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Noncanonical Hedgehog Signaling

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Abstract

The notion of noncanonical hedgehog (Hh) signaling in mammals has started to receive support from numerous observations. By noncanonical, we refer to all those cellular and tissue responses to any of the Hh isoforms that are independent of transcriptional changes mediated by the Gli family of transcription factors. In this chapter, we discuss the most recent findings that suggest that Patched1 can regulate cell proliferation and apoptosis independently of Smoothened (Smo) and Gli and the reports that Smo modulates actin cytoskeleton-dependent processes such as fibroblast migration, endothelial cell tubulogenesis, axonal extension, and neurite formation by diverse mechanisms that exclude any involvement of Gli-dependent transcription. We also acknowledge the existence of less stronger evidence of noncanonical signaling in *Drosophila*.

I. Definition

The main players of the so-called “canonical” hedgehog (Hh) signaling pathway in mammals have been the objects of intense study in the last decade. We have learnt that in the absence of any Hh ligand (Sonic (Shh), Indian (Ihh), or Desert (Dhh)), the receptor Patched1 (Ptc1) prevents activation of the seven-transmembrane protein Smoothened (Smo) by inhibiting its translocation into the primary cilium. In the presence of Hh, Smo accumulates in the cilium in an active conformation that initiates a complex signaling network that results in activation of the Gli family of transcription factors (Riobo and Manning, 2007). However, a vast number of recent studies have demonstrated that not all Hh signaling proceeds through Gli activation, and we have named this subset of Gli-independent responses “noncanonical” Hh signaling (Jenkins, 2009).

In this chapter, we review the current evidence for the existence of at least two distinct classes of noncanonical Hh signaling (Fig. 3.1): a Type I, which works through functions of Ptc1 that are unrelated to its inhibitory activity on Smo, and a Type II, which operates through Smo functions (or activities) beyond Gli regulation.

II. Type I Noncanonical Signaling: Pathways Engaged Exclusively by Ptc1

A. Apoptosis

Apoptosis, or programmed cell death, is an essential process for development, tissue homeostasis, and tumor suppression. Hh opposes apoptosis by at least three mechanisms: increased expression of pro-survival genes (such as Bcl-2) through the modulation of Gli transcription (Cayuso *et al.*, 2006; Katoh and Katoh, 2009), activation of the survival kinase Akt (Riobo *et al.*, 2006a), and Type I noncanonical pathway.

Growing evidence suggests that Ptc1 can promote or induce apoptosis independently of the canonical pathway (Chang *et al.*, 2010; Chinchilla *et al.*, 2010; Mille *et al.*, 2009; Thibert *et al.*, 2003). In the absence of an Hh ligand, ectopic expression of Ptc1 induces apoptosis in

293T and neuroepithelial cells (Thibert *et al.*, 2003). This process is independent of the downstream elements of the canonical Hh pathway, as overexpression of Smo cannot prevent cell death. Soft-agar assays also showed that Ptc1 function as a tumor suppressor might be related to its capacity to induce apoptosis. In line with those findings, we found that siRNA-mediated depletion of Ptc1 in endothelial cells extends cell survival in the absence of serum and to limit caspase-3 activation (Chinchilla *et al.*, 2010). We proposed that the pro-apoptotic effect of Ptc1 is exerted through a Type I noncanonical pathway for the following reasons: (1) lack of detectable canonical Hh signaling in endothelial cells both *in vitro* and *in vivo* (Chinchilla *et al.*, 2010; Lavine *et al.*, 2008; Pola *et al.*, 2001); (2) lack of effect of the Smo antagonists SANT-1, cyclopamine, and KAAD-cyclopamine to prevent the Shh-dependent reduction in caspase-3 activity (Fig. 3.2); and (3) inability of the Smo agonist (SAG) to mimic the antiapoptotic effect of Shh (Fig. 3.2).

Ptc1 has been proposed to function as a dependence receptor, meaning that cell survival is dependent on the presence of the ligand when the receptor is expressed (Bredesen *et al.*, 2004; Mehlen and Thibert, 2004). There are more than 10 dependence receptors, most of which are involved in neural development and tumor suppression. Ptc1 shares the common features for the dependent receptors: (i) in the presence of ligand, receptors transduce signal, leading to the activation of the canonical signaling transcription pathway; (ii) in the absence of ligand, dependence receptors activate a different (noncanonical) pathway and induce apoptosis, which can be reverted by reintroducing the respective ligand; (iii) all dependence receptors contain the DART (dubbed dependence-associated receptor transmembrane) motif (del Rio *et al.*, 2007); and (iv) in order to induce cell death, the receptor needs to be cleaved by a caspase to expose a pro-apoptotic domain. Thibert *et al.* showed that the C-terminal cytoplasmic domain of Ptc1 is a substrate for caspase-3, -7, and -8 (Thibert *et al.*, 2003). Cleavage of Ptc1 is essential for induction of apoptosis because mutation of the caspase site (D1392N) abolishes Ptc1-induced cell death. Moreover, a truncated mutant constitutively exposing the caspase site Ptc1 (1–1392) induces cell death regardless of the presence of Shh, while full-length Ptc1 pro-apoptotic activity is exquisitely sensitive to Shh, demonstrating the functional importance of caspase cleavage. Further studies shed light on this mechanism, revealing that Ptc1 induces apoptosis by recruitment of a pro-apoptotic complex that includes caspase-9, DRAL, and TUCAN-1 to the C-terminal tail (Mille *et al.*, 2009). However, it is still unknown whether caspase-9 is involved in the generation of the pro-apoptotic Ptc1 (1–1392) and whether recruitment of the complex is a requisite for or a consequence of the cleavage at D1392.

