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Review

Decoding the Hedgehog signal in animal development

J. Jia[a] and J. Jiang[b,] *

[a] Sealy Center for Cancer Cell Biology and Department of Biochemistry and Molecular Biology, University of Texas Medical Branch, Galveston, Texas 77555 (USA)
[b] Center for Developmental Biology and Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390 (USA), Fax: +1 214 648 1960, e-mail: Jin.Jiang@UTSouthwestern.edu

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Abstract. The Hedgehog (Hh) family of secreted proteins plays essential roles in a myriad of developmental processes via a complex signaling cascade conserved in species ranging from insects to mammals. In many developmental contexts, Hh acts as long-range morphogen to control distinct cellular outcomes as a function of its concentration. Here we review the current understanding of the Hh signaling mechanisms that govern the establishment of the Hh gradient and the transduction of the Hh signal with an emphasis on the intracellular signaling cascade from the receptor to the nuclear effector. We discuss how graded Hh signals are transduced to govern distinct developmental outcomes.

Keywords. Hedgehog signaling, Wnt, patched, smoothened, Ci/Gli transcription factor, kinase, pattern formation, development.

Introduction

A central problem in developmental biology is how diverse cell types are generated and how cells of different types are organized to form appropriate body patterns. A common theme from studies using model organisms is that cells choose fates and form patterns based on the positional information they receive, which is often provided by evolutionarily conserved families of secreted signaling molecules. The Hedgehog (Hh) family of secreted proteins represents one such family.

hh was initially discovered by Christiane Nusslein-Volhard and Eric Wieschaus in their saturation genetic screens for mutations that affect patterning of the *Drosophila* larval cuticle [1]. *hh* was cloned simultaneously by several laboratories and was shown to encode a secreted protein [2–4]. Shortly thereafter, multiple vertebrate homologs of Hh were identified in several organisms [5–8]. The important role of the *hh* family in vertebrate development was immediately realized by the provocative expression of one family member, *Sonic hedgehog* (*Shh*), in a number of well-characterized embryonic organizing centers including the notochord and zone of polarizing activity (ZPA) of the limb bud [5–8]. Embryological and genetic studies demonstrated that Shh indeed accounts for the long-sought organizing activities emanating from these signaling centers [5–9]. Moreover, accumulating experimental evidence has demonstrated that Shh, as well as the *Drosophila* Hh, can function as morphogens that regulate cell patterning as a function of their concentrations [10–14]. These observations raise several important questions: how is the Hh morphogen gradient generated? How are different levels of the Hh morphogen transduced within each cell to generate distinct developmental outcomes?

Answers to these questions rely on a complete understanding of the Hh signaling pathway. In the past two decades, many Hh pathway components have been identified, mainly through genetic studies in *Drosophila*. These include components that control production, propagation, reception, and transduction of the Hh signal (Table 1).

* Corresponding author.

Table 1. Components of the Hedgehog pathway.

Drosophila Gene	Protein	Function	Vertebrate counterpart
hedgehog (hh)	secreted protein	ligand	Shh, Ihh, Dhh
skinny hedgehog (skn)	acyltransferase	Hh palmitoylation	Skn
dispatched (disp)	multiple-span transmembrane protein	releasing Hh	Disp
tout-velu (ttv)	glycosyltransferase	heparan sulfate polymerization, Hh spreading	EXT1
sister of tout-velou (sotv)	glycosyltransferase	heparan sulfate polymerization, Hh spreading	EXT2
brother of tout-velou (botv)	glycosyltransferase	heparan sulfate polymerization, Hh spreading	EXT3
dally	core protein of HSPGs	Hh movement	Glypican
dally-like (dlp)	core protein of HSPGs	Hh movement	Glypican
shifted (shf)	secreted protein	Hh spreading	WIF
patched (ptc)	multiple-span transmembrane protein	Hh receptor	Ptc1, Ptc2
smoothened (smo)	seven-transmembrane protein	Hh signal transducer	Smoothened
protein kinase A (PKA)	Ser/Thr kinase	Ci processing and Smo activation	PKA
casein kinase 1 (CKI)	Ser/Thr kinase	Ci processing and Smo activation	CKI
shaggy (sgg)	Ser/Thr kinase	Ci processing	GSK3β
supernumerary limbs (slimb)	F-box protein	substrate recognition subunit of ubiquitin E3 ligase	β-TRCP
costal2 (cos2)	kinesin-related protein	scaffold for Ci phosphorylation and Hh signal transduction	KIFs
fused (fu)	Ser/Thr kinase	activation of full-length Ci	Fu
suppressor of fused (su(fu))	PEST domain protein	repression of full-length Ci	Su(fu)
cubitus interruptus (ci)	Zinc finger transcription factor	transcriptional activator and repressor of Hh target genes	Gli1, Gli2, Gli3

The biochemical properties of many Hh pathway components have been revealed and several physical links among signaling components have been discovered. The overall theme of the Hh pathway appears to be conserved from *Drosophila* to mammals. For example, in both *Drosophila* and vertebrates, Hh binds to its receptor Patched (Ptc), which leads to activation of the signal transducer Smoothened (Smo). Smo then transduces the Hh signal into the cytoplasm to activate the latent transcription factor Ci/Gli. However, important differences between the *Drosophila* and the vertebrate Hh pathways exist. Here, we review our current understanding of the mechanisms that govern the establishment of Hh gradients and the transduction of Hh signals with an emphasis on the intracellular signaling cascade. We discuss how graded Hh signals are transduced to control distinct developmental outcomes in several developmental contexts.

Hh in pattern formation and cell growth control

In *Drosophila*, *hh* is required at multiple developmental stages and is responsible for patterning embryonic segments as well as adult appendages including wings and legs [15]. One of the best systems for studying Hh signaling is the *Drosophila* wing. In the developing wing imaginal disc, posterior (P) compartment cells express and secrete Hh proteins that act upon neighboring anterior (A) compartment cells to induce the expression of target genes including *decapentaplegic* (*dpp*), which encodes a member of the TGFβ/BMP family of secreted proteins (Fig. 1a) [16, 17]. Dpp then diffuses bidirectionally into both A and P compartments and functions as a long-range morphogen to control the growth and patterning of cells throughout the entire wing [18, 19]. In addition to its long-range influence through Dpp, Hh acts as a short-range morphogen to direct cell patterning at the A/P boundary by activating other genes including *patched* (*ptc*), *collier* (*col*), and *engrailed* (*en*) (Fig. 1a) [11, 20]. Low levels of Hh suffice to induce the expression of *dpp* whereas higher levels of Hh signaling are required to activate *ptc* and *col*. The induction of *en* appears to require the highest doses of Hh signaling activity [11].

