The Hedgehog Signal Transduction Network

David J. Robbins,1,2,3* Dennis Liang Fei,1 Natalia A. Riobo**

Hedgehog (Hh) proteins regulate the development of a wide range of metazoan embryonic and adult structures, and disruption of Hh signaling pathways results in various human diseases. Here, we provide a comprehensive review of the signaling pathways regulated by Hh, consolidating data from a diverse array of organisms in a variety of scientific disciplines. Similar to the elucidation of many other signaling pathways, our knowledge of Hh signaling developed in a sequential manner centered on its earliest discoveries. Thus, our knowledge of Hh signaling has for the most part focused on elucidating the mechanism by which Hh regulates the Gli family of transcription factors, the so-called “canonical” Hh signaling pathway. However, in the past few years, numerous studies have shown that Hh proteins can also signal through Gli-independent mechanisms collectively referred to as “noncanonical” signaling pathways. Noncanonical Hh signaling is itself subdivided into two distinct signaling modules: (i) those not requiring Smoothened (Smo) and (ii) those downstream of Smo that do not require Gli transcription factors. Thus, Hh signaling is now proposed to occur through a variety of distinct context-dependent signaling modules that have the ability to crossstalk with one another to form an interacting, dynamic Hh signaling network.

Introduction

The Hedgehog (Hh) family of proteins plays an evolutionarily conserved, instructional role during the development of many metazoans (1, 2). Hh also plays an important role in the maintenance of many adult structures that include proliferating cell populations, perhaps by regulating the various progenitor cells that give rise to these structures (3, 4). The specific roles Hh plays in these processes are diverse because it functions as a morphogen, a mitogen, a survival factor, and as an instructive, pattern-forming molecule. For example, Hh proteins regulate the development of a wide range of metazoan embryonic and adult structures, and disruption of Hh signaling pathways results in various human diseases. Here, we provide a comprehensive review of the signaling pathways regulated by Hh, consolidating data from a diverse array of organisms in a variety of scientific disciplines. Similar to the elucidation of many other signaling pathways, our knowledge of Hh signaling developed in a sequential manner centered on its earliest discoveries. Thus, our knowledge of Hh signaling has for the most part focused on elucidating the mechanism by which Hh regulates the Gli family of transcription factors, the so-called “canonical” Hh signaling pathway. However, in the past few years, numerous studies have shown that Hh proteins can also signal through Gli-independent mechanisms collectively referred to as “noncanonical” signaling pathways. Noncanonical Hh signaling is itself subdivided into two distinct signaling modules: (i) those not requiring Smoothened (Smo) and (ii) those downstream of Smo that do not require Gli transcription factors. Thus, Hh signaling is now proposed to occur through a variety of distinct context-dependent signaling modules that have the ability to crossstalk with one another to form an interacting, dynamic Hh signaling network.

Hh Receptors

Patched (Ptc) functions as the core Hh receptor, binding to Hh ligands with an affinity in the low nanomolar range (30, 31). The identification of Ptc as a Hh receptor was supported by Drosophila genetics, which had already implicated Ptc as a pivotal regulator of Hh signaling that functioned downstream of Hh but upstream of the other known sig-
naling components (13, 32–34). The predicted membrane topology of Ptc suggests 12 transmembrane domains and two large extracellular loops. On the basis of this topology, and its primary sequence, Ptc is most homologous to the resistance-nodulation-division (RND) family of bacterial transporters (35). These transporters are found almost exclusively in bacteria, in which they function to pump lipophilic toxins and heavy metals out of the cell in a manner driven by a proton gradient. Although there are few examples of such gradients in eukaryotic cells, mutating highly conserved amino acids in Ptc that are absolutely required for RND transporter activity attenuates Ptc activity (36). Ptc also contains a sterol-sensing domain, an ~180-amino-acid motif found in many cholesterol-binding proteins (37–40). Missense mutations that alter conserved amino acids within the sterol-sensing domain of Ptc affect its ability to regulate the expression of Hh target genes without affecting its ability to bind Hh. Besides binding Hh to initiate signaling, Ptc also modulates the extracellular gradient of Hh observed in a developing field of cells and does so in a manner that is functionally distinct from its ability to modulate Hh signal transduction (41). Mammals have a second distinct Ptc gene, Ptc2, which encodes a slightly smaller protein with topology and domains similar to Ptc1 (42–44). Ptc1 and Ptc2 expression patterns show some overlap but also display many expression domains with minimal overlap. Transcription of Ptc genes is directly regulated by Gli transcription factors, and both Ptc1 and Ptc2 are up-regulated in response to Hh (45–47).