It is worth noting the importance of the C-terminal domain (CTD) of Ptc1, containing 273 aa, in the regulation of cell death. The CTD alone (soluble) or a fragment truncated at the caspase cleavage site, Ptc1 (1165–1392), induces cell death in 293T and neuroepithelial cells at similar extent as the full-length Ptc1 (Thibert *et al.*, 2003). Mutation of the cleavage site (D1392N) acts as a dominant negative inhibitor for Ptc1-induced apoptosis. The CTD was suggested to also be a critical regulator of degradation and localization of Ptc1 (Chang *et al.*, 2010; Kawamura *et al.*, 2008; Lu *et al.*, 2006). In addition, it was recently reported that Ptc1 undergoes proteolytic processing at the C-terminus and the soluble CTD translocates to the nucleus and mediates a new form of signal transduction (Kagawa *et al.*, 2011). In *Drosophila*, the CTD is indispensable for repression of the canonical pathway when Hh is absent (Johnson *et al.*, 2000). However, studies using mammalian cells and mouse models suggest that in mammals, the CTD is not required for the canonical signal transduction but most likely has a distinct function in apoptosis and/or regulation of proliferation (Makino *et al.*, 2001; Mille *et al.*, 2009; Nieuwenhuis *et al.*, 2007; Sweet *et al.*, 1996; Thibert *et al.*, 2003). A spontaneous Ptc1 mouse mutant has a frameshift in the CTD that gives rise to retention of only the first 53 aa after the last transmembrane domain and addition of 63 nonsense residues. Named mesenchymal dysplasia (*Ptc1^{mes}*), the mice exhibit

epidermal hyperplasia, increased musculature and preaxial polydactyly (Makino *et al.*, 2001; Nieuwenhuis *et al.*, 2007; Sweet *et al.*, 1996). Compared with $Ptc1^{-/-}$ homozygotes, which die between embryonic days 9 and 10.5 with severe neural tube defects as a result of hyperactivation of the canonical Hh signaling (Goodrich *et al.*, 1997), the $Ptc1^{mes/mes}$ embryos exhibit normal spinal cord development, suggesting that most of the CTD is absolutely dispensable for canonical signaling in mammalian embryogenesis. Studies using $Ptc1^{-/mes}$ mice (Makino *et al.*, 2001) also demonstrated that $Ptc1^{mes}$ can substitute a wild-type allele with regard to the canonical Hh pathway (Nieuwenhuis *et al.*, 2007). However, the same report also indicates that the CTD plays an important role in body size control and skin development since the $Ptc1^{mes/mes}$ animals show a marked increase in proliferation of the basal cell layer of the skin (Nieuwenhuis *et al.*, 2007). While they did not observe changes in cell death, the result is inconclusive due to the technical limitations on measuring the apoptosis in that system.

A different mouse model to study the function of Ptc-1 CTD in tumor suppression is a natural polymorphism (T1267N) responsible for a 600-fold change in susceptibility to develop Ras-induced skin squamous cell carcinomas in FVB versus C57BL/6 mice. The polymorphism was shown to reduce the binding of Ptc1 to Tid1, a tumor suppressor protein (Wakabayashi *et al.*, 2007). Direct interaction of Ptc1 with other potential apoptosis-related proteins has also been reported, as mentioned above (Mille *et al.*, 2009). Shh also activates the ERK pathway independently of Smo, probably through modulating the interaction of the SH3 interacting motif of Ptc1 CTD with SH3-containing proteins like Grb2 (Chang *et al.*, 2010). Taken together, these findings strongly support the notion that the mammalian Ptc1 CTD functions in the induction of cell death independently of the canonical Hh signaling pathway.

B. Cyclin B1 and cell cycle regulation

A large number of studies have demonstrated that the canonical Hh signaling pathway plays an essential role in proliferation of some cell types, the best-characterized example being postnatal proliferation of cerebellar granule precursor cells (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Induction of *n-myc* and *cyclin D1* by Gli-dependent transcription mediates the mitogenic effect of Shh in those cells (Kenney and Rowitch, 2000; Kenney *et al.*, 2003). Cyclins are a family of proteins and act as regulatory subunits for cyclin-dependent kinases, and these complexes are responsible for regulating cell cycle progression through the cell cycle by directly affecting a cell's transcriptional regime (Murray, 2004). Dynamic modulation of the expression, phosphorylation, interactions, and subcellular localization of these complexes and their individual subunits all influence the activity of these complexes, and thus cell cycle progression. The Cyclin B1–Cdk1 complex (also known as M-phase promoting factor (MPF)) is specifically involved in the G2/M checkpoint, and its activation is essential for mitotic progression (Takizawa and Morgan, 2000). The activated form of this complex contains a series of phosphorylated serine residues on Cyclin B1. Interestingly, noncanonical Hh signaling also regulates the cell cycle at the level of Cyclin B1. An early report detected Ptc1 in a yeast two-hybrid assay designed to identify binding partners of phosphorylated Cyclin B1 (Barnes *et al.*, 2001). Biochemical analysis revealed that Ptc1 interacts with phospho-Cyclin B1, but not with the unphosphorylated protein, through the large intracellular loop between transmembrane domains 6 and 7. Overexpression of Ptc1 in 293T cells resulted in a redistribution of Cyclin B1 from the nucleus to the cytoplasm and reduced cell proliferation, which was restored by addition of a Cyclin B1 mutant that cannot be phosphorylated. In a later study, the same group demonstrated that the $Ptc1^{Q688X}$, a truncation mutant found in some basal cell carcinomas (BCCs), is unable to interact with Cyclin B1 and, unlike wild-type Ptc1, did not reduce the mitotic index (Barnes *et al.*, 2005).

The identification of Ptc1 as a binding partner of Cyclin B1 suggested a new mode of cell cycle regulation by Hh signaling. Indeed, the presence of Shh disrupts Ptc1 interaction with Cyclin B1, allowing its nuclear translocation and promoting completion of mitosis. The regulation of Cyclin B1 by Shh/Ptc1 was not limited to overexpression experiments but was also evident with the endogenous proteins. These results support the notion that Shh regulates proliferation by modulating the interaction between Ptc1 and phosphorylated Cyclin B1 via a noncanonical signaling pathway.

