P. W. and McMahon, A. P. (2001) Hedgehog signaling in animal development: paradigms and principles. *Genes & Dev*. **15,** 3059–3087.
5. Pasca di Magliano, M., and Hebrok, M. (2003) Hedgehog signalling in cancer formation and maintenance. *Nature Rev. Cancer* **3,** 903–911.
6. Ogden, S. K., Ascano, M. Jr., Stegman, M. A., Suber, L. M., Hooper, J. E., and Robbins, D. J. (2003) Identification of a functional interaction between the transmembrane protein Smoothened and the kinesin-related protein Costal2. *Curr. Biol*. **13,** 1998–2003.
7. Zhang, W., Zhao, Y., Tong, C., et al. (2005) Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of Cubitus interruptus. *Dev. Cell* **8,** 267–278.
8. Burke, R., Nellen, D., Bellotto, M., et al. (1999) Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* **99,** 803–815.
9. Hacker, U., Nybakken, K., and Perrimon, N. (2005) Heparan sulphate proteoglycans: the sweet side of development. *Nature Rev. Mol. Cell Biol*. **6,** 530–541.
10. Gorfinkiel, N., Sierra, J., Callejo, A., Ibanez, C., and Guerrero, I. (2005) The Drosophila ortholog of the human Wnt inhibitor factor Shifted controls the diffusion of lipid-modified Hedgehog. *Dev. Cell* **8,** 241–253.
11. Yao, S. Lum, L., and Beachy, P. A. (2006) The Ihog cell-surface proteins bind Hedgehog and mediate pathway activation. *Cell* **125,** 343–357.
12. Huangfu, D. and Anderson, K. V. (2006) Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. *Development* **133,** 3–14.
13. Osterlund, T. and Kogerman, P. (2006) Hedgehog signalling: how to get from Smo to Ci and Gli. *Trends Cell Biol*. **16,** 176–180.
14. Zhang, W., Kang, J. S., Cole, F., Yi, M. J., and Krauss, R. S. (2006) Cdo functions at multiple points in the Sonic Hedgehog pathway, and Cdo-deficient mice accurately model human holoprosencephaly. *Dev. Cell* **10,** 657–665.
15. Tenzen, T., Allen, B. L., Cole, F., Kang, J. S., Krauss, R. S. and McMahon, A. P. (2006) The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev. Cell* **10,** 647–656.
16. Varjosalo, M., Li, S. P., and Taipale, J. (2006) Divergence of hedgehog signal transduction mechanism between Drosophila and mammals. *Dev. Cell* **10,** 177–186.
17. Svard, J., Henricson, K. H., Persson-Lek, M., et al. (2006) Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev. Cell* **10,** 187–197.
18. Tay, S. Y., Ingham, P. W., and Roy, S. (2005) A homologue of the Drosophila kinesin-like protein Costal2 regulates Hedgehog signal transduction in the vertebrate embryo. *Development* **132,** 625–634.
19. Scholey, J. M. and Anderson, K. V. (2006) Intraflagellar transport and cilium-based signaling. *Cell* **125,** 439–442.