Several other Hh binding proteins have now been shown to function as Hh co-receptors (49). Furthermore, although many of these co-receptors are highly conserved between mammals and Drosophila, the ability of these homologs to bind Hh proteins is regulated in distinct ways in different classes of animals. The first identified Hh co-receptor was isolated in a screen that used a library of small interfering RNAs (siRNAs) to modulate Hh signaling in a Drosophila cell line and was called interference hedgehog (Ihog) for this reason (49). Ihog functions as a positive regulator in Hh signaling because decreased Ihog function reduced Hh signaling. A homologous protein, brother of Ihog (Boi), was also identified in Drosophila. Ihogs are single-pass transmembrane proteins that contain a small nonconserved intracellular domain and an extracellular domain characterized by four immunoglobulin-like domains and two fibronectin type III (FN3) domains. This topology is reminiscent of other extracellular ligand receptors, providing the initial insight that Ihogs might function as co-receptors for Hh. Ihog associates with both Hh and Ptc through its FN3 domains, with the first FN3 domain binding to Hh and the second FN3 domain associating with Ptc (30, 51). The FN3 domains of Ihog were unable to associate directly with purified Hh in vitro unless heparin was included, suggesting that heparin is necessary for formation of the Drosophila Hh receptor complex in vivo. The phenotype of Drosophila lacking either Ihog or Boi is relatively mild, but flies lacking both gene products display a severe reduction of Hh signaling in embryos and defects in Hh-regulated development of numerous adult structures (51). Thus, Boi functions redundantly with Ihog in the fly, forming discrete receptor complexes that bind Hh through one FN3 domain and interact with Ptc through the other FN3 domain, in order to initiate the Hh signaling cascade.

Two vertebrate orthologs of Ihog have been identified, cell adhesion molecule down-regulated by oncogenes (Cdo) and brother of Cdo (Boc), which function as co-receptors for Hh proteins in a manner similar to that of Ihogs (49, 52–54). Mice lacking either Cdo or Boc are viable but display phenotypes consistent with minor disruptions in Hh signaling (52–58). Mice lacking both Cdo and Boc exhibit more severe Hh loss-of-function phenotypes, which is consistent with their acting in a redundant fashion. However, the phenotypes of mice lacking both Cdo and Boc indicated that signaling was not completely abrogated in some Hh-dependent structures. This residual Hh activity was provided by a third, vertebrate-specific, co-receptor, growth arrest–specific gene 1 (Gas1) (55–57, 59). Gas1 has little primary sequence homology to Ihogs and lacks a true transmembrane domain, associating with membranes through a glycosylphosphatidylinositol anchor (48, 60). Similar to what was observed between Ihog and Hh, the purified Hh binding domains of the mammalian co-receptors were unable to bind mammalian Hh proteins. Further, unlike what was observed with the Drosophila proteins, the addition of heparin did not increase the binding affinity between the vertebrate Hh proteins and their co-receptors (61). Instead, it was noted that the direct association between Hh and the vertebrate co-receptors required high concentrations of calcium ions (Ca²⁺). When Shh was cocryrstallized with the various co-receptor Hh-binding domains, it was found that Shh coordinates two Ca²⁺ molecules along the surface that binds to the various co-receptors. The binding affinity of Shh for Cdo or Boc increased dramatically in the presence of Ca²⁺, and mutation of those amino acids in Shh responsible for Ca²⁺ coordination reduced its affinity for co-receptor binding. Missense mutations of the same amino acids were identified as the underlying cause of two distinct, inherited developmental disorders, highlighting the importance of this Ca²⁺-binding surface (61).

Another mechanistic difference between Drosophila and vertebrate Hh co-receptors is the presence of a third FN3 domain in the vertebrate co-receptors (53, 61). Unexpectedly, although the first FN3 domains of Cdo and Boc share extensive homology with the first, Hh-binding FN3 domain of Ihog and Boi, Cdo and Boc associated with Shh through the third FN3 domain rather than through the first FN3 domain. The identification of a functionally inactive Shh mutant that can bind to Ptc1 but is unable to bind to Cdo, Boc, or Gas1 illustrated the importance of co-receptor formation for signaling (57). Missense mutations in Cdo or Gas1 were also identified in patients with holoprosencephaly, a developmental disorder previously shown to result from decreased Hh activity (54, 58, 62). These mutant gene products displayed a reduced association with Ptc1 but still bound Shhh similarly to the wild-type co-receptors, providing genetic support for the importance of Hh co-receptors during human development (57, 62, 63).