Vertebrates have at least three Hh family members whose functions are largely non-overlapping. For example, Indian hedgehog (Ihh) is mainly involved in bone morphogenesis whereas Desert hedgehog (Dhh) is essential for testis development [21–24]. However, in certain developmental contexts including the regulation of left/right asymmetry by the mouse node, Ihh acts redundantly with Shh [25]. Shh is involved in a much broader spectrum of developmental processes [15]. In the limb bud, *Shh* is ex-

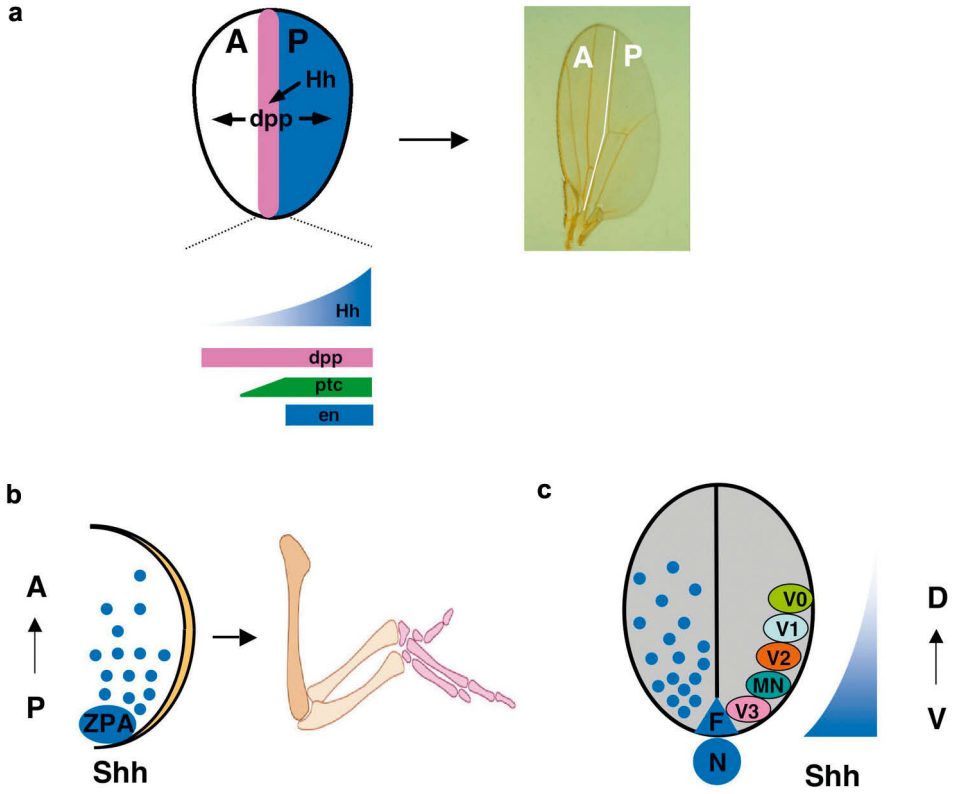

Figure 1. Hh signaling in development. (*a*) In the *Drosophila* wing imaginal disc, Hh proteins produced by posterior (P) compartment cells (blue) diffuse into the anterior (A) compartment and induce neighboring A compartment cells (pink) to express *dpp*, which encodes a TGFβ/BMP family member of secreted protein. Dpp then diffuses into both A and P compartments to control cell growth and patterning in the entire wing. Near the A/P boundary, Hh acts as a local morphogen to specify patterning by setting up different spatial limits of target gene expression. (*b*) In vertebrate limb development, Shh proteins produced by ZPA diffuse anteriorly and form a concentration gradient to direct digit formation along the A/P axis. (*c*) In neural tube development, Shh proteins emanating from the notochord (N) and floor-plate (F) form a ventral (V) to dorsal (D) concentration gradient that directs the formation of multiple neuronal subtypes at precise positions along the D/V axis. Increasing levels of Shh progressively specify progenitors giving rise to neuronal subtypes of more ventral character. MN, motor neuron; V0–V3, V0–V3 interneurons.

pressed in the ZPA, a population of apical, posterior mesenchyme cells that polarize the digits along the A/P axis (Fig. 1b) [26]. Ectopic expression of *Shh* in the anterior limb bud induces supernumerary digits in a concentration-dependent manner with higher levels of signal inducing more posterior digits. In the neural tube, Shh activity emanating from the notochord and floor-plate directs the formation of multiple neuronal subtypes at precise positions along the dorsal-ventral axis [15, 27]. Accumulating evidence suggests that Shh acts as a direct and long-range morphogen with increasing levels progressively specifying progenitors that give rise to neuronal subtypes of more ventral character (Fig. 1c) [9, 12, 28–32].

In addition to controlling cell fate and patterning, Hh family members have also been implicated in regulating cell proliferation, cell migration, axon guidance, and stem cell renewal [33–36]. In the developing cerebellum, Shh secreted by the Purkinje cells stimulates the proliferation of granule neuron precursors in the overlying external germinal cell layer [37–40]. In skin development, Shh cooperates with other signals to organize a series of growth and morphogenetic events during embryonic hair follicle development and adult hair cycling [41]. An early genetic study in *Drosophila* suggested that Hh signaling is required for somatic stem cell proliferation in the ovary [34]. Recent studies implied that Shh acts as an important regulator of neural stem cell proliferation and maintenance in postnatal rodent brains [42–45]. Given the diverse roles of Hh in cell growth and patterning as well as tissue homeostasis, it is not surprising that malfunction of Hh signaling activity has been linked to numerous human disorders including birth defects and cancers [46, 47]. The central role of the Hh family in development and disease has stimulated tremendous interest in studying Hh signaling mechanisms.

Sending and propagating the Hh signal

As Hh family members function either as short-range inducers or long-range morphogens depending on developmental contexts, the secretion and propagation of Hh

signals must be tightly controlled to ensure the formation of appropriate activity gradients. Here we only highlight several major mechanisms that regulate the formation of the Hh gradient. For a more comprehensive review of this topic, please refer to the recently published review by Guerrero and colleagues [48].

Lipid modification of Hh

In Hh-producing cells, the full-length Hh is cleaved into N-terminal (HhN) and C-terminal fragments through autocleavage, and a cholesterol moiety is covalently linked to the C terminus of HhN [49]. Subsequently, palmitoylation, which is catalyzed by the acyltransferase dubbed Skinny hedgehog (Skn) (also known as Cmn, Rasp, and Sightless), occurs at the N-terminal region of HhN (Fig. 2) [50–54]. In both *Drosophila* and mammals, Hh secretion by the signal-sending cells requires the activity of Dispatched (Disp), a multiple-span transmembrane protein structurally related to Ptc [52, 55–57]. At least in *Drosophila*, Disp is only required in the signal-sending cells to release the cholesterol-modified form of Hh [52, 55].

Although cholesterol modification was initially thought to restrict the spreading of Hh ligand [58], recent studies have provided evidence that such modification is essential for its long-range propagation and signaling in both *Drosophila* and mammals [59, 60]. Palmitoylation modulates Hh signaling activity in *Drosophila* development [50, 52, 53], but does not seem to regulate Hh movement in *Drosophila* embryos [60]. In the vertebrate, however, such modification appears to be essential for the long-range Shh signaling activity [51].