While those studies were conducted *in vitro*, several histological observations are in line with those findings. Work characterizing Shh expression pattern in human urinary tract development revealed a correlation between Shh expression and Cyclin B1 localization: higher levels of Shh correlated to cytoplasmic and nuclear Cyclin B1, while when Shh levels go down Cyclin B1 relocates to the apical membranes together with Ptc1 (Jenkins *et al.*, 2007). Another study showed that epidermal ablation of Ptc1 in mice coincided with an increase in nuclear Cyclin B1, when compared to normal epidermis (Adolphe *et al.*, 2006). Wang *et al.* demonstrated that epidermal tumors that arise in Ptc1^{+/-} mice are derived from hair follicle progenitor cells and are characterized by increased nuclear Cyclin B1. This is different from tumors developed in the SmoM2 mice model, which express a constitutively active form of Smo (Wang *et al.*, 2011; Youssef *et al.*, 2010). They suggested that nuclear Cyclin B1 and not increased Smo activity results in hair follicle-derived tumors. These data allude to a possible mechanism by which loss of Ptc1 can lead to tumorigenesis independently of the canonical Hh pathway, though more work will need to be done to determine if this alternative pathway is sufficient to induce tumor formation or synergizes with enhanced Gli1 activity.

Finally, studies in zebrafish provided some mechanistic insight into the regulation of Ptc1–Cyclin B1 interaction (Jiang *et al.*, 2009). The authors reported that dissociation of the Ptc1–Cyclin B1 complex after Shh activation involves the formation of another complex between Ptc1 and G-protein receptor kinase-2 (GRK2). GRK2 silencing by siRNA abrogated the ability of Shh to redistribute Cyclin B1 to the nucleus, and exogenous GRK2 expression was able to rescue the antiproliferative phenotype associated with Ptc1 overexpression. Taken together, these results suggest that Shh stimulation may induce a conformational change in Ptc1 that increases its affinity for GRK2 at the expense of its interaction with Cyclin B1.

In summary, there is significant evidence for a noncanonical role for Ptc1 in cell cycle regulation through Cyclin B1 in an apparently Smo- and Gli-independent manner (Type I noncanonical signaling). The relative contribution of this noncanonical pathway on overall cell growth and survival mediated by Hh signaling remains to be established.

III. Type II Noncanonical Signaling: Pathways Engaged by Smoothened

A. Small GTPases

The discovery that Smo is a functional G-protein-coupled receptor (GPCR) with selectivity toward heterotrimeric G_i proteins had profound implications on Hh signaling (Riobo *et al.*, 2006b). Several GPCRs have been shown to activate small GTPases, which play key roles in cytoskeletal reorganization (Ridley, 2006). Small GTPases are monomeric G proteins that act as molecular switches and, thus, rapidly regulate cellular processes (Vetter and Wittinghofer, 2001). Activated by guanine exchange factors (GEFs), they bound GTP in their “on” state, when they are able to interact with and activate their molecular targets. GTPases are then inactivated by nucleotide hydrolysis to GDP, an intrinsically slow process, enhanced by GTPase-activating proteins (GAPs). Small GTPases can be divided into four families: Ras, Rho, Arf, and Rab. The Rho family, which has been shown to mediate cytoskeletal reorganization, is further divided into three subfamilies: Rho, Rac, and Cdc42.

These subfamilies differentially modulate distinct cytoskeletal rearrangements through polymerization of actin filaments, and these unique rearrangements can then regulate coordinated movement of the cell.

Recent work demonstrated that Hh proteins promote actin stress fiber formation and endothelial cell tubulogenesis in a Smo-dependent manner in endothelial cells (Chinchilla *et al.*, 2010). Interestingly, while endothelial cells express all key proteins of the Hh signaling pathway, they are unable to activate the canonical pathway in response to Hh ligands, as measured by activation of the marker Gli-target genes *gli1* or *ptch1*. However, the human umbilical vein endothelial cells (HUVECs) and human cardiac microvascular endothelial cells (HMVECs) utilized in this study exhibit strong morphological changes in response to Shh, Ihh, or Dhh stimulation. In this system, stimulation by Hh ligands results in the formation of actin stress fibers within minutes of treatment, thus suggesting a noncanonical pathway based on the time course and the lack of detectable Gli-dependent transcription. The acute cytoskeletal changes were shown to depend on Smo- and G_i-protein-mediated activation of the small GTPase RhoA (Fig. 3.3). In addition to short-term changes, stimulation of endothelial cells by the three Hh ligands resulted in a significantly increase in tubulogenesis after several hours by a process that was also the result of Smo-mediated, G_i-dependent activation of RhoA. Interestingly, this study also demonstrated that, unlike the canonical paradigm whereby the different Hh ligands exhibit distinct potencies for Gli activation (Shh>>Ihh>Dhh) (Pathi *et al.*, 2001), endothelial cells responded equally to all three ligands in terms of stress fiber formation, RhoA activation, and tubulogenesis.

Another study demonstrated that Shh stimulates fibroblast migration via activation of small Rho GTPases (Polizio *et al.*, 2011). In this system, both Shh and purmorphamine (PUR, a potent SAG) enhance migration of NIH-3T3 fibroblasts but have no effect on Smo^{-/-} MEFs. Inhibition experiments demonstrated that activation of both G_i and PI3K by Smo was necessary for cell migration and activation of RhoA and Rac1. To confirm that these effects were indeed noncanonical, the canonical pathway was inhibited by the expression of Gli3R, the repressor form of the Gli3 transcription factor. Effective inhibition of the canonical pathway was confirmed, but expression of Gli3R did not perturb Shh- or PUR-mediated migration, indicating that fibroblast migration is affected by a noncanonical Hh signaling pathway.