**Smoothened**

*Drosophila Smoothened (Smo)* encodes a protein predicted to belong to the seven-transmembrane G protein–coupled receptor (GPCR) superfamily (64). Activation of Smo occurs in two steps: translocation of Smo from intracellular vesicles to the cell surface and subsequent phosphorylation. Translocation of Smo to the cell surface in an inactive conformation can be induced by the Smo inhibitor cyclopamine, but not by other Smo inhibitors, suggesting that translocation alone is not sufficient for activation. Unlike the majority of these family members, Smo contains a distinctively long carboxyl-terminal (C-terminal) domain (C-tail) that is absolutely necessary for Hh-dependent signaling (64–66). This domain
contains four conserved clusters of basic amino acids embedded within a region containing six Ser-Thr phosphorylation sites. *Drosophila* Smo exists as a constitutive multimer through homomeric association of its N-terminal regions, and Hh binding to Ptc promotes phosphorylation of the C-tail, which neutralizes the net positive charge of the basic residue clusters, allowing the C-tails to move closer to one another (67, 68). Despite the low overall sequence homology between *Drosophila* and mammalian Smo, these self-interaction domains, phosphoregulatory motifs, and regulated conformational changes are conserved, and phosphorylated Smo translocates to the plasma membrane in *Drosophila* or to the plasma membrane of the primary cilium in mammals (69–72). The majority of *Drosophila* cells do not have primary cilia, but this organelle is absolutely required for Hh signaling in vertebrates. *Drosophila* Smo is phosphorylated by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA), casein kinase 1 (CK1), casein kinase 2 (CK2), and G protein–coupled receptor kinase 2 (GRK2) in the presence of Hh (73–76). In the absence of Hh, several distinct protein phosphatases—including protein phosphatase 2A (PP2A), PP1, and PP4—maintain a low steady state of Smo phosphorylation (77, 78). Differential phosphorylation of the Smo C-tail correlates with graded activation of Smo, which is consistent with such a mechanism being required to respond to distinct concentrations of Hh (67, 72, 75, 78). Phosphorylation of the C-tail also reduces the ubiquitin-activating enzyme 1 (UBA1)–mediated ubiquitination of multiple sites within this domain, thus inhibiting endocytosis and subsequent proteasomal and lysosomal degradation of Smo (79, 80). CK1β, GRK2, and another still-unidentified protein kinase phosphorylate the C-tail of mammalian Smo in the presence of Hh proteins (72). Deletion of the basic amino acid motifs of mammalian Smo is sufficient to induce Smo accumulation in the primary cilia (68). GRK2-mediated phosphorylation of vertebrate Smo allows Smo to bind to β-arrestins 1 or 2 (81, 82). β-Arrestin 2 was subsequently shown to bridge Smo to the kinesin motor Kif3a to promote cilary accumulation of Smo in mammalian cells (82).

Consistent with its predicted topology, Smo couples to a specific family of inhibitory G proteins (Giα) to regulate Hh signaling (83, 84). Heterotrimeric G proteins are subdivided into four families according to the sequence similarity of their α subunit and the effectors they stimulate. Giα inhibit adenylylate cyclase (AC) and activate some potassium (K+) channels and phospholipase Cγ (85). Because PKA negatively regulates the Gli family of transcription factors (86–89), it was proposed that Smo might signal through activation of Gi proteins to reduce PKA activity. Early studies used pertussis toxin (PTX) to discern whether a Gα-dependent mechanism modulated Hh signaling. All mammalian Gα, family members, with the exception of Gαq, and the single *Drosophila* Gαi, isofrom, are sensitive to inhibition by PTX (90). Expression of a transgene encoding PTX in zebrafish embryos results in a phenotype similar to loss-of-function *Hh* mutations (91). This phenotype is rescued by coexpression of a construct encoding a dominant negative form of PKA, but not by coexpression of *Hh*, suggesting that one or more Gα family members act upstream of PKA and downstream of Hh. Another study showed that expression of *Smo* in frog melanophore cells induces a change in melanosome aggregation consistent with decreased cAMP, and this aggregation is attenuated by PTX. These results suggest that Smo could induce a decrease in cAMP concentration, either directly through coupling to Gi or indirectly by promoting coupling to another GPCR (92). More recent observations have substantiated that Smo acts as a Gα-coupled GPCR in some contexts both in vertebrates and in *Drosophila* (83, 84, 90, 93, 94). To demonstrate coupling, vertebrate Smo and different combinations of transgenes encoding G protein α subunits representing the four G protein families plus the βi and γi subunits were expressed in the S9 insect cell line. In these cells, which lack Ptc, Smo increased the rate of guanosine triphosphate (GTP) exchange of all G1 protein family members in a dose-dependent manner but had no effect on members of other G protein families (83). Moreover, three distinct Smo antagonists blocked Gα activation with a similar potency as that with which they can inhibit activation of a Gli reporter gene. Furthermore, PTX impaired Gli activation downstream of Smo in mouse fibroblast NIH3T3 cells and did so in a manner that could be rescued by a constitutively active Gαq mutant (83). RNA interference (RNAi)–mediated knockdown of the single Gαi isoform produced in a *Drosophila* cell line also reduced the activation of several Hh-induced posttranslational modifications of downstream signaling components, allowing Gαi activity to be placed downstream of Smo but upstream of the *Drosophila* Gli family member Cubus interruptus (Ci) (84).

In addition, a substantial reduction in cAMP abundance was observed in cultured insect S2 cells within minutes of treatment with Hh and was dependent on both Smo and Gαi. In flies, loss-of-function Gαi mutants also revealed phenotypes and reduced patterns of Hh target gene expression consistent with a role of Gαi in Hh signaling (84). In support of Hh regulating the steady-state abundance of cAMP through Gαi, increasing cAMP production by reducing the gene dosage of the phosphodiesterase *Dunce* or by expressing a transgene encoding a viral AC enhanced Smo loss-of-function phenotypes (84, 93).

