
Preface

Posttranslational Modifications and Secretion of the Hedgehog (Hh) Proteins

Mammalian genomes contain three homologs of the *Drosophila* Hh protein—Sonic (Shh), Indian (Ihh), and Desert (Dhh), encoded by separate genes [1]. All Hh proteins are auto-processed and posttranslationally modified in a similar manner before being secreted by the producing cell and received by surrounding tissues. Coincidentally, Hh proteins share a high homology in their C-terminal half, similar to the intein regions of self-splicing proteins of bacteria that undergo intramolecular processing. Autoprocessing occurs in the endoplasmic reticulum where the signal sequence of the Hh precursor is first cleaved. The C-terminal autoprocessing domain of Hh catalyzes the self-cleavage by an internal Cys nucleophilic attack on a peptide bond, which is resolved by addition of a cholesterol moiety that separates the Hh protein in two fragments, with the N-terminal fragment (HhN) covalently bound to cholesterol. The C-terminal fragment (HhC) is rapidly degraded by the proteasome [2], while HhN is further posttranslationally modified by addition of a palmitic acid group by skinny hedgehog (SKI) at its N-terminus before being targeted to the plasma membrane. Thus, active Hh proteins are dually lipidated, which makes them extremely hydrophobic. Release of HhN, from now on called simply Hh, to the extracellular milieu requires the activity of two proteins, a 12-pass transmembrane protein with homology to the Hh receptor called Dispatched (DispA in vertebrates), and the secreted glycoprotein Scube2, a member of the signal peptide, CUB, domain, epidermal growth factor-related family that is secreted together with Hh [3–5]. Scube2 interaction with Hh requires cholesterol modification of Hh, distinct from the interaction between Dispatched and Hh. The palmitic acid moiety enhances the Scube2-Hh interaction while also enhancing the rate of release from the producing cell. Although Scube2 is absent in *Drosophila*, alternative mechanisms appear to regulate its secretion. The lipid-binding protein Shifted (Shf), an ortholog of the Wnt Inhibitory Factor-1 (WIF-1) protein, works in a similar fashion as Scube2 by associating with cholesterol-modified HhN to promote its stability and release [6]. In addition, uptake of Hh proteins into lipoprotein-like particles promotes long-range signaling in *Drosophila*.

Lipidated Hh tethers to the plasma membrane with high affinity, but it must also signal over long distances in order to exert its morphogenetic role on cellular differentiation during embryonic development. Cholesterol-unmodified, full-length Hh is active, but not to the same extent as when lipidated. Moreover, signaling by unmodified Hh leads to ectopic expression of Hh target genes and ultimately developmental defects. A mechanism to explain the release of Hh from the producing cell must address the issues of solubility, stability, and activity of the protein once it is released. To address solubility, Hh may form oligomers that occlude the lipidated Hh in a “micelle-like” structure. A visible result of oligomerization is the formation of larger Hh-containing structure called visible clusters. In Chapter 1, a method for stable delivery of soluble Hh proteins in cell culture that can be adapted for studies in vivo will be described. Other carriers of soluble cholesterol-modified Hh have been identified and include lipoprotein particles and exosomes [5]. In addition, specialized filopodia known as cytonemes have been discovered as transporters of Hh, but not hubs for Hh signal transduction [7]. We will describe a detailed protocol for imaging of cytonemes in *Drosophila* wing discs in Chapter 2. To address stability, a subfamily of

heparin sulfate proteoglycans (HSPGs) known as glypicans may be involved at least in *Drosophila* where the glypican Dally is involved in stabilizing Hh at the cell surface of Hh-producing cells. In vertebrates, the gene *tout-velu* (ttv) is required for the generation of HSPG chains and also provides a link between glypicans and Hh signaling [8]. In fact, glypicans play multiple roles in Hh signaling. For example, the glycosaminoglycan (GAG) chains of Dally and another *Drosophila* glypican, Dlp, mediate oligomerization or inclusion of lipid-modified Hh in lipoprotein particles in the *Drosophila* imaginal wing disc [9]. The signaling potency of Hh in vertebrates increases when lipidated. Conversely, lipidated Hh can only initiate juxtacrine signaling via direct cell-cell contact as it remains on the outer leaflet of the plasma membrane of the producing cell unless it is released into the extracellular environment. The distance over which the Hh signal is transmitted is intimately associated with Hh secretion. In Chapter 3 we present a mathematical model for studying Hh proteins gradient formation in tissues.