While these studies provide solid evidence that Smo-dependent, noncanonical Hh signaling elicits specific cellular responses via the activation of small GTPases, the mechanism by which Smo or G_i activates RhoA and Rac1 has not been yet established. A recent report looking at Hh signaling during dendritic spine formation sheds some light on this issue (Sasaki *et al.*, 2010). Tiam1 (T-lymphoma invasion and metastasis) is a GEF specific for Rac1 that localizes to dendritic spines via interactions with spine-specific proteins. The Tiam1–Rac1 cascade is activated in response to Shh stimulation, without parallel activation of the canonical Hh pathway. Results indicated that both stimulation by Hh ligand and depletion of Smo via siRNA resulted in an increase in spine formation. Subsequent binding studies demonstrated that Smo interacts directly with Tiam1 and sequesters it, thus preventing Rac1 activation. This complex dissociates upon Smo activation, which in turn allows Tiam1 to activate Rac1. While these studies do not ascertain the requirement of G_i activity, they do provide a direct mechanism by which Smo activation leads to Rac1 activation. It will be interesting to see if these types of interactions are common to Smo-mediated activation of small GTPases and whether or not these events are G_i-dependent.

In summary, there is considerable evidence for Smo-dependent, noncanonical Hh signaling that is mediated through the activation of small GTPases. Future studies designed to elucidate the exact mechanisms by which Smo activates these molecular switches have the

potential to identify specific molecular targets for modulating noncanonical Hh signaling responses as well as provide insights into the diverse phenotypes observed when Hh signaling is dysregulated.

B. Src activation in the axonal growth cone

The identification of a noncanonical Hh signaling pathway playing a role in cytoskeleton remodeling is reminiscent of the identification, over the past few years, of noncanonical Wnt and BMP signaling pathways. Before the identification of these noncanonical signaling pathways, all of the functions of Hh isoforms were thought to be executed by alteration of gene expression by the Gli family of transcription factors. Recently, it has been demonstrated that Shh can signal through stimulating the activity of Src family kinase (SFK) members in a Smo-dependent manner to control axon guidance (Yam *et al.*, 2009). In this study, axons of dissociated commissural neurons placed in a Shh gradient turned rapidly toward increasing concentrations of Shh in a transcriptional-independent manner. Instead, Shh stimulates the activity of SFK members (Src and Fyn) in a Smo-dependent fashion. This study established the existence of a transcription-independent Shh signaling pathway, which acts locally at the axonal growth cone. Through this pathway, Shh gradients can elicit a rapid and spatially polarized response within the growth cone, without retrograde signaling to elicit transcription in the nucleus to induce global gene expression changes in the cell. It has been proposed that a graded activation of SFKs in response to a Shh gradient mediates changes in the actin cytoskeleton (Liu *et al.*, 2007; Robles *et al.*, 2005; Suter and Forscher, 2001). However, the regulation of Src by a noncanonical Hh pathway appears to be even more complex. In a screening for Ptc1-interacting proteins containing SH3 domains, it was found that the C-terminal tail of Ptc1 (1163–1435) binds to Src (Chang *et al.*, 2010). These findings define a novel signaling cascade operating through SH3 domain-containing proteins that could be directly stimulated by the Hh ligands. Together, both studies suggest that Shh is able to stimulate Src activity via both Type I and Type II noncanonical signaling pathways, and that the latter is preferred in axonal guidance.

Contributions of the canonical Hh pathway to Src activation are unlikely since (1) Hh-responsive transcriptional reporters have failed to identify Src or Fyn as components of the canonical Hh signaling pathway (Lum *et al.*, 2003; Nybakken *et al.*, 2005; Varjosalo *et al.*, 2008) and (2) SFK mutant mice lack phenotypes reminiscent of dysregulated canonical Shh signaling (Kuo *et al.*, 2005).

C. Arachidonic acid metabolites

Arachidonic acid (ARA) is an important signaling molecule that mediates many cellular functions including inflammation and actin remodeling (Glenn and Jacobson, 2002; Peppelenbosch *et al.*, 1993). ARA is synthesized in the cell upon receptor stimulation and can act directly as a second messenger or can be further metabolized into leukotrienes and prostaglandins by the action of lipoxygenase or cyclooxygenase, respectively (Funk, 2001).

Recent studies have found that ARA metabolites, particularly leukotrienes synthesized by the lipoxygenase pathway, are involved in Gli-independent Hh signal transduction. In fibroblasts, Shh induces the formation of lamelli-podia and promotes migration (Bijlsma *et al.*, 2007; Polizio *et al.*, 2011). A role of leukotrienes was proposed since these responses were partially inhibited by treatment with 5-lipoxygenase inhibitors, could be mimicked by addition of ARA to cell culture medium, and the ARA metabolite leukotriene was upregulated in the cells following Shh stimulation (Bijlsma *et al.*, 2007). These results strongly support the speculation that ARA, together with trimeric G-protein-dependent activation of RhoA and Rac1, is one of the signals that mediate Shh-induced cytoskeletal rearrangements. This study also demonstrated that Shh-induced cell migration is Smo

dependent but Gli independent (Type II noncanonical pathway). The authors claimed the following reasons for their conclusion: (1) acute response (only minutes after addition of Shh) and (2) insensitivity to overexpression of Sufu, a negative regulator of the Gli transcription factors.

The same group also reported that leukotrienes mediate the formation of neurite projections in motor neurons differentiated from embryonic stem cells (ESCs) treated with Shh (Bijlsma *et al.*, 2008). Neurite formation is also Gli independent and mediated by Smo, since this response occurs in wild type and ESC deficient in various combinations of Gli transcription factors, but not in Smo-deficient ESCs. In conclusion, these studies point to ARA and leukotrienes as novel players in Type II noncanonical Hh signaling in fibroblast migration and neuronal development.

D. Calcium transients

Calcium ions regulate a vast number of neuronal cell functions, including proliferation, differentiation, apoptosis, and migration (Berridge, 1998; Komuro and Rakic, 1996; LoTurco *et al.*, 1995). Stimulation of transient elevations of intracellular calcium (Ca^{2+}) activates protein kinases, regulates transcription, and influences motility and morphology (Catterall, 2010). It is not surprising that Ca^{2+} has also been shown both to influence expression of some Hh pathway genes and to mediate some cellular responses to Hh signaling.