GPCRs are activated by binding of one or more ligands to their extracellular domain (64, 85). However, the identification of a definitive physiological ligand for Smo has proven to be elusive. An elegant study of Ptc-mediated Smo repression supports a catalytic rather than stoichiometric mechanism of action for Ptc, leading to the speculation that Ptc functions by regulating the intracellular concentration of endogenous small-molecule Smo modulators (96). The homology of Ptc to RND proteins and the presence of a sterol-sensing domain in Ptc suggested that such a modulator could be a sterol or lipid. Numerous groups have now identified distinct classes of oxysterol Smo modulators, a subset of which activates Smo and a subset of which inhibits it. *Ptc1*–transfected cells secrete 3β-hydroxysteroid (pro-vitamin D3), which inhibits Smo function. Inhibiting the synthesis of provitamin D3 impairs Ptc1-mediated repression of Smo (97). Two independent groups identified oxysterol agonists of Smo (98, 99), showing that inhibition of cholesterol biosynthesis abolishes activation of Gli proteins in medulloblastoma cells and that this could be rescued by the addition of cholesterol or various oxysterol (OHC) metabolites, including 20α-OHC, 22(S)-OHC, 24-OHC, and 25-OHC. Furthermore, these oxysterols were potent Smo-dependent stimulators of maximal Gli-dependent transcription. More recently, 20(S)-OHC has been shown to bind directly to Smo and activate Gli-dependent transcription synergistically with the Smo agonist (SAG), suggesting positive allosteric modulation of Smo by oxysterols (100). However, another report proposed that Ptc represses Smo activity by promoting a reduction in the abundance of phos-
increased concentration of PI4P. Consistent with this suggestion, PI4P also regulates the membrane translocation and activation of Smo. Moreover, depletion of PI4P kinase in NIH3T3 cells also impairs activation of a Gli-luciferase reporter gene by Shh (101).
through a series of self-contained degradation domains known as degrons (111, 112). Ubiquitination of proteins is controlled by a series of enzymatic activities (E1 to E4), with the specificity of ubiquitination dictated by a large family of E3 ubiquitin ligases (113, 114). Ci stability is controlled by two multiprotein E3 ligases: (i) the Skp1-Cul5-I-F-box (SCF) complex and (ii) the Cul3-HIB complex, which includes the scaffolding protein Cul3 and the ubiquitin ligase HIB (Hh-induced MATH and BTB domain-containing protein), which is also known as SPOP (Speckle-type POZ protein) (112, 115). In the absence of Hh, SCF promotes the processing of Ci\textsubscript{FL} into Ci\textsubscript{R} (116). The Cul1 degrons of Ci consist of Ser- and Thr-rich stretches of amino acids that require sequential phosphorylation by PKA, glycogen synthase kinase-3 (GSK3), and CK1 for recognition of Ci\textsubscript{R} by Cul1 (86–88, 117–121). Increased phosphorylation of Ci\textsubscript{R} renders it a better substrate for the F-box protein Slumb, which acts as a substrate recognition subunit to target Ci\textsubscript{R} for ubiquitination (116). This ubiquitination of Ci renders it a substrate for the proteasome. However, unlike most proteasome substrates, Ci is not completely proteolyzed. Instead, only the C-terminal half of Ci\textsubscript{R} is proteolyzed, thus yielding the truncated Ci\textsubscript{R} form. This unusual partial processing of Ci\textsubscript{R} requires its zinc finger region and Lys750 (122). In cells exposed to high concentrations of Hh, Ci\textsubscript{R} is quite labile (106) and is completely proteolyzed through a mechanism linked to its transcriptional activity. This complete degradation of Ci\textsubscript{R} is mediated by two distinct N- and C-terminal degrons that are targeted by HIB (115, 123, 124). Further, HIB acts as a feedback regulator of Ci activity, because Hh stimulation up-regulates expression of the gene encoding HIB. Thus, in response to moderate amounts of Hh, the loss of Ci\textsubscript{R} and accumulation of Ci\textsubscript{R} results in moderate amounts of Ci transcriptional regulatory activity, and in response to large amounts of Hh, the short-lived, highly active Ci\textsubscript{R} form is produced.

Mammals possess three Ci homologs—Gli1, Gli2, and Gli3—and the combinatorial activity of these regulate a diverse set of Hh target genes (2). Like Ci, Gli activity is controlled through a combination of regulated expression, protein stability, and modulation of specific activities, much of which is directed by a diverse array of posttranslational modifications. Whereas Gli1 acts only as a transcriptional activator, Gli2 is the principal Hh-regulated transcriptional activator (Gli\textsubscript{A}), and Gli3 is the principal Hh-regulated repressor (Gli\textsubscript{R}) (89, 125–138). In the absence of Hh signaling, Gli2\textsubscript{R} and Gli3\textsubscript{R} are partially proteolyzed in a manner that removes and degrades the C-terminal activation domain, yielding proteins that are essentially transcriptional repressors (Gli\textsubscript{R}). In the presence of Hh, this proteolytic processing is blocked, allowing Gli2\textsubscript{A} and Gli3\textsubscript{A} to accumulate and be subsequently converted into potent, nuclear-enriched transcriptional activators (Gli\textsubscript{A}). The transcription of Gli1 increases in response to Hh (127, 139), and increased abundance of Gli1 is a widely used biomarker for increased canonical Hh signaling activity (140). Thus, the proteolytic processing, stability, and transcriptional potency of the three Gli transcription factors combine to regulate basal and Hh-stimulated expression of Hh-responsive genes and do so in a manner that varies with the amount of Hh to which cells are exposed. Many of the proteins that play pivotal roles in regulating the activity, nuclear localization, or stability of Ci are conserved in mammals (Fig. 1B) (2, 7). Remarkably, the relative importance of these orthologous Gli modulators in Hh signaling varies between classes of animals, differing between flies, fish, and mammals (141–144).