Hedgehog Reception and Signaling

In the absence of Hh, the 12-pass transmembrane receptor Patched1 (Ptch1) inhibits the activity of a G protein-coupled receptor (GPCR) and core transducer of the pathway, Smoothened (Smo), by keeping it in an inactive state via an unknown mechanism. The inhibitory role of Ptch1 is reversed by binding of an Hh ligand with the aid of at least one co-receptor. Three redundant co-receptors for Hh proteins have been identified: CAM-related/downregulated by oncogenes (Cdon), brother of Cdon (Boc), and growth arrest-specific 1 (Gas1). They are absolutely and collectively required for binding to Hh synergistically with Ptch1 to transmit the Hh signal, as *Cdo*^{-/-};*Boc*^{-/-};*Gas1*^{-/-} triple knock-out mice display phenotypes strongly associated with defective canonical Hh signaling and are unresponsive to Shh [10, 11]. Cdon and Boc are single-pass transmembrane proteins belonging to the immunoglobulin superfamily and share sequence homology with Interference Hedgehog (Ihog) and Brother of Ihog (Boi), respectively, in *Drosophila* [12, 13]. However, according to crystallographic and biochemical studies, their mode of Hh binding is completely unlike that of the vertebrate co-receptors [14]. Cdon and Boc share redundant functions in most cell types such as in cerebellar granule neuron precursor (CGNP) cells that require Boc and Gas1, but not Cdon, for proliferation [15]. Gas1 is a glycosyl phosphatidylinositol (GPI)-anchored cell surface protein. The co-receptors act upstream of Smo, as upregulation of Smo activity rescues Hh pathway-mediated proliferation in *Boc*^{-/-};*Gas1*^{-/-} CGNPs [15]. Glypicans are also GPI-anchored proteins that act as co-receptors for either promoting or inhibiting Hh signaling [8]. GPC1 and GPC3 are negative regulators of the Hh pathway, both interact with low-density lipoprotein receptor-related protein-1 (LRP1), and the interaction is stabilized in the presence of Hh. This stabilization induces endocytosis and degradation of the glypican–Hh complex. In addition, GPC3 competes with Ptch1 for Hh binding. GPC5 and GPC6 are positive regulators of the Hh pathway, as loss-of-function mutations in GPC6 contributes to autosomal recessive omodysplasia, and upregulation of GPC5 contributes to rhabdomyosarcoma, an Hh-dependent type of cancer [8].

Sequence similarity and crystallographic evidence support the notion that Smo belongs to the GPCR superfamily. The spatial organization of Smo's 7-pass transmembrane helical bundle resembles that of class A GPCRs; however, Smo lacks conserved residues in helix VI that are critical for facilitating the active state conformation in this GPCR subfamily [16, 17].

Interestingly, Smo shows high sequence homology to the Frizzled (FZD) family of receptors, members of class F GPCRs, in particular with the FZD cysteine-rich domain (CRD) that contains a hydrophobic groove to which cholesterol-like moieties bind [17]. Thus, Smo has been classified as a member of the class F GPCR subfamily [18]. *Drosophila* Smo also shares the GPCR-like heptahelical bundle; however the structures of vertebrate and invertebrate Smo proteins have diverged as they share low sequence homology [19].

Both *Drosophila* and vertebrate Smo couple to heterotrimeric G inhibitory proteins (Gi) [20, 21]. Evidence of Smo-Gi coupling in vertebrates derived from direct measurements of Smo-catalyzed exchange of GDP for GTP in all members of the Gi family [20]. These experiments showed constitutive activity of Smo toward Gi proteins in the absence of ligand and demonstrated that several Smo inhibitors act as inverse agonists preventing activation of Gi by constitutively active Smo [20]. A detailed description of the protocol to quantify the level of Smo coupling to G proteins is provided in Chapter 4. In addition, studies in cell culture using NIH3T3 cells, fibroblasts that respond readily to Hh proteins and that will be discussed in several chapters in this book, showed that pertussis toxin (PTX), a protein that disrupts coupling of GPCRs to Gi proteins, prevents many cellular responses to Shh and serves as a tool to study Smo-Gi-dependent processes. The most studied function of Gi proteins is inhibition of adenylyl cyclase and concomitant reduction in cytosolic cAMP levels. Accordingly, activation of Gi by Smo decreases the concentration of intracellular cAMP to a comparable extent than other prototypical Gi-coupled GPCRs [22]. The relevance of GPCRs and G proteins in Hh signaling continues to be more appreciated as recent evidence supports the role of the orphan GPCR Gpr161 in the cell-specific regulation of basal Hh signaling repression [23].