How lipid-modified Hh moves over a long distance is not clear, but soluble Shh multimers that contain lipids and have high signaling potency have been detected [61], suggesting that Shh may diffuse as soluble multimeric, lipid-containing complexes. Consistent with this idea, palmitoylation-deficient Shh no longer forms soluble complexes and fails to signal over long distance [51]. A recent study in *Drosophila* provided evidence that lipoprotein particles may carry lipid-modified proteins such

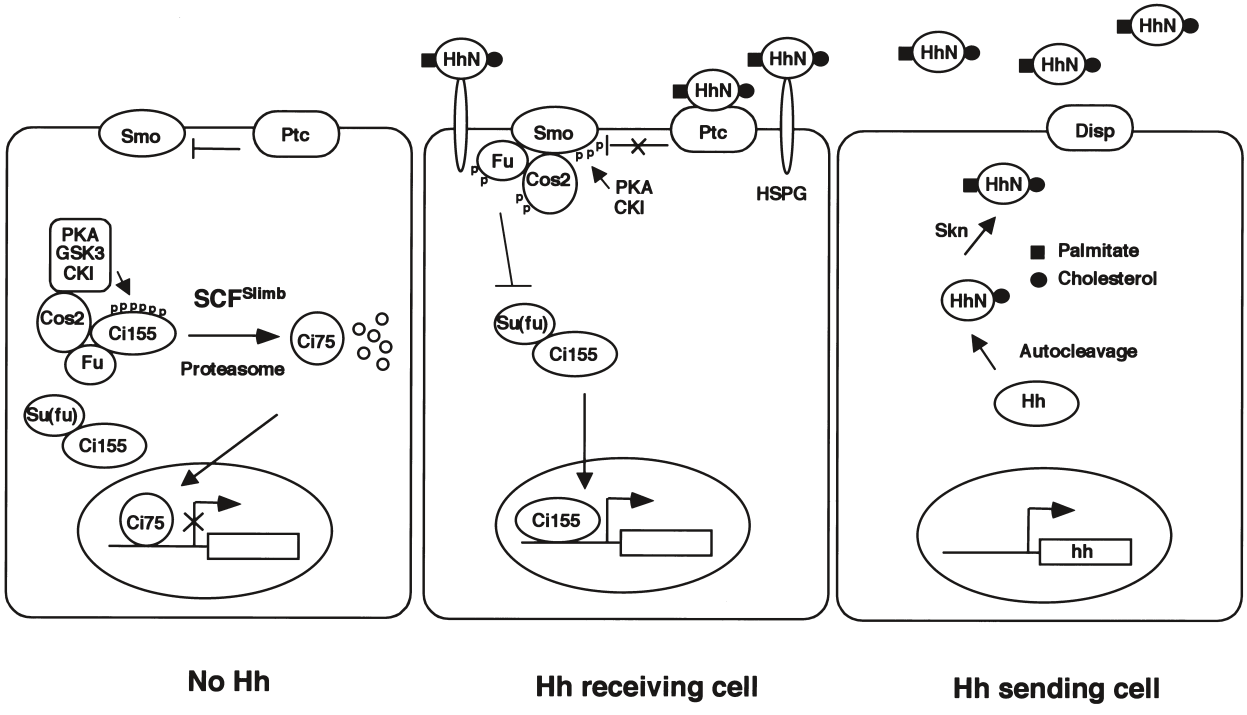

Figure 2. Hh signaling pathway in *Drosophila.* In Hh-sending cells, full-length Hh undergoes autocleavage to generate an N-terminal fragment (HhN) modified by cholesterol. HhN is further modified with palmitoylation through the action of Skn. Secretion of cholesterol-modified Hh requires Disp. Movement of Hh requires heparan sulfate proteoglycans (HSPGs). In A compartment cells distant from the A/P boundary where there is no Hh, Ptc inhibits Smo. Full-length Ci (Ci155) forms complexes with Cos2, Fu, and Su(fu). Cos2 recruits PKA, GSK3 and CKI to effectively phosphorylate Ci. Hyperphosphorylated Ci is recognized by SCF^{Slimb} that targets Ci for proteolysis through the ubiquitin/proteasome pathway to generate a truncated repressor form (Ci75). In A compartment cells near the A/P boundary, Hh binding to Ptc leads to phosphorylation of Smo by PKA and CKI. Phosphorylation of Smo results in its cell surface accumulation and activation. Smo recruits the Cos2-Fu-Ci complex to the plasma membrane and dissociates multiple kinases from Cos2, leading to inhibition of Ci phosphorylation and processing. In addition, Hh signaling induces nuclear translocation of Ci155 and stimulates its transcriptional activator activity by antagonizing Su(fu). In response to Hh, both Cos2 and Fu are also phosphorylated although the kinases involved and the biological significance of these phosphorylation events are largely unknown.

as Hh and Wingless (Wg) and transport them over a long distance [62].

Regulation of Hh signaling by heparan sulfate proteoglycans

In addition to lipid modification regulating Hh movement, genetic studies in *Drosophila* suggest that Hh movement and signaling also require extracellular matrix macromolecules such as heparan sulfate proteoglycans (HSPGs), which contain a protein core with covalently attached heparan sulfate (HS) glycosaminoglycan (GAG) chains [63]. The initial clue that HSPG could be involved in Hh movement came from the observation that clones of mutant cells deficient for *tout-velu* (*ttv*), which encodes the EXT family of glycosyltransferase required for HS polymerization, impede Hh spreading [64]. Two other members of the EXT family of glycosyltransferase, Sister of Ttv (Sotv) and Brother of Ttv (Botv) have also been implicated in Hh signaling [65–67]. Mutations in the human *EXT* genes, *EXT1* and *EXT2*, are found in hereditary multiple exostoses, a disease associated with overgrowth of bone [68]. A recent study provided evidence that mouse EXT1 regulates the range of Ihh signaling in chondrocyte development [69], supporting the view that HSPGs may also control the range of Hh signaling in vertebrates. Whether HSPGs also regulate other Hh family members in vertebrate development remains to be determined.

Genetic studies in *Drosophila* identified two genes encoding core proteins of HSPGs, *dally* and *dally-like* (*dlp*), as important regulators of Hh signaling activity. Dlp appears to be specifically required for Hh signaling in embryonic development and in a tissue culture system, whereas Dally acts redundantly with Dlp in wing discs [70–72]. How HSPGs regulate Hh signaling is not fully understood but the observation that Dlp physically interacts with Hh suggests that Dlp may function as a co-receptor for Hh [70]. Dlp could enrich Hh on the cell surface, thus facilitating its interaction with Ptc. Dlp could also function as a stepping stone to facilitate intercellular movement of Hh [63].

Feedback regulation by Ptc

A hallmark of Hh signaling is the upregulation of *ptc* transcription in response to the signal, which functions as a negative feedback that restricts the spreading of Hh. Replacement of endogenous *ptc* with a *ptc* transgene that was no longer upregulated by Hh resulted in a broader Hh signaling range in wing discs [73]. Hh binds directly to Ptc, which internalizes Hh through Dynamin-dependent endocytosis and targets Hh to lysosomes for degradation [74–76]. Hence, by removing Hh from the cell surface, Ptc plays an important role in shaping the Hh gradient.

Species-specific extracellular regulators of Hh

Whereas the aforementioned mechanisms may operate in both invertebrates and vertebrates, species-specific mechanisms also exist. For example, the Hh-interacting protein (Hip), a vertebrate-specific membrane-bound glycoprotein induced by Shh, acts in parallel to Ptc to attenuate Hh signaling [77–79]. On the other hand, two recent studies identified the *shifted* (*shf*) gene product, a secreted protein homologous to the human Wnt inhibitory factor (WIF), as an important regulator of Hh spreading in *Drosophila* wing imaginal discs [80, 81].