**Canonical Hh Signaling**

*Drosophila* Smo regulates the activity and amounts of Ci through a series of membrane-associated protein complexes that contain the protein kinase Fused (Fu), the kinesin-related protein Costal2 (Cos2), and a genetic suppressor of Fu called Suppressor of Fused (Sufu) (145). Cos2 serves as a scaffold for Fu, Sufu, Ci, and Smo enrichment (146–150). Cos2 also associates with many of the protein kinases and phosphatases that regulate Ci activity in the absence of Hh (118) and subsequently regulate Smo activity in the presence of Hh (78, 151). Fu regulates Ci activity primarily through phosphorylation-mediated regulation of Cos2 and Sufu activity (150, 152–163). A Cos2-nucleated complex associates with Smo on vesicular membranes in the absence of Hh and releases Ci upon Hh stimulation (147–150). Cos2 associates with Smo in two distinct regions, one of which reflects constitutive binding and one that is highly regulated by Hh, in a manner that may be regulated by Fu kinase activity (146, 150, 161, 162, 164). However, the estimated 12-fold stoichiometric excess of Cos2 over Smo suggests that, at steady state, the bulk of Cos2 in cells is not associated with Smo (165). These other Cos2 complexes associate with the microtubule cytoskeleton in highly motile vesicles whose motility is dependent on microtubules, adenosine 5'-triphosphate (ATP), and the adenosine triphosphatase (ATPase) domain of Cos2 (157). In response to Hh stimulation, the pool of Cos2 associated with Smo moves from intracellular vesicular membranes to the plasma membrane (147, 149). As Smo abundance is stabilized in response to Hh (71), an increase in the Smo-associated Cos2 complex is also observed. Smo can regulate the majority of the Cos2 complexes (166, 167), presumably by interacting with these Cos2 complexes in a dynamic manner that allows the bulk of Cos2 to interact with Smo at the plasma membrane.

The Fu gene encodes a protein with an N-terminal serine-threonine protein kinase domain (168) and a large noncatalytic C-terminal domain (the Fu tail) (169) that serves as a scaffold for many protein-protein interactions (155). Fu plays a role in converting Ci\textsubscript{R} into both Ci\textsubscript{A} and Ci\textsubscript{R}. Whereas the kinase activity of Fu is required for Ci\textsubscript{A} formation and to attenuate the processing of Ci\textsubscript{R} to Ci\textsubscript{R} (106, 161–163, 170, 171), the processing of Ci\textsubscript{R} to Ci\textsubscript{R} requires only the Fu tail (163, 169, 172). Targeting Fu to the plasma membrane renders it constitutively active (173), but the regulation of Fu activity is complex, as is its genetics, which is consistent with its pivotal role in regulating the functionally distinct forms of Ci (14). In the absence of Hh, the two domains of Fu self-associate to generate a form that interacts with Cos2 (172). In response to Hh, Fu forms a constitutively active dimer that is phosphorylated both within the kinase activation loop and the Fu tail domain, and these phosphorylation events are likely mediated by both autophosphorylation and by CK1 (161–163). Furthermore, Fu phosphorylation increases in response to increasing Hh, promoting Fu-mediated phosphorylation of Cos2, Sufu, and perhaps an additional undetermined substrate (161–163). As well as functioning downstream of Smo, Fu may also act in a positive feedback loop to further activate Smo (151, 173). Although Fu is absolutely required in *Drosophila* Hh signaling, its role in mammalian Hh signaling is less clear—possibly because of its redundancy with other Hh-regulated protein kinases (143, 174, 175).
Originally identified as a genetic suppressor of the phenotype resulting from decreased Fu function (176), Sufu has proven to be one of the more enigmatic components of the Hh signaling pathway (177). Contributing to our lack of understanding of Sufu is that fact that it contains few identifiable primary sequence motifs, and null mutations in Sufu cause only minimal effects on Hh-induced patterning in Drosophila (178). However, Sufu does play an important role in Hh signaling, albeit one that is genetically redundant with Cos2 (153). Sufu also associates directly with Fu and Ci in a manner analogous to that of Cos2 (152, 156). Sufu is an abundant protein that is 10- to 100-fold enriched as compared with other members of this signaling complex (165). However, when Sufu is isolated from a membrane-enriched Cos2 signaling complex, it is present in approximately equimolar amounts with Fu, Cos2, and Ci (165). The reason for this vast stoichiometric excess is not clear, but may reflect its requirement for attenuating the activity of any Ci that is not complexed with Cos2 (150) because Sufu is capable of physically sequestering Ci in the cytoplasm (170, 179–181). Although the bulk of Sufu is cytoplasmic (156), small amounts have also been observed in the nucleus (181). Thus, Sufu has been proposed to modulate the expression of Ci target genes by attenuating Ci activity. Although Drosophila lacking Sufu are viable (176), vertebrates lacking Sufu exhibit many developmental defects, which are severe in mice (141, 182) because Sufu plays an important functional role in controlling the stability of Gli2FL and Gli3FL in vertebrates (115, 183–187).