The canonical Hh pathway regulates the activity of the transcription factor Cubitus interruptus (Ci) in *Drosophila* and its three vertebrate orthologs glioma-associated oncogene homolog (Gli) transcription factors according to the degree of Smo activation [1]. A direct consequence of increasing Hh concentration is the stepwise phosphorylation of the Smo C-terminal tail (C-tail) by casein kinase 1 alpha (CK1 α) and G protein-coupled receptor kinase 2 (GRK2) in vertebrates and by CK1 α and protein kinase A (PKA) in *Drosophila* [24, 25]. A protocol for studying the phosphorylation events and its functional consequences is provided in Chapter 5. Phosphorylation of the C-tail of Smo leads to a conformational change that brings the C-tails of two Smo monomers together to form a functional dimer [26]. A method for analysis of Smo conformational changes is presented in Chapter 6. Activation of Smo correlates with its trafficking from intracellular vesicles to the plasma membrane in *Drosophila* and to the primary cilium in vertebrates [27, 28]. Smo translocation to the primary cilium requires the intraflagellar transport (IFT) protein complex component Kif3a, along with β -arrestins [29]. GRK2 phosphorylation creates docking sites for recruitment of β -arrestin 2 (β arr2) to active Smo at the plasma membrane [30]. Smo ciliary localization is not sufficient for activation of Gli, as it must undergo a second unknown activation step before it signals the activation of Gli at the primary cilium [31, 32]. Once activated by Smo, full-length activated Gli (Gli^A) isoforms translocate to the nucleus where they activate the transcription of Hh target genes. Hh responsive genes are dependent either on Gli^A activity or on the de-repression of Gli^R activity [33]. The activation of Gli is intimately associated with the presence of the primary cilium, as full-length Gli must accumulate at the primary cilium along with the ciliary localization of active Smo in order for Gli activation to occur [34, 35]. The accumulation of Gli at primary cilia is PKA sensitive [34]. In Chapters 7 and 8, we present protocols for evaluation of transcriptional activity of the Gli transcription factors in cell culture and to determine the expression level of

endogenous Gli proteins, which in the case of Gli1 serves as readout of canonical pathway activation, since it is the most sensitive Hh-target gene.

Ci activation in *Drosophila* requires the interaction between the C-terminal tail of the active Smo dimer and a Hh signaling complex (HSC) containing the kinase fused (Fu), the microtubule-binding protein costal2 (Cos2), suppressor of fused (Sufu), and Ci [36]. Interaction between Smo and the HSC results in: (1) autoactivation of Fu, (2) phosphorylation of Cos2, and (3) dissociation of Ci from the HSC. Autoactivation of Fu is required to antagonize the inhibitory activity of Sufu, whereas the dissociation of Ci from the HSC leads to its nuclear translocation by an unknown mechanism to activate Ci-target genes [37]. Thus, the relative stoichiometry among the HSC proteins is expected to govern Ci activation. A protocol for absolute quantification of different proteins of the Hh pathway is presented in Chapter 9.