Calcium has repeatedly reported to induce expression of Hh ligands. It was reported that Ca^{2+} chelation inhibits *ihh* gene expression in chick chondrocytes (Zuscik *et al.*, 2002). Also, classical protein kinase C (PKC) isoforms, which are activated by Ca^{2+} release, sustain *shh* gene expression in the chick wing bud (Lu *et al.*, 2001). In mouse primary gastric cultures, gadolinium-, thapsigargin-, and carbachol-mediated release of intracellular Ca^{2+} induces Shh expression as well (El-Zaatari *et al.*, 2010). In contrast, Ca^{2+} -chelation with BAPTA-EGTA reduced Shh expression. Thus, during gastric acid secretion, intracellular calcium release and PKC activation stimulate *shh* gene expression.

On the other hand, since Smo was proved to act as a GPCR, and stimulation of GPCRs often engages second messengers such as Ca^{2+} , the interplay between Ca^{2+} transient and Shh signaling was recently investigated. Belgacem and Borodinsky reported that recombinant N-terminal Shh peptide acutely increases Ca^{2+} spike activity in a dose-dependent manner in the developing spinal cord (Belgacem and Borodinsky, 2011). This effect is mimicked by a SAG and is prevented by cyclopamine, a Smo antagonist. They further demonstrated that this effect depends on both extracellular Ca^{2+} and intracellular Ca^{2+} stores and on Pertussis toxin (PTX)-sensitive heterotrimeric G_i proteins. While the exact mechanisms are not yet clear, they proposed that activation Smo, resulting in the activation of a PTX-sensitive G_i protein, leads to activation of Phospholipase C- γ (PLC) and increases IP_3 generation. Opening of IP_3 -operated stores will deplete intracellular Ca^{2+} stores, which leads to the subsequent activation of transient receptor potential channel 1 (TRPC1) and voltage-gated channels (Cav), resulting in increased Ca^{2+} spike activity. This hypothesis is supported by the observed inhibition of Ca^{2+} spike activity by inhibitors of PLC, IP_3 R, and TRCP1 channels. Also in support, the authors found that acute stimulation of Smo with SAG results in sequential IP_3 and Ca^{2+} transients in the primary cilia of neurons, and that this effect is abolished by the Smo inhibitor cyclopamine.

This provoking finding suggests that Hh signaling might regulate a cohort of physiological processes involving fluctuations of Ca^{2+} that acutely affect membrane potential and Ca^{2+} -dependent signaling pathways.

IV. Noncanonical Hh Signaling in *Drosophila*

The concept of noncanonical Hh signaling has not been formally extended to *Drosophila*, despite the existence of an old controversy about a subset of Hh target genes that seem to be independent of the Gli ortholog Cubitus interruptus (Ci). A large number of studies on Hh signaling in *Drosophila* focus on the effect of mutations that alter the body segment pattern at the larva stage. Each segment has a denticle-cover anterior part and a naked cuticle posterior part. *Drosophila* Hh is expressed and secreted from two rows of cells in the posterior compartment, from where it patterns the expression of *wingless* (*wg*), the Wnt ortholog, and of *rhomboid* (*rho*), ortholog of the mammalian EGFR protein. Absence of Hh results in lack of expression of *wg* and *rho* resulting in abnormal segmentation and lack of specification of the naked cuticle, leading to an “Hh-like” larva. If all Hh signaling in *Drosophila* is channeled through Ci activation, thus absence of Ci should result in a phenotype identical to that of the *hh* mutant. However, Gallet *et al.* showed that a null allele of Ci (*ci94*) has a much weaker phenotype than the Hh null (*hhAC*) (Gallet *et al.*, 2000). Moreover, overexpression of Hh in a Ci-deficient background induces early stage expression of *wg* and a stronger expression of *rho* than in the presence of Ci, indicating that these are noncanonical effects. This group further demonstrated that expression of *rho* is mediated by the C2H2-zinc finger transcription factor Teashirt (*tsh*), which would act redundantly with Ci to transduce Hh signaling in the fly.

Another group presented a different point of view: instead of activating the pathway by Hh overexpression, they used two *ptc*-null alleles (*ptcIIW* and *ptcS2*) in the Ci-null background and analyzed the cuticle phenotype but not *wg* and *rho* expression (Méthot and Basler, 2001). While they agreed that the cuticle phenotype of *ci94* is different from *hhAC*, they showed that an allele encoding just the repressor form of Ci (*ciCell*) resembles more closely the Hh-null phenotype. Notwithstanding, some stripes of naked cuticle are still present, suggesting that Ci-independent signaling may occur. In addition, this group analyzed the requirement for Ci in Hh-dependent imaginal wing disc patterning using somatic recombination techniques. In this model, clones that lack Ci cannot induce expression of *engrailed* (*en*) or *ptc*, when the Hh pathway was activated by loss of function of Ptc. The authors concluded that all Hh signaling in *Drosophila* requires Ci activity, either the activator or the repressor. However, an alternative explanation is that, as demonstrated in vertebrates, some Ci-independent signaling could be independent of Smo, for example, mediated by other Hh-interacting proteins (iHog, Boi, Dsp, etc.) and thus cannot be induced by removal of Ptc but is evidenced by Hh overexpression.

Another aspect of fly development that appears to utilize a noncanonical Hh pathway is the formation of the Bolwig’s organ, a light-sensing organ at the larval stage. Hh induces expression of *atonal* (*ato*) in the precursors of the Bolwig’s organ and that *ato* expression can be induced by loss of Ptc and blocked in a *smo* mutant (Susuki and Saigo, 2000). Elegant genetic analysis revealed that (1) loss of Ci does not affect Bolwig’s organ formation or *ato* expression, (2) an activator form of Ci (*ciZnC*) cannot rescue *ato* expression in an Hh mutant, and (3) the Ci repressor (*ciCell*) also fails to rescue the Hh mutants. These observations strongly argue in favor of the existence of at least some form of Ci-independent Hh signaling in *Drosophila*.