Primary Cilia and Hh Signaling

It is now clearly established that maximal vertebrate Hh signaling requires the primary cilium (188), an organelle previously thought to be vestigial (189). Most vertebrate cells contain one nonmotile primary cilium, a membrane-encased protrusion located on the apical side of polarized cells. Primary cilia are composed of nine microtubule pairs anchored to the basal body and a specialized septin-like region at the base that limits access to the body of the primary cilium. They are distinct from motile cilia, lacking both the central pair of microtubules and radial spokes required for motility, and their formation is intimately tied to the cell cycle. Trafficking through primary cilium occurs in a microtubule-dependent manner and is regulated by two large, non-membrane-bound, multiprotein complexes. The trafficking of these multiprotein complexes, from the basal body to the tip of the primary cilium and from the tip of the cilium back to the basal body, is regulated by several anterograde and retrograde molecular motors.

Ptcl is enriched in primary cilia in the absence of Hh and moves out of cilia upon binding to Hh (190). Several other components of the Hh signaling pathway—including Smo (70, 191–193), Sufu (194), Gli2, and Gli3 (184, 193–198)—are thought to traffic into and back out of primary cilia in the absence of Hh signaling, continuously sampling the environment of this organelle (188, 199). Basal trafficking of Sufu-Gli complexes through primary cilia occurs in a Smo-independent manner (183, 186). The basal trafficking of the Sufu-Gli2FL and Sufu-Gli3FL complexes through cilia allows the Gli genes to be hyperphosphorylated at their Cull1-dependent degrons. These phosphorylation events may be regulated by the mammalian Cos2 ortholog Kif7, which acts as a scaffolding protein for PKA, GSK3, and CK1, similarly to the role of Cos2 in Drosophila Hh signaling. Subsequent to ubiquitination, the Sufu-Gli2FL association is reduced by a mechanism that is not well understood, and Gli2FL and Gli3FL are partially cleaved by proteasomes, such as those enriched at the basal body (183). The liberated Gli2FL and Gli3FL then leave the primary cilium and translocate to the nucleus, where they repress Hh target genes. In response to Hh, Smo becomes enriched along the membrane of the primary cilium, whereas Kif7 moves from the basal body to the tip of the cilium, and this latter translocation requires the molecular motor activity of Kif7 (183, 200). Gli2FL, Gli3FL, and Sufu then translocate to the tip of the primary cilium in a manner dependent on the molecular motors Kif7 and Kif3A.

The Sufu-Gli2FL and Sufu-Gli3FL complexes accumulate in primary cilia within minutes of exposure to Hh, and they do so independently of new protein synthesis (183). These observations suggested that the trafficking of Gli2FL and Gli3FL into primary cilia is an immediate and direct response to Hh signaling. Gli2FL and Gli3FL then become differentially phosphorylated and dissociate from Sufu. This differential phosphorylation of Gli proteins requires primary cilium trafficking. These modifications allow Gli2FL and Gli3FL to be converted into Gli2 and Gli3, translocate from the primary cilium into the cytoplasm, then translocate into the nucleus, where they promote expression of Hh target genes. Nuclear-enriched Gli2 and Gli3 are quite labile and are quickly degraded by the proteasome (112, 123). Dissociation of Sufu-Gli complexes serves two purposes: (i) attenuating Gli2 and Gli3 processing to Gli2 and (ii) promoting the formation of Gli3. In the absence of Hh, formation of complexes with Sufu promotes processing of Gli3 through a Cul3 ubiquitin proteasome system. In the presence of Hh, these complexes dissociate, leading to activation of Gli2 and Gli3 and subsequent complete degradation through a Cul3 ubiquitin proteasome system. Gli3 formation occurs independently of Hh in cells lacking Sufu, and this occurs whether the cells have a primary cilium or not (184, 201). This is distinct from the Hh-independent activation observed in Ptcl− /− mouse embryonic fibroblasts (MEFs), in which constitutive Gli activity requires the primary cilium (202). These observations suggest that the main function of the primary cilium in Hh signaling is to attenuate Sufu function. It has also been proposed that Sufu might associate with newly translated Gli1, which would contribute to primary cilium an additional level of control over Gli1 activity (183).