Receiving and transducing the Hh signal

Hh exerts its biological influence via a complex signal transduction cascade (Fig. 2). Conceptually, the Hh pathway can be viewed as centering around two nodes: one at the junction between the plasma membrane and cytoplasm that involves the regulation of the seven-transmembrane (7-TM) receptor-like protein Smo, and the other at the interface between the cytoplasm and nucleus that involves the regulation of the Cubitus interruptus (Ci)/Gli family of Zn finger transcription factors. At both levels, the system employs an 'anti-repression' mechanism. For example, at the cell surface, the signaling activity of Smo is blocked by Ptc and is derepressed upon Hh binding to Ptc [73, 82, 83]. Inside the cell, intracellular signaling complexes inhibit the activator activity of Ci/Gli and Hh alleviates this blockage [15]. In addition, Ci and Gli3 are converted into truncated repressor forms by proteolytic processing, which is blocked by Hh.

Hh signaling at the cell surface

The reception system of the Hh signal is unusual and consists of two transmembrane proteins: Ptc and Smo [15]. Ptc is the receptor for Hh whereas Smo is the signal transducer. In the absence of Hh, Ptc inhibits Smo through a poorly understood mechanism. Based on the observation that overexpressed Ptc and Smo in cultured cells can be coimmunoprecipitated, Ptc was initially proposed to block Smo by forming a stable complex [83]. However, attempts to detect stable Ptc-Smo complexes failed in various systems [84–86]. Moreover, immunostaining of *Drosophila* imaginal discs indicated that Ptc and Smo are largely segregated [87]. In cultured cells, Ptc can inhibit Smo at a stoichiometry as low as 1:50, suggesting that Ptc acts catalytically to inhibit Smo [86].

The precise mechanism by which Ptc exerts its inhibitory role is still unknown. Ptc contains a sterol-sensing domain (SSD) and an overall structure similar to the resistance-nodulation-division (RND) family of prokaryotic permeases [86, 88]. Mutations of conserved residues in either the SSD or the RND-like structure impair Ptc function [86, 89, 90]. Another protein containing both SSD and

RND domains is the Niemann-Pick disease type C1 protein (NPC1) [91]. NPC1 regulates cholesterol trafficking, and upon expression in bacteria can transport acrifalvine and oleic acid across the membranes [92, 93]. Based on these and other observations, Ptc has been proposed to function as a permease to facilitate transmembrane movement of a small molecule(s) that acts as Smo agonist or antagonist [86].

How Smo activity is regulated also remains poorly understood. In *Drosophila*, Hh induces cell surface accumulation and phosphorylation of Smo [87]. Recent studies from several laboratories demonstrated that protein kinase A (PKA), which was initially identified as a negative regulator of the Hh pathway [94–97], acts together with casein kinase I (CKI) to phosphorylate Smo, leading to its cell surface accumulation and activation [98–100]. Blocking PKA or CKI activity prevents Hh-induced Smo accumulation and attenuates pathway activity, whereas increasing PKA activity promotes Smo accumulation and pathway activation [98]. PKA and CKI phosphorylate the intracellular C-terminal tail of Smo at three clusters of sites *in vitro*, most of which are also phosphorylated *in vivo* [98–100]. Phosphorylation-deficient forms of Smo in which PKA or CKI sites are mutated to alanine are defective in Hh signaling. Phosphorylation appears to be sufficient to activate Smo as phosphorylation-mimicking Smo variants in which PKA and CKI sites are converted to acidic residues such as aspartate and glutamate exhibit constitutive signaling activity [98, 100].

Intracellular trafficking of Smo

How phosphorylation activates Smo is not fully understood but there is a good correlation between phosphorylation-induced cell surface accumulation and activity [98]. Smo lacking PKA or CKI sites failed to accumulate on the cell surface in response to Hh whereas phosphorylation-mimicking Smo exhibited constitutive cell surface accumulation, suggesting that phosphorylation regulates Smo activity at least in part by promoting its cell surface accumulation. Consistent with this notion, an earlier study indicated that forced cell surface localization of Smo increased its activity, whereas endoplasmic retention of an activated form of Smo blocked its signaling activity [101].

A transmission electron microscopic study of *Drosophila* imaginal discs indicated that Smo is localized primarily in the lysosomes of A compartment cells but is enriched on the plasma membrane of P compartment cells [102]. In *Drosophila* salivary gland cells, blocking endocytosis using a dominant negative form of Rab5 promotes Smo cell surface accumulation [101]. Antibody uptake experiments using cultured cells suggested that Hh regulates Smo cell surface accumulation by blocking endocytosis and/or promoting recycling of Smo through phosphorylation [98]. Taken together, these observations suggest that in the absence of Hh, Ptc keeps Smo in an unphosphorylated or hypophosphorylated state and Smo is removed from the cell surface via endocytosis and degraded in lysosomes. Upon Hh stimulation, the phosphorylation level of Smo increases and hyperphosphorylation either blocks endocytosis and/or promotes recycling of Smo, leading to its cell surface accumulation.

Regulation of intracellular trafficking has also been observed for mammalian Smo. In cultured cells, both Smo and Ptc are internalized and colocalized in endosomal compartments, and Hh induces segregation of Smo away from Hh-Ptc complexes destined for lysosome degradation [85]. However, vertebrate Smo proteins lack the PKA and CKI phosphorylation consensus sites found in *Drosophila* Smo, implying that their trafficking and activity are not regulated by PKA and CKI phosphorylation. Nevertheless, there is evidence that mammalian Smo is phosphorylated by GRK2 in cultured cells [103]. Intriguingly, phosphorylation by GRK2 appears to promote association of Smo with β-arrestin 2 and endocytosis of Smo via clathrin-coated pits [103]. In support of the functional significance of Smo/β-arrestin 2 interaction, morpholino knockdown of β-arrestin 2 activity in zebrafish embryos resulted in phenotypes indicative of Hh signaling defects [104]. A more definitive proof that β-arrestin-2-mediated endocytosis of Smo plays an essential role in vertebrate Hh signaling awaits genetic study in mice.

Hh signal transduction by intracellular signaling complexes

In *Drosophila*, Hh signal transduction culminates at the activation of the latent transcription factor Ci (Fig. 2) [15, 105]. Ci activity is regulated by multiple mechanisms including proteolytic processing, phosphorylation, cytoplasmic retention, and protein-protein interactions. In imaginal disc development, Ci plays dual roles that are performed by two distinct forms. In the absence of Hh, full-length Ci (Ci155) undergoes proteolytic processing to generate a truncated form (Ci75) that functions as a repressor to block the expression of Hh-responsive genes such as *dpp* [106, 107]. Ci processing requires hyperphosphorylation by multiple kinases including PKA, GSK3β, and members of the CKI family including CKIα and CKIε [108–113]. These kinases phosphorylate Ci155 at multiple sites in its C-terminal region and hyperphosphorylation promotes Ci processing (Fig. 3) [113–115]. Loss-of-function of any of these three families of kinases results in a complete blockage of Ci processing [108–113].