In both *Drosophila* and vertebrates, in the absence of Hh ligands, Ci/Gli are kept in an inactive state by partial processing into transcriptional repressor forms, Ci^R/Gli^R. Phosphorylation by PKA at several sites initiates Gli^R formation at the base of the primary cilium, and Sufu forms a complex with Gli to prevent its translocation of Gli at the ciliary tip [38]. In *Drosophila*, Ci sequestration in the HSC allows PKA to initiate Ci^R formation. In order to signal partial proteolysis, PKA must phosphorylate Ci/Gli in several phosphorylation site clusters as complete loss of PKA catalytic activity is correlated with ligand-independent Hh pathway activation, and elevated intracellular cAMP levels are correlated with suppression of the Hh pathway [1, 33]. Phosphorylation of the first four out of six conserved Ser residues at the carboxyl side of the DNA-binding domain of Ci/Gli by PKA leads to further posttranslational modifications by glycogen synthase kinase-3 (GSK3) and casein kinase-1 (CK1) [39–41] and partial proteasomal processing. A protocol for determination of inhibitory and stimulatory phosphorylation of Gli2 is presented in Chapter 10, and a protocol for generating Gli2 and Gli3 with mutated phosphorylation sites expressed at near-endogenous levels is detailed in Chapter 11.

Full-length Ci/Gli in their active form are short-lived [33, 39]. In vertebrates, the Itch E3 ubiquitin ligase of the HECT family (also known as AIP4) ubiquitylates and targets Gli1 for total proteasomal degradation [42, 43]. The high promiscuity among E3 ligases and substrates predicts that in a context-specific manner, other E3 ligases might also regulate the turnover of the Ci/Gli transcription factors. In Chapter 12, we detail a protocol to investigate ubiquitylation of Gli family proteins by Itch that can be adapted to test other ligases and the type of ubiquitin chain branching. Another modification that inhibits Gli1 and Gli2 transcriptional activity in a reversible manner is acetylation by histone acetyltransferases (HATs) [44]. A method for evaluating the degree of Gli1 and Gli2 acetylation/deacetylation is detailed in Chapter 13.

Evaluation of Hh pathway activity in mammalian embryonic tissues has mostly been accomplished by in situ hybridization of Hh-target genes, such as *gli1* and *ptc1*, and by immunostaining of Hh ligands in those tissues that allow proper permeabilization maintaining the morphology. A novel method for evaluation of Ihh expression in the bone in chick embryos is presented in Chapter 14. In mice, the generation of a Ptc1^{+/lacZ} heterozygote model served as reporter of canonical Hh signaling activity using β -gal staining of tissues as readout, since *ptc1* is a classical target gene. This was extremely useful to study Hh-responsive tissues during embryogenesis but has not been exploited as a common method to study Hh signaling in adult mouse tissues. Here we present a method for dual staining of β -gal and Shh in hematoxylin-eosin counterstained skin sections from Ptc1^{+/lacZ} mice (Chapter 15).

Noncanonical Hh Signaling

Smo and Ptch1 are central components of the Hh pathway that leads to slow, graded activation of Gli (canonical signaling) but they can also initiate fast, nontranscriptional cellular responses collectively known as noncanonical Hh signaling [1, 45]. Overexpression of Ptch1 promotes cell death through its C-terminal domain and retains cyclin B1 out of the nucleus through its third intracellular loop, effectively slowing cell cycle progression [46, 47]. These functions of Ptch1 are independent of Smo and the Gli transcription factors and are classified as “Type I” noncanonical signaling [45]. The seventh intracellular domain of Ptch1, its C-terminal tail, recruits adaptor proteins and pro-caspase-9, which self-activates by oligomerization and induces apoptosis [48]. Importantly, Shh disrupts the Ptch1-proapoptotic complex interaction and promotes survival, as seen in endothelial cells [49].

Noncanonical Hh signaling Type II is mediated by Smo but is independent of Gli activation/repression. The ability of Smo to couple to Gi proteins is central to this branch of Hh signaling. It appears that Smo can activate Gi both within and outside of the primary cilium. For instance, Smo-Gi coupling in adipocytes and skeletal muscle stimulates aerobic glycolysis, glucose uptake, and calcium increase [50]. A protocol to study these metabolic effects of noncanonical Hh signaling is provided in Chapter 16. A similar primary-cilium localized activation of Gi by Smo regulates calcium spike activity in spinal neuron precursors [51]. In contrast, Smo-Gi coupling can occur outside of the primary cilium, as has been reported during activation of small Rho GTPases in fibroblasts and endothelial cells and of Src family kinases in neurons [52–54].

A comprehensive understanding of the Hh pathway and its functions in development and disease needs the consideration of both canonical and noncanonical responses. This Edition of Hedgehog Signaling Protocols provides novel protocols for the study of newly discovered functions and modifications of components of the Hh pathway.

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