Type I Noncanonical Hh Signaling

Ptcl can function as a dependence receptor (Fig. 2), regulating cell survival in a ligand-dependent manner (203), as illustrated by the finding that overexpression of Ptcl induces apoptosis in a variety of ex vivo and in vitro assays (204). Like other dependence receptors, Ptcl contains a dependence-associated receptor C-terminal motif that is cleaved by caspases at a conserved aspartic acid (Asp198) in the absence of Shh, to expose a proapoptotic domain (204, 205). Mutation of this cleavage site (Ptcl198Asp) abolished Ptcl-induced cell death, and this mutant receptor acted as a dominant negative inhibitor of endogenous Ptcl-induced apoptosis. Furthermore, a C-terminally truncated Ptcl mutant that mimics caspase-cleaved Ptcl induced cell death regardless of the presence of Shh, demonstrating the functional importance of caspase cleavage. Ptcl associates with a multiprotein complex that is required for induction of cell death, and its association with this proapoptotic complex was disrupted in the presence of Hh (206). All three vertebrate Hh ligands exhibited an antiapoptotic effect in cultured cells, which was not blocked by various Smo antagonists, was not mimicked by Smo overexpression or
the Smo agonist SAG, and was independent of Gli transcriptional activity (207). Thus, these findings suggest that this signaling mechanism was dependent on both Hh and Ptc1 but independent of Smo.

Canonical Hh signaling plays an essential role in cell proliferation through induction of the genes encoding cyclin D1 and N-Myc (208–210). Interestingly, a Ptc1-dependent noncanonical signaling activity also regulates the cell cycle through modulation of the subcellular localization of cyclin B1. Ptc1 is a binding partner of phosphorylated cyclin B1, and their association is regulated at the G2/M checkpoint (211, 212). Ptc1 ablation or Shh binding to Ptc1 disrupted this interaction, allowing nuclear translocation of cyclin B1 and subsequent completion of mitosis (Fig. 2). Conversely, in the absence of Shh, overexpression of Ptc1 in human embryonic kidney 293 T cells resulted in redistribution of cyclin B1 from the nucleus to the cytoplasm and decreased cell proliferation. A truncated mutant form of Ptc1 that is unable to interact with cyclin B1 was identified in a subset of basal cell carcinomas, and epidermal ablation of Ptc1 in mice increased nuclear cyclin B1, which is consistent with cyclin B1 driving mitosis in these Hh-dependent tumors (213). Interestingly, in response to Shh, GRK2 could displace cyclin B1 in its association with Ptc1 (214). Furthermore, RNAi-mediated knockdown of GRK2 abrogated the Shh-induced redistribution of cyclin B1 to the nucleus, and this effect could be rescued by expressing wild-type GRK2. Taken together, these results suggest that Shh stimulation induces a conformational change in Ptc1 that increases its affinity for GRK2 at the expense of its interaction with cyclin B1. The direct association of cyclin B1 and GRK2 with Ptc1 and the modulation of this association by Shh suggest that this mechanism does not require Smo.

Type II Noncanonical Hh Signaling

A large number of studies have implicated a noncanonical Hh signaling pathway in regulating the actin cytoskeleton through activation of the Rho family of small GTPases, such as RhoA and Rac1 (Fig. 3). In cultured endothelial cells, all Hh ligands promote actin stress fiber formation and tubulogenesis in a Smo-dependent manner though activation of RhoA (207, 215). In fibroblasts, stimulation of RhoA and Rac1 by Smo is required for migration in response to Shh (216). Pharmacological analysis has demonstrated that stress fiber formation and tubulogenesis depend on Smo- and Gβγ-mediated activation of RhoA. Activation of small GTPases by Hh proteins is not limited to endothelial cells and fibroblasts. For example, Shh increases the number of dendritic spines in hippocampal neurons, but apparently through an alternative mechanism (217). This latter work suggested that inactive Smo prevents Rac1 activation by interacting with the Rac guanine nucleotide exchange factor (GEF) T-lymphoma invasion and metastasis 1 (Tiam1). This Smo-Tiam1 complex dissociates upon Shh-mediated activation of Smo, thus allowing Tiam1 to activate Rac1. The rapid time course of these responses, the lack of detectable Gli-dependent transcriptional activity, and the inability of Gli3R to prevent these responses suggest that this is a Gli-independent Hh signaling pathway. Thus, there is considerable evidence supporting Smo-dependent, Gli-independent, noncanonical Hh signaling mediated by activation of small GTPases.