How phosphorylation of Ci regulates its processing has not been fully resolved, but this process requires the activity of an F-box/WD40-containing protein, Slimb, which acts downstream of PKA [110, 116]. The verte-

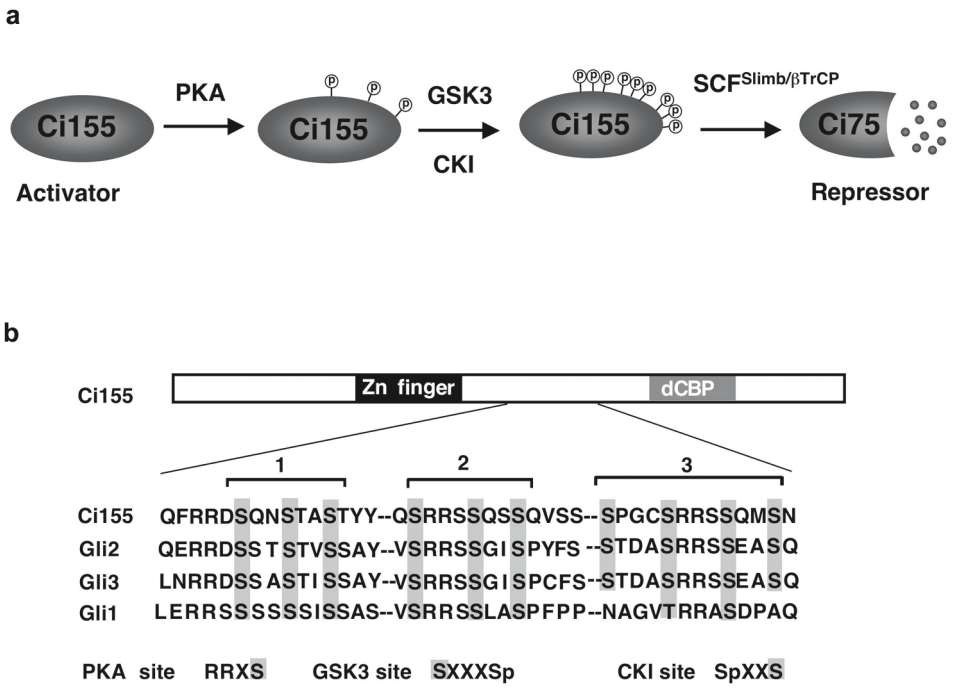

Figure 3. Regulation of Ci processing by multiple kinases. (*a*) Ci155 is phosphorylated at multiple sites in its C-terminal half by PKA, which primes further phosphorylation by GSK3β and CKI. Sequential phosphorylation by these three kinases creates multiple docking sites that recruit SCF^{Slimb}, followed by proteasome-mediated proteolysis to generate Ci75. (*b*) A schematic drawing of Ci155. The Zn finger DNA-binding domain and dCBP-binding domain are indicated as black and gray boxes, respectively. Sequence surrounding the three phosphorylation clusters and its alignment to the corresponding regions in Gli proteins is shown underneath. PKA, GSK3β, and CKI sites are shaded and their consensus sequences are indicated.

brate homolog of Slimb, β-TRCP, functions as a substrate recognition subunit of the so-called SCF (Skp1, Cdc53, and F-box) ubiquitin ligase complex that normally targets phosphorylated substrates such as Iκ-B and β-catenin for ubiquitination, followed by proteasome-mediated proteolysis [114]. Slimb binds hyperphosphorylated Ci *in vitro*, and substitution of the three phosphorylation clusters with a canonical Slimb/β-TRCP recognition motif found in β-catenin renders Slimb binding and Ci processing independent of CKI phosphorylation, suggesting that hyperphosphorylation of Ci recruits $SCF^{Slimb/\beta\text{-TRCP}}$ [113, 117].

Ci155 exists in a large protein complex that includes the kinesin-related protein Costal2 (Cos2) and the Ser/Thr kinase Fused (Fu) (Fig. 2) [118, 119]. Complex formation impedes nuclear translocation of Ci155 through microtubule dependent and -independent mechanisms [120–123]. In addition, Cos2 directly associates with PKA, GSK3β, and CKI and acts as a scaffold between these kinases and Ci to facilitate Ci phosphorylation [124]. Ci155 also forms a complex with Su(fu), which appears to be largely devoid of Cos2 [125]. Su(fu) impedes Ci nuclear import [120, 121], and inhibits Ci155 activity in the nucleus [121, 126], possibly through recruiting a co-repressor complex [127].

Hh signaling appears to inhibit Ci phosphorylation and processing by dissociating the Cos2-Ci-kinase complex [124]. In addition, high levels of Hh convert Ci155 into a hyperactive but labile transcriptional activator by antagonizing Su(fu) through the Ser/Thr kinase Fu [126]. The hyperactive form of Ci155 then induces high-threshold Hh responses including activation of *ptc* and *en* [107, 128]. *fu* mutations only affect high-threshold Hh responses and the defects caused by *fu* mutations is rescued by *Su(fu)* mutations [129, 130].

In vertebrates, three Gli proteins, Gli1, Gli2, and Gli3, function as the transcriptional mediators of Hh signaling. Genetic and biochemical studies suggest that Gli2 and Gli3 are the primary mediators of Hh signaling, whereas Gli1 is a transcriptional target of Hh signaling and feeds back positively to reinforce the Hh signaling activity [131–138]. Gli2 mainly functions as an activator whereas Gli3 does so as a repressor although in some developmental contexts, repressor activity of Gli2 and activator activity of Gli3 have also been detected [135, 139–142]. Consistent with their ability to function as both activator and repressor, Gli2 and Gli3 are proteolytically processed to truncated forms, and there is evidence that at least Gli3 processing is inhibited by Hh [143–145]. The multiple clusters of phosphorylation sites found in Ci are also present in Gli proteins (Fig. 3) [112, 113]. A recent study demonstrated that Gli3 processing is also regulated by hyperphosphorylation-mediated binding of β-TRCP [146]. Hence, the biochemical mechanism underlying

Ci/Gli3 processing is conserved between *Drosophila* and vertebrates.

Although Gli1 is not processed, PKA phosphorylation appears to regulate its transcriptional activity [143]. A recent study showed that PKA phosphorylation of Gli facilitates β-TRCP binding, which regulates Gli protein stability [147]. Hence, β-TRCP-mediated ubiquitination targets Gli1 for complete degradation as opposed to selective proteolysis, raising an interesting question of what determines complete degradation versus selective proteolysis. The study by Huntzicker et al. [147] also uncovered a β-TRCP-independent but proteasome-dependent mechanism for Gli1 degradation. This degradation is mediated by a signal (degron) located in the N-terminal region of Gli1. Interestingly, related sequence can also be found in other Gli family proteins including Ci, raising a possibility that the dual degradation mechanism may regulate other Gli family members as well. Indeed, a previous study in *Drosophila* demonstrated a Slimb-independent but Cul3-dependent mechanism for degrading Ci in the posterior region of eye imaginal discs [148]. Of interest would be to determine if the N-terminal degron of Gli1 is recognized by a member(s) of the Cul3-based E3 ubiquitin ligases, which utilize BTB proteins as their substrate recognition subunits in an analogous fashion to Cul1-based SCF complexes utilizing F-box proteins [149].

Similar to Ci, the Gli proteins are also regulated by Su(fu) via direct protein-protein interaction [150–153]. Su(fu) impedes nuclear import of Gli1 and may also inhibit Gli transcriptional activity by recruiting corepressor complexes [127, 150, 151, 153]. The negative function of Su(fu) was further demonstrated by *Su(fu)* mutant mouse embryos that exhibit dramatic phenotypes similar to *ptc* mutant embryos [154, 155]. This is in contrast to Su(fu) mutant flies, which are viable and exhibit only mild defects [126]. These findings suggest that mammalian Su(fu) may have evolved as a major intracellular inhibitor of Gli activities.