V. Concluding Remarks

We have presented ample evidence that noncanonical Hh signaling regulates key physiological functions in mammals during development and after birth. The recognition of those Gli-independent functions has been delayed due to the earlier consensual notion in the research community that true Hh-dependent effects are those requiring Smo and Gli activity,

which was initially assessed using genetic models and later with pharmacological and molecular biology tools. The availability of recombinant active Hh isoforms and more specific inhibitors leads to discredit such belief and opened up a new area of research that teaches us that Hh signaling is more complex and regulates more physiological processes than previously thought.

Abbreviations

ARA	arachidonic acid
Ci	Cubitus interruptus
CTD	C-terminal domain
Hh	hedgehog
Ptc1	Patched1
Shh	sonic hedgehog
Smo	Smoothened

References

- Adolphe C, Hetherington R, Ellis T, Wainwright B. Patched1 functions as a gatekeeper by promoting cell cycle progression. *Cancer Res.* 2006; 66:2081–2088. [PubMed: 16489008]
- Barnes EA, Kong M, Ollendorff V, Donoghue DJ. Patched1 interacts with cyclin B1 to regulate cell cycle progression. *EMBO J.* 2001; 20:2214–2223. [PubMed: 11331587]
- Barnes EA, Heidtman KJ, Donoghue DJ. Constitutive activation of the shh-ptc1 pathway by a patched mutation identified in BCC. *Oncogene.* 2005; 24:902–915. [PubMed: 15592520]
- Belgacem YH, Borodinsky LN. Sonic hedgehog signaling is decoded by calcium spike activity in the developing spinal cord. *Proc Natl Acad Sci USA.* 2011; 108:4482–4487. [PubMed: 21368195]
- Berridge MJ. Neuronal calcium signaling. *Neuron.* 1998; 21:13–26. [PubMed: 9697848]
- Bijlsma MF, Borensztajn KS, Roelink H, Peppelenbosch MP, Spek CA. Sonic hedgehog induces transcription-independent cytoskeletal rearrangement and migration regulated by arachidonate metabolites. *Cell Signal.* 2007; 19:2596–2604. [PubMed: 17884337]
- Bijlsma MF, Peppelenbosch MP, Spek CA, Roelink H. Leukotriene synthesis is required for hedgehog-dependent neurite projection in neuralized embryoid bodies but not for motor neuron differentiation. *Stem Cells.* 2008; 26:1138–1145. [PubMed: 18292210]
- Bredesen DE, Mehlen P, Rabizadeh S. Apoptosis and dependence receptors: A molecular basis for cellular addiction. *Physiol Rev.* 2004; 84:411–430. [PubMed: 15044679]
- Catterall WA. Signaling complexes of voltage-gated sodium and calcium channels. *Neurosci Lett.* 2010; 486:107–116. [PubMed: 20816922]
- Cayuso J, Ulloa F, Cox B, Briscoe J, Martí E. The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. *Development.* 2006; 133(3):517–528. [PubMed: 16410413]
- Chang H, Li Q, Moraes RC, Lewis MT, Hamel PA. Activation of Erk by sonic hedgehog independent of canonical hedgehog signalling. *Int J Biochem Cell Biol.* 2010; 42:1462–1471. [PubMed: 20451654]
- Chinchilla P, Xiao L, Kazanietz MG, Riobo NA. Hedgehog proteins activate pro-angiogenic responses in endothelial cells through non-canonical signaling pathways. *Cell Cycle.* 2010; 9:570–579. [PubMed: 20081366]
- Dahmane N, Ruiz i Altaba A. Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development.* 1999; 126(14):3089–3100. [PubMed: 10375501]
- Del Rio G, Kane DJ, Ball KD, Bredesen DE. A novel motif identified in dependence receptors. *PLoS One.* 2007; 2:e463. [PubMed: 17520022]

- El-Zaatari M, Zavros Y, Tessier A, Waghray M, Lentz S, Gumucio D, Todisco A, Merchant JL. Intracellular calcium release and protein kinase C activation stimulate sonic hedgehog gene expression during gastric acid secretion. *Gastroenterology*. 2010; 139:2061–2071. [PubMed: 20816837]
- Funk CD. Prostaglandins and leukotrienes: Advances in eicosanoid biology. *Science*. 2001; 294:1871–1875. [PubMed: 11729303]
- Gallet A, Angelats C, Kerridge S, Therond PP. Cubitus interruptus-independent transduction of the Hedgehog signal in *Drosophila*. *Development*. 2000; 127:5509–5522. [PubMed: 11076770]
- Glenn HL, Jacobson BS. Arachidonic acid signaling to the cytoskeleton: The role of cyclooxygenase and cyclic AMP-dependent protein kinase in actin bundling. *Cell Motil Cytoskeleton*. 2002; 53:239–250. [PubMed: 12211105]
- Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science*. 1997; 277:1109–1113. [PubMed: 9262482]
- Jenkins D. Hedgehog signalling: Emerging evidence for non-canonical pathways. *Cell Signal*. 2009; 21:1023–1034. [PubMed: 19399989]
- Jenkins D, Winyard PJ, Woolf AS. Immunohistochemical analysis of Sonic hedgehog signaling in normal human urinary tract development. *J Anat*. 2007; 211:620–629. [PubMed: 17850284]
- Jiang X, Yang P, Ma L. Kinase activity-independent regulation of cyclin pathway by GRK2 is essential for zebrafish early development. *Proc Natl Acad Sci USA*. 2009; 106:10183–10188. [PubMed: 19502428]
- Johnson RL, Milenkovic L, Scott MP. In vivo functions of the patched protein: Requirement of the C terminus for target gene inactivation but not Hedgehog sequestration. *Mol Cell*. 2000; 6:467–478. [PubMed: 10983992]
- Kagawa H, Shino Y, Kobayashi D, Demizu S, Shimada M, Ariga H, Kawahara H. A novel signaling pathway mediated by the nuclear targeting of C-terminal fragments of mammalian Patched 1. *PLoS One*. 2011; 6:e18638. [PubMed: 21533246]
- Katoh Y, Katoh M. Hedgehog target genes: Mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. *Curr Mol Med*. 2009; 9:873–886. [PubMed: 19860666]
- Kawamura S, Hervold K, Ramirez-Weber FA, Kornberg TB. Two patched protein subtypes and a conserved domain of group I proteins that regulates turnover. *J Biol Chem*. 2008; 283:30964–30969. [PubMed: 18768465]
- Kenney AM, Rowitch DH. Sonic hedgehog promotes G(1) cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol Cell Biol*. 2000; 20(23):9055–9067. [PubMed: 11074003]
- Kenney AM, Cole MD, Rowitch DH. Nmyc upregulation by sonic hedgehog signaling promotes proliferation in developing cerebellar granule neuron precursors. *Development*. 2003; 130(1):15–28. [PubMed: 12441288]
- Komuro H, Rakic P. Intracellular Ca²⁺ fluctuations modulate the rate of neuronal migration. *Neuron*. 1996; 17:275–285. [PubMed: 8780651]
- Kuo G, Arnaud L, Kronstad-O'Brien P, Cooper JA. Absence of Fyn and Src causes a reeler-like phenotype. *J Neurosci*. 2005; 25:8578–8586. [PubMed: 16162939]
- Lavine KJ, Kovacs A, Ornitz DM. Hedgehog signaling is critical for maintenance of the adult coronary vasculature in mice. *J Clin Invest*. 2008; 118(7):2404–2414. [PubMed: 18568073]
- Liu G, Li W, Gao X, Li X, Jurgensen C, Park HT, Shin NY, Yu J, He ML, Hanks SK, Wu JY, Guan KL, et al. p130CAS is required for netrin signaling and commissural axon guidance. *J Neurosci*. 2007; 27:957–968. [PubMed: 17251438]
- LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR. GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. *Neuron*. 1995; 15:1287–1298. [PubMed: 8845153]
- Lu HC, Swindell EC, Sierralta WD, Eichele G, Thaller C. Evidence for a role of protein kinase C in FGF signal transduction in the developing chick limb bud. *Development*. 2001; 128:2451–2460. [PubMed: 11493562]
- Lu X, Liu S, Kornberg TB. The C-terminal tail of the Hedgehog receptor Patched regulates both localization and turnover. *Genes Dev*. 2006; 20:2539–2551. [PubMed: 16980583]