It is now well established that Shh can also act as an axon guidance cue (218). Shh signaling acts as an attractive cue for commissural axons and a repulsive cue for retinal axons during embryonic development. Commisural axons extend from the dorsal neural tube and project toward and then across the floor plate guided by the concerted effects of several molecular cues. Shh acts in a Smo-dependent manner as a chemorepellant in this process: Cultured commissural neurons turn their axons toward a source of Shh within minutes of exposure, and this response requires the presence of Smo (220). This rapid response provided the first suggestion that Shh-induced axon turning would not require transcription. Consistent with this signaling mechanism being independent of Gli-mediated transcriptional regulation, expression of Gli3R did not attenuate Shh-induced axon turning. Instead, Shh rapidly and locally stimulated phosphorylation of the Src family kinase...
and opening of IP₃ neurons through Gₛ kinase family (SFK) members Src and Fyn in neurons. In addition, Smo stimulates calcium release from the endoplasmic reticulum (ER) in spinal neurons through G₃, and PLC-γ-catalyzed generation of IP₃ and opening of IP₃-dependent channels. (226, 227), and this effect required the presence of Smo. Both the reduction in cAMP and the retraction of growth cones occurred transiently 15 to 30 min after the addition of Shh. This rapid response to Shh appears incompatible with a mechanism requiring Gli-mediated transcriptional regulation but is consistent with Shh activating Gₛ proteins, which in turn inhibit AC to reduce axonal cAMP.

Calcium ions regulate a vast number of processes in neuronal and neuronal precursor cells, including proliferation, differentiation, apoptosis, and migration (228, 229). Recently, Shh was shown to induce a dose-dependent increase in Ca²⁺ spikes in neural tube explants (230). This effect was mimicked by treatment with a Smo agonist and attenuated by cotreatment with a Smo antagonist or pretreatment with PTX, suggesting that Smo activation in this context promoted the activity of a PTX-sensitive Gₛ protein. Furthermore, this work suggested that the Giβγ subunits released upon Gₛ activation activated phospholipase C-γ (PLC-γ) to produce inositol 1,4,5-triphosphate (IP₃), which would subsequently increase intracellular Ca²⁺ abundance. Consistent with this hypothesis, inhibitors of PLC-γ and of IP₃ receptors reduced Ca²⁺ spike activity. In addition, acute stimulation of Smo with SAG resulted in sequential IP₃ and Ca²⁺ transients in the primary cilium of neurons, and Smo antagonists abolished this effect. Although the involvement of Gli-dependent transcription was not formally ruled out in these studies, the rapid activation of these responses is inconsistent with a transcription-based mechanism. This provocative finding suggests that Hh signaling might regulate a cohort of other physiological processes through Ca²⁺-dependent signaling pathways.

**Hh Signaling Networks—A Consolidated View**

Historically, many of the components initially proposed as constituting the signaling pathway for a newly discovered ligand have depended on how the initial pieces of that puzzle were identified and on the scientific interests of the individuals assembling the puzzle. Thus, whether a researcher is, for
example, a geneticist, a pharmacologist, or a biochemist will influence the experimental approaches and therefore what will eventually come to be known as the “canon- nal” signaling pathway for that ligand. Eventually, however, other researchers with different backgrounds and other model systems will begin to uncover context-specific modifications of this “canonical” signaling pathway. Further, some of these context-specific differences may eventually coalesce into “noncanonical” signaling pathways. Differences and similarities between distinct experimental models will also contribute to how these signaling modules are viewed, although context-specific signaling variations may also confound such analyses. And as is the case with all scientific disciplines, the difficulty in interpreting new findings as they are reported in any rapidly moving field will distort many of these models—at least temporarily. To date, many of these issues have also played key roles in the genesis of our understanding of Hh signaling. An additional hurdle imposed on these models of Hh signaling has been the discovery that primary cilia are of central importance for Hh signaling in mammals, but they are irrelevant to Hh signaling in Drosophila (231). When Hh signaling is examined from a global perspective, several generalities emerge, many of which also apply to other signaling pathways. Hh signaling should be viewed as a signaling network rather than as a simple linear pathway (Fig. 4). This network has multiple distinct signaling modules that can function independently or in an overlapping manner, depending on cellular context. Signaling through the network may branch into different modules at the level of Ptc, in a manner that may or may not be dependent on various Ptc co-receptors. Another pivotal branch point for these signaling modules is Smo, which can regulate both G protein and non-G protein–mediated signaling modules. Last, regulation of the stability and activity of Gli transcription factors by Hh proteins serves as the dominant transcriptional nexus of Hh activity. These major signaling modules thus combine in different ways to initiate signaling upon Hh binding to Ptc. However, once this signaling network is initiated, a series of additional feedback, feedforward, and crosstalk mechanisms serve to further modulate this network. Because the early elucidation of the core Hh signal transduc tion components was dominated by geneti- cists, in many cases these latter signaling events have been difficult to separate from the initial events in Hh signaling because of the involvement of multiple feedback and feedforward regulatory mechanisms. One could also envision cellular contexts in which initial early events induced by Hh signaling are very rapid and, therefore, transcription-independent but are followed by a series of transcription-dependent events that are subject to additional feedback and feedforward regulation. Such temporally regulated signaling modules might also begin to explain the slow rate of Gli target gene expression, which appears slower than one might posit, given the rapid rates of early developmental processes regulated by Hh. This is especially true when comparing Gli target gene expression to the immedi ate-early genes transcribed in response to serum growth factors (232). Thus, as our knowledge of Hh signaling matures, we anticipate a clearer understanding of the spatial, temporal, and cell-contextual regulation of the various signaling modules that comprise the Hh signaling network.

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