Fu and a Cos2-related protein appear to play conserved roles in zebrafish [156, 157]; however, similar roles in mammalian systems have not yet been demonstrated. Surprisingly, mice lacking Fu do not exhibit any discernible Hh-related phenotypes [158, 159], suggesting that either there is functional redundancy between Fu and a distantly related kinase(s) or the Hh pathway might have diverged downstream of Smo.

In addition to the aforementioned regulatory mechanisms, several new components that control Gli activities have been identified by genetic studies in vertebrates. For example, a genetic screen in zebrafish identified *iguana* (*igu*), which encodes a zinc finger/coiled-coil domain protein, as a novel regulator of the Hh pathway [160, 161]. Interestingly, Igu has both positive and negative roles in the Hh pathway, and appears to act downstream of PKA to regulate nuclear translocation of Gli proteins.

Another interesting new component in the mammalian Hh signaling pathway is Rab23, a member of the Rab family of GTPases involved in vesicular trafficking. *Rab23*, also called *open brain*, was identified as a negative regulator of the Hh pathway in an N-ethyl-N-nitrosourea screen in mice [162]. Genetic epistasis studies placed Rab23 downstream of Smo and upstream of Gli. Although the mechanism of action remains unknown, Rab23 appears to suppress the Gli activator activity and promote the production of Gli3 repressor [162, 163].

Relaying the Hh signal to the intracellular signaling complex by Smo

How Smo transduces the Hh signal to the intracellular signaling components remained an enigma until recently. Smo is related to G-protein-coupled receptors; however, evidence for an involvement of trimeric G proteins in Hh signaling in physiological settings has been lacking [125]. Several recent studies demonstrated that Smo transduces the Hh signal by physically interacting with the Cos2-Fu-Ci complex [125, 164–166]. The association of Smo with the Cos2-Fu-Ci complex appears to be mediated by direct interaction between the Smo C-terminal intracellular tail (SmoCT) and the Cos2 cargo-binding domain. Smo lacking its C tail failed to transduce Hh signal whereas membrane-tethered forms of SmoCT have constitutive albeit low levels of Hh signaling activity [125, 164, 167].

Cell surface recruitment of intracellular signaling complexes through accumulated Smo is thought to cause dissociation of the Cos2-Ci-kinase complex and hence inhibition of Ci phosphorylation and processing [124]. Indeed, overexpressing a myristoylated SmoCT can cause dissociation of the Cos2-kinase complex and block Ci processing [124, 164, 167]. In addition, cell surface recruitment of Cos2-Fu-Ci may also lead to phosphorylation of Cos2, Fu, and Su(fu) [105]. Phosphorylation of Fu could activate its kinase activity [168], which in turn may phosphorylate Su(fu) and Cos2 to block their ability to downregulate Ci [70, 169]. The finding that Smo transduces the Hh signal by recruiting the Cos2 complex to the plasma membrane explains, at least in part, the positive role of Cos2 in the Hh pathway observed earlier [121, 122].

Not clear is whether vertebrate Smo utilizes a similar mechanism to transduce the Hh signal. Although a zebrafish homolog of Cos2 is involved in Hh signaling, it has not been shown to interact with Smo [156, 157]. The C-terminal region of SmoCT, which has been implicated in Cos2 binding in *Drosophila*, diverges considerably in vertebrates [83], raising a possibility that vertebrate Smo might not act by interacting with Cos2-related proteins. In contrast to what has been demonstrated for *Drosophila* Smo, a membrane-tethered form of vertebrate SmoCT does not have detectable Hh signaling activity [J. Briscoe,

personal communication]. Hence, how vertebrate Smo communicates with intracellular signaling components awaits further investigation.

Cilia and mammalian Hh signaling

Genetic studies in mice revealed that a number of components of the intraflagellar transport (IFT) machinery are required for Hh signaling and act between Smo and Gli proteins in neural tube patterning and limb development [170–172]. IFT proteins are essential for the assembly and maintenance of cilia and flagella [173]; hence, the finding that IFT components are required for Hh signaling implies that cilia mediate Hh signal transduction. Indeed, recent studies revealed that several mammalian Hh signaling components including Smo, Su(fu), and all three Gli proteins are localized to cilia [174–176]. In addition, cilia localization of Smo is disrupted in IFT mutants. These observations suggest that IFT proteins may play a permissive role in Hh signaling by concentrating multiple Hh pathway components to a specialized subcellular compartment, possibly for more efficient protein-protein interactions. However, one cannot rule out the possibility that some of the IFT proteins could also directly participate in Hh signal transduction independent of their roles in cilia formation. Cilia localization of Smo is stimulated by Hh and inhibited by an Hh-pathway-specific inhibitor [174]. In support of the functional significance of cilia localization of Smo, an oncogenic form of Smo is constitutively localized in cilia, whereas a mutant form of Smo lacking a conserved cilia localization motif fails to support Hh signaling [174–176]. These results suggest that Hh activates Smo at least in part by promoting its cilia localization. Hence, in mammals, cilia may function as a signaling center for Hh signal transduction. In the absence of Hh, cilia localization of Gli3 could be essential for its processing to generate Gli repressors, as Gli repressor function and Gli3 processing are compromised in IFT mutants [171, 172]. Hh induces Smo translocation to cilia, allowing Smo to engage in a productive signaling complex with downstream pathway components to inhibit the production of Gli repressors and stimulate the activity of Gli activators.

In *Drosophila*, mutants lacking IFT function are viable and do not exhibit any patterning defects associated with Hh signaling except that they exhibit sensory behavior defects, consistent with the role of IFT in the forma-