- Lum L, Yao S, Mozer B, Rovescalli A, Von Kessler D, Nirenberg M, Beachy PA. Identification of Hedgehog pathway components by RNAi in *Drosophila* cultured cells. *Science*. 2003; 299:2039–2045. [PubMed: 12663920]
- Makino S, Masuya H, Ishijima J, Yada Y, Shiroishi T. A spontaneous mouse mutation, mesenchymal dysplasia (mes), is caused by a deletion of the most C-terminal cytoplasmic domain of patched (ptc). *Dev Biol*. 2001; 239:95–106. [PubMed: 11784021]
- Mehlen P, Thibert C. Dependence receptors: Between life and death. *Cell Mol Life Sci*. 2004; 61:1854–1866. [PubMed: 15289929]
- Méthot N, Basler K. An absolute requirement for Cubitus interruptus in Hedgehog signaling. *Development*. 2001; 128:733–742. [PubMed: 11171398]
- Mille F, Thibert C, Fombonne J, Rama N, Guix C, Hayashi H, Corset V, Reed JC, Mehlen P. The Patched dependence receptor triggers apoptosis through a DRAL-caspase-9 complex. *Nat Cell Biol*. 2009; 11:739–746. [PubMed: 19465923]
- Murray AW. Recycling the cell cycle: Cyclins revisited. *Cell*. 2004; 116:221–234. [PubMed: 14744433]
- Nieuwenhuis E, Barnfield PC, Makino S, Hui CC. Epidermal hyperplasia and expansion of the interfollicular stem cell compartment in mutant mice with a C-terminal truncation of Patched1. *Dev Biol*. 2007; 308:547–560. [PubMed: 17631878]
- Nybakken K, Vokes SA, Lin TY, McMahon AP, Perrimon N. A genome-wide RNA interference screen in *Drosophila melanogaster* cells for new components of the Hh signaling pathway. *Nat Genet*. 2005; 37:1323–1332. [PubMed: 16311596]
- Pathi S, Pagan-Westphal S, Baker DP, Garber EA, Rayhorn P, Bumcrot D, Tabin CJ, Blake Pepinsky R, Williams KP. Comparative biological responses to human Sonic, Indian, and Desert hedgehog. *Mech Dev*. 2001; 106:107–117. [PubMed: 11472839]
- Peppelenbosch MP, Tertoolen LG, Hage WJ, de Laat SW. Epidermal growth factor-induced actin remodeling is regulated by 5-lipoxygenase and cyclooxygenase products. *Cell*. 1993; 74:565–575. [PubMed: 8348619]
- Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake Pepinsky R, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. *Nat Med*. 2001; 7(6):706–711. [PubMed: 11385508]
- Polizio AH, Chinchilla P, Chen X, Kim S, Manning DR, Riobo NA. Heterotrimeric Gi proteins link Hedgehog signaling to activation of Rho small GTPases to promote fibroblast migration. *J Biol Chem*. 2011; 286:19589–19596. [PubMed: 21474452]
- Ridley AJ. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol*. 2006; 16:522–529. [PubMed: 16949823]
- Riobo NA, Manning DR. Pathways of signal transduction employed by vertebrate Hedgehogs. *Biochem J*. 2007; 403(3):369–379. [PubMed: 17419683]
- Riobo NA, Lu K, Ai X, Haines GM, Emerson CP Jr. Phosphoinositide 3-kinase and Akt are essential for Sonic Hedgehog signaling. *Proc Natl Acad Sci USA*. 2006a; 103(12):4505–4510. [PubMed: 16537363]
- Riobo NA, Saucy B, Dilizio C, Manning DR. Activation of heterotrimeric G proteins by Smoothened. *Proc Natl Acad Sci USA*. 2006b; 103(33):12607–12612. [PubMed: 16885213]
- Robles E, Woo S, Gomez TM. Src-dependent tyrosine phosphorylation at the tips of growth cone filopodia promotes extension. *J Neurosci*. 2005; 25:7669–7681. [PubMed: 16107653]
- Sasaki N, Kuriso J, Kengaku M. Sonic hedgehog signaling regulates actin cytoskeleton via Tiam1-Rac1 cascade during spine formation. *Mol Cell Neurosci*. 2010; 45:335–344. [PubMed: 20654717]
- Susuki T, Saigo K. Transcriptional regulation of atonal required for *Drosophila* larval eye development by concerted action of eyes absent, sine oculis and hedgehog signaling independent of fused kinase and cubitus interruptus. *Development*. 2000; 127:1531–1540. [PubMed: 10704398]
- Suter DM, Forscher P. Transmission of growth cone traction force through apCAM-cytoskeletal linkages is regulated by Src family tyrosine kinase activity. *J Cell Biol*. 2001; 155:427–438. [PubMed: 11673478]