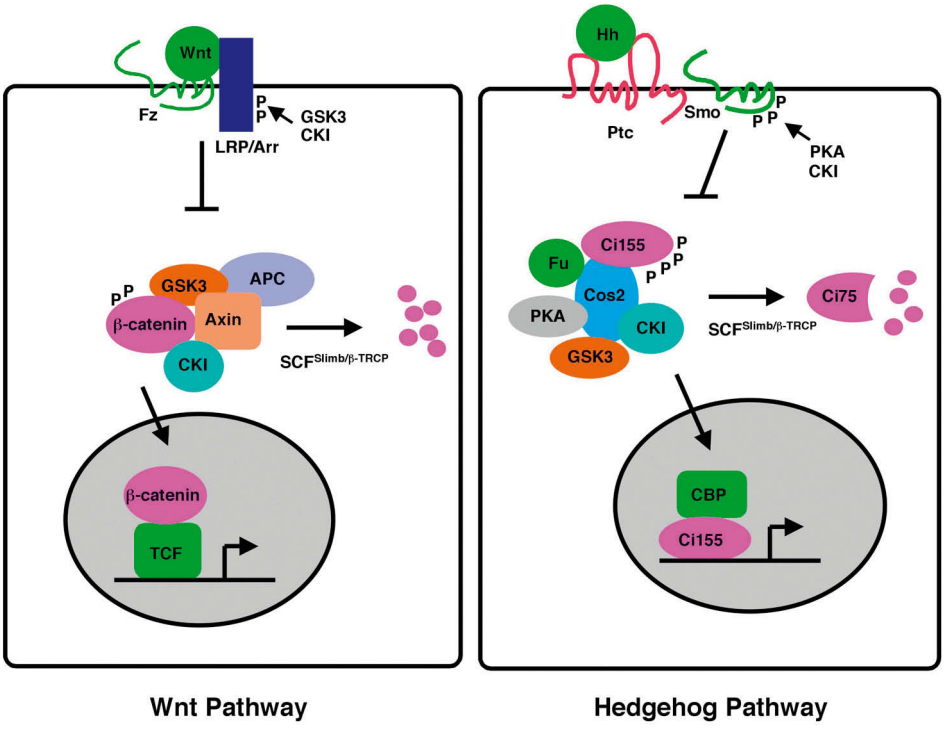

Figure 4. Similarities between Hh and Wnt pathways. In the absence of Wnt, Axin recruits CKI and GSK3β to phosphorylate β-catenin, which promotes β-catenin degradation through the $SCF^{Slimb/\beta\text{-}TrCP}$-mediated ubiquitin/proteasome pathway. In the presence of Wnt, Wnt induces the formation of the Fz/LRP receptor complex and LRP phosphorylation by GSK3β and CKI, leading to the recruitment of Axin complex to the plasma membrane and the inhibition of β-catenin phosphorylation and degradation. In the absence of Hh, Cos2 recruits PKA, GSK3β, and CKI to phosphorylate Ci, which targets Ci for $SCF^{Slimb/\beta\text{-}TrCP}$ proteasome-mediated proteolysis. Binding of Hh to Ptc induces phosphorylation of Smo by PKA and CKI, which results in cell surface accumulation and activation of Smo. Smo recruits Cos2 complex to the plasma membrane, leading to the disassembly of the Cos2-kinase complex. As a consequence, Ci phosphorylation and processing are inhibited.

tion of ciliated sensory neurons [177, 178]. Morpholino knockdown experiments as well as mutagenesis studies in zebrafish also failed to reveal any role for IFT components in Hh signaling [179, 180]. Although one cannot rule out the possibility that a maternal contribution could have masked the effect of zygotic mutations in zebrafish IFT components, the involvement of cilia in Hh signaling could be a recently evolved mechanism restricted to mammals.

Similarities between Hh and Wnt pathways

The canonical Wnt pathway is another majoring signaling pathway widely used in animal development [181]. Wnt binds its receptor Frizzled (Fz), which is a 7-TM protein related to Smo, and coreceptor LRP5/6 (Arrow in *Drosophila*) to initiate a signaling cascade [182]. Despite the difference in their ligand-receptor relationships, Hh and Wnt pathways bear several interesting parallels in their signal transduction cascades because they not only share a number of common components but also share a similar logic of pathway regulation (Fig. 4). The first identified common pathway component is Slimb/β-TRCP, whose loss-of-function mutations led to activation of both pathways [116]. GSK3β, a negative component in the Wnt pathway, also plays a negative role in the Hh pathway [111, 112]. Both Hh and Wnt pathways employ CKI at multiple levels of their signal transduction cascades [112, 113, 183, 184]. In addition, there is evidence that Su(fu) also regulates the Wnt pathway by binding to β-catenin [185].

In quiescent cells, both pathways employ large protein complexes to bring kinases and their substrates in close proximity, resulting in phosphorylation and proteolysis of the transcription factor (Ci) or transcriptional effector (β-catenin) [124, 184, 186]. In response to ligand stimulation, both pathways recruit the cytoplasmic signaling complex to the cell surface to cause disassembly of the complex, leading to dephosphorylation and stabilization of the transcription factor/effector [124, 182, 187]. Even though both pathways use common kinases to control the phosphorylation and proteolysis of their transcription factor/effector, these kinases, together with their substrates, form distinct signaling complexes assembled by pathway-specific scaffolding proteins. Pathway activation is achieved by a specific interaction between the receptor system and a scaffolding protein. Hence, each pathway only controls the pool of kinases in the same complex with the pathway effector, leading to pathway-specific regulation of substrate phosphorylation.

Another interesting parallel between the *Drosophila* Hh and Wnt pathways is that the same set of kinases play opposing roles and phosphorylate distinct sets of substrates depending on signaling states. In the *Drosophila* Hh pathway, PKA and CKI phosphorylate Ci in the absence of Hh to inhibit pathway activation [112, 113]. In the presence of Hh, PKA and CKI phosphorylate Smo to stimulate its signaling activity [98–100]. Similarly, in the absence of Wnt, GSK3β and CKI phosphorylate β-catenin to inhibit Wnt signaling. Upon Wnt stimulation, GSK3β and CKI phosphorylate the Wnt coreceptor LRP5/6 to activate signaling [188, 189]. These observations have raised the important question of how different substrates are chosen by the same kinases in different signaling states. One solution, as suggested by the studies on the Wnt pathway, is that each pathway may utilize two distinct pools of kinases, one in close proximity to the transcription factor/effector and the other to the membrane reception system [188, 189].

How are different thresholds of Hh activity interpreted by signal-receiving cells?

In *Drosophila* wing development, threshold responses to the Hh morphogen appear to be mediated by differential regulation of the two forms of Ci. Accumulation of Ci155 and expression of *dpp* occur in broader domains than activation of *ptc* and *en*, suggesting that low levels of Hh suffice to block Ci processing whereas higher levels of Hh are required to stimulate the activity of Ci155 to turn on *ptc* and *en* (Fig. 1a, 5c). Blockage of Ci processing in *slimb* mutant clones induces *dpp* but not *ptc*, whereas *slimb Su(fu)* double-mutant cells activate *ptc*, suggesting that activation of Ci155 is achieved, at least in part, by alleviating the repression imposed by Su(fu) [110]. In addition, only high levels of Hh appear to antagonize Su(fu) to stimulate the maturation of Ci155 into a labile hyperactive form (Fig. 5c) [126]. Activation of Ci155 may also involve dephosphorylation of Ci155, as phosphorylation of Ci by PKA not only promotes Ci processing but also inhibits the activity of Ci155 independent of Ci processing [110]. Hence increasing levels of Hh signal may cause decreasing levels of Ci repressor activity and increasing levels of Ci activator activity (Fig. 5a).

A similar model has been proposed for regulating Gli proteins by a graded Shh signal [190, 191]. In the neural tube, loss of Gli2, a major source of Gli activator, results in loss of progenitor cells of most ventral character whereas progenitor cells of ventral-lateral and lateral characters still form [132, 133]. Ventral-lateral and lateral neural progenitors lost in *Shh* mutants are partially restored in *Shh Gli3* double mutants [192, 193]. These results suggest that high levels of Shh specify the ventral-most progenitors via stimulating Gli2 (and perhaps Gli3) activator, whereas low levels of Shh specify ventral-lateral and lateral progenitors through inhibiting Gli3 repressor. Hence, the Shh gradient could generate a gradient of Gli activator activity and a reverse gradient of Gli repressor activity (Fig. 5a).