- Sweet HO, Bronson RT, Donahue LR, Davisson MT. Mesenchymal dysplasia: A recessive mutation on chromosome 13 of the mouse. *J Hered.* 1996; 87:87–95. [PubMed: 8830098]
- Takizawa CG, Morgan DO. Control of mitosis by changes in the subcellular location of cyclin-B1-Cdk1 and Cdc25C. *Curr Opin Cell Biol.* 2000; 12:658–665. [PubMed: 11063929]
- Thibert C, Teillet MA, Lapointe F, Mazelin L, Le Douarin NM, Mehlen P. Inhibition of neuroepithelial patched-induced apoptosis by sonic hedgehog. *Science.* 2003; 301:843–846. [PubMed: 12907805]
- Varjosalo M, Bjorklund M, Cheng F, Syvanen H, Kivioja T, Kilpinen S, Sun Z, Kallioniemi O, Stunnenberg HG, He WW, Ojala P, Taipale J. Application of active and kinase-deficient kinome collection for identification of kinases regulating hedgehog signaling. *Cell.* 2008; 133:537–548. [PubMed: 18455992]
- Vetter IR, Wittinghofer A. The guanine nucleotide-binding switch in three dimensions. *Science.* 2001; 294:1299–1304. [PubMed: 11701921]
- Wakabayashi Y, Mao JH, Brown K, Girardi M, Balmain A. Promotion of Hras-induced squamous carcinomas by a polymorphic variant of the Patched gene in FVB mice. *Nature.* 2007; 445(7129): 761–765. [PubMed: 17230190]
- Wallace VA. Purkinje-cell-derived Sonic hedgehog regulates granule neuron precursor cell proliferation in the developing mouse cerebellum. *Curr Biol.* 1999; 9(8):445–448. [PubMed: 10226030]
- Wang GY, Wang J, Mancianti ML, Epstein EH Jr. Basal cell carcinomas arise from hair follicle stem cells in *Ptch1*^{+/-} mice. *Cancer Cell.* 2011; 19:114–124. [PubMed: 21215705]
- Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron.* 1999; 22(1):103–114. [PubMed: 10027293]
- Yam PT, Langlois SD, Morin S, Charron F. Sonic hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. *Neuron.* 2009; 62:349–362. [PubMed: 19447091]
- Youssef KK, Van Keymeulen A, Lapouge G, Beck B, Michaux C, Achouri Y, Sotiropoulou PA, Blanpain C. Identification of the cell lineage at the origin of basal cell carcinoma. *Nat Cell Biol.* 2010; 12:299–307. [PubMed: 20154679]
- Zuscik MJ, D'Souza M, Ionescu AM, Gunter KK, Gunter TE, O'Keefe RJ, Schwarz EM, Puzas JE, Rosier RN. Growth plate chondrocyte maturation is regulated by basal intracellular calcium. *Exp Cell Res.* 2002; 276:310–319. [PubMed: 12027460]

Figure 3.1.

Schematic representation of the two types of noncanonical Hh signaling. Type I requires only binding of an Hh isoform to Ptc1 and is mediated by novel functions of Ptc1 unrelated to Smo repression, and it is by definition insensitive to Smo modulators. Type II is dependent on Smo and in some cases it has been shown to rely on signaling through G_i proteins, and it is both mimicked by Smo agonists and inhibited by Smo antagonists.

Figure 3.2.

Apoptosis was assessed by caspase-3 activity measurement of serum starved HUVECs in the absence (control) or in the presence of 2.5 μ g/ml Shh (Shh), 0.5 μ M Smo agonist (SAG), or Shh plus the Smo inhibitors SANT-1, cyclopamine (CP), or KAAD-cyclopamine (KAAD) (Chinchilla *et al.*, 2010).

Figure 3.3.

The three Hh isoforms promote tubulogenesis in endothelial cells by a G_i protein and RhoA-dependent mechanism. (A) HUVECs were cultured in a 3D collagen Type I matrix for 24h in the absence (control) or presence of Shh, Ihh, or Dhh (all at $2.5\mu\text{g/ml}$). Photographs are representative tube densities. (B) Quantification of tube density in the absence (control) or presence of Shh, Ihh, and Dhh, and in the presence of Shh after preincubation with $0.5\mu\text{M}$ KAAD-cyclopamine (KAAD, Smo inhibitor) or 100ng/ml Pertussis toxin (PTX; inhibitor of G_i protein activation). (C) HUVECs were serum starved for 24h and stimulated with Shh, Ihh, or Dhh (all at $2.5\mu\text{g/ml}$) during 15 min. Active RhoA (RhoA-GTP) was pulled down from whole cell lysates with a Rhotekin-binding domain-GST fusion protein coupled to GSH-sepharose beads. Active and total RhoA were evaluated by Western blot, and the densitometric values are shown in the bar graph ($n=3$). (D) RhoA pull-down assay as in (C) in cells preincubated with $0.5\mu\text{M}$ KAAD-cyclopamine (KAAD) or 100ng/ml PTX ($n=3$) (Chinchilla et al., 2010).