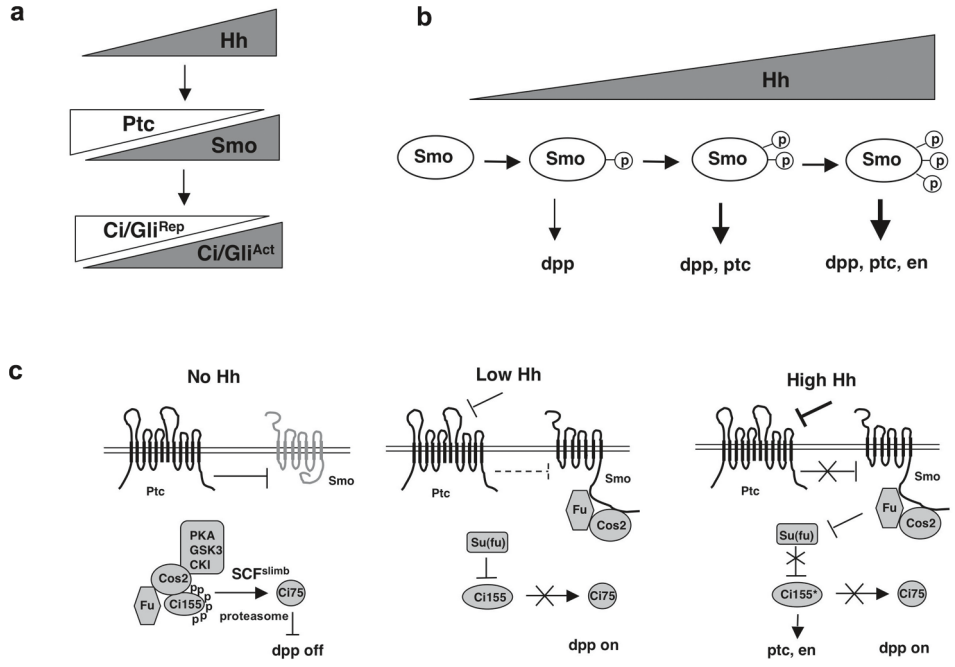

Figure 5. Transduction of different levels of Hh signal. (*a*) Increasing levels of Hh cause diminishing Ptc activities, resulting in increasing Smo activities, which in turn leads to decreasing levels of Ci/Gli repressor and increasing activities of Ci/Gli activator in the nucleus. (*b*) A proposed model for regulating *Drosophila* Smo activity by differential phosphorylation. Increasing levels of Hh could cause increasing levels of Smo phosphorylation, leading to increasing Smo activities. (*c*) In the absence of Hh, Ptc inhibits Smo. The majority of Ci155 undergoes proteolytic processing to generate Ci75. The inhibition of Smo by Ptc is partially alleviated by low levels of Hh (indicated by the dashed line), Smo recruits the Cos2-Fu complex to the plasma membrane and inhibits Ci processing. As a consequence, Ci75 is not produced and *dpp* is derepressed. However, the transcriptional activity of Ci155 is still inhibited by Su(fu). High levels of Hh completely block Ptc, resulting in a further increase in Smo signaling activity. Hyperactive forms of Smo stimulate phosphorylation and activity of bound Fu, which in turn antagonizes Su(fu) to activate Ci155, leading to high-threshold responses such as the expression of *ptc* and *en*.

In vertebrate limb development, Shh controls the polarity of digits mainly through the regulation of Gli3 repressor activity whereas Gli activator activity is dispensable [138, 194, 195]. In this context, the graded influence of Shh on digit identity is mediated by an anterior-posterior gradient of Gli repressor activity. Indeed, direct biochemical measurement revealed that Gli3 processing is differentially regulated by Shh along the anterior-posterior axis so that an anterior high and posterior low Gli3 repressor gradient forms in the developing limb [144].

How are distinct levels of Hh sensed and transmitted at the cell surface? In *Drosophila* wing discs, Hh induces cell surface accumulation and activation of Smo through phosphorylation [87, 98–100]. Interestingly, the extent of Smo phosphorylation appears to determine both the abundance of Smo at the cell surface and its signaling potency, raising an interesting possibility that different thresholds of Hh are transduced by differentially phosphorylated isoforms of Smo (Fig. 5b) [98]. Low Hh only induces low levels of Smo phosphorylation, which suffices to activate low threshold responses such as *dpp* expression, whereas high Hh induces high levels of Smo phosphorylation, leading to activation of high threshold responses including *ptc* and *en* expression. Increasing Smo phosphorylation and cell surface accumulation might facilitate Smo dimerization/oligomerization, which could be essential for transducing high levels of Hh signaling activity. For example, Smo dimerization/oligomerization might trigger phosphorylation and activation of Fu, which is required for high-threshold Hh responses [126, 129]. Consistent with this idea, membrane-associated SmoCT stimulates low-threshold responses but prevents high-threshold responses, as SmoCT might not be able to dimerize/oligomerize and hence could lock the bound Fu in an inactive state [164, 167].

Graded Smo activity is likely to reflect a reverse Ptc activity gradient (Fig. 5a). How does increasing Hh activity generate a reverse Ptc activity gradient? Hh blocks Ptc activity by direct binding; hence, the level of Ptc activity was thought to be determined by the number of unbound Ptc molecules [86]. However, a recent study in *Drosophila* demonstrated that Hh-bound Ptc could influence the inhibitory activity of unbound Ptc [196]. By expressing various combinations of Ptc variants that either no longer bind Hh or constitutively bind Hh, Casali and Struhl [196] showed that a roughly twofold change in the ratio of liganded to unliganded Ptc can distinguish between on and off states of the Hh pathway, suggesting that the Hh gradient is measured by the ratio of Hh-bound to unbound Ptc proteins rather than the absolute number of unbound

Ptc. Accordingly, increasing Hh leads to an increasing ratio of bound to unbound Ptc and diminishing Ptc activity even though the absolute level of Ptc increases in response to Hh. How liganded Ptc titrates out the activity of unliganded Ptc is unknown but an attractive model, as suggested by Casali and Struhl, is that Ptc may function as a multimer to catalytically inhibit Smo, and Hh binding to any Ptc molecule in a given multimer could block the activity of the entire multimer. Alternatively, Hh-bound Ptc could counteract the catalytic activity of unbound Ptc or titrate out limited downstream effector of Ptc.

Conclusion and future prospects

Studies in the past two decades have provided a wealth of information regarding the Hh pathway, which serves as a framework for understanding how Hh morphogen gradients are generated and how different concentrations of Hh morphogen are interpreted to generate distinct developmental programs. However, important questions regarding many aspects of the Hh signaling mechanism and its physiological roles still remain. For example, how does Ptc exert its negative role on Hh signaling? Does Ptc function as a small molecule transporter to regulate the distribution of an as yet unidentified Smo agonist or antagonist? If so, identifying such a physiologically relevant Smo agonist or antagonist may pose a big challenge. How Smo is regulated and how Smo activates the intracellular signaling complex is still not fully understood. There is increasing evidence that these mechanisms may differ between *Drosophila* and mammals. For example, cilia localization of Smo appears to be important for mammalian Hh signaling but dispensable for *Drosophila* Hh signaling. What is the trafficking machinery that regulates Smo localization in cilia? Is it related to the mechanism responsible for regulating Smo cell surface accumulation in *Drosophila*? Does cilia localization facilitate a productive interaction between Smo and intracellular signaling complexes? The mammalian Hh signal transduction pathway is still not well defined and the lack of any Hh-related phenotypes in mice lacking Fu suggests that the Hh signal transduction pathway may have diverged downstream of Smo. The biological effects of Hh signaling could be cell fate determination, pattern formation, cell proliferation, or axon guidance, depending on the developmental contexts. Moreover, increasing evidence suggests that Hh signaling responses may also change with time [197, 198]. The mechanisms underlying the spatial (tissue-specific) and temporal (developmental-stage-specific) regulation of Hh responses are poorly understood. Future studies will undoubtedly address these and many other unsolved puzzles in the Hh signaling pathway.

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