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Decoding the phosphorylation code in Hedgehog signal transduction

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Hedgehog (Hh) signaling plays pivotal roles in embryonic development and adult tissue homeostasis, and its deregulation leads to numerous human disorders including cancer. Binding of Hh to Patched (Ptc), a twelve-transmembrane protein, alleviates its inhibition of Smoothened (Smo), a seven-transmembrane protein related to G-protein-coupled receptors (GPCRs), leading to Smo phosphorylation and activation. Smo acts through intracellular signaling complexes to convert the latent transcription factor Cubitus interruptus (Ci)/Gli from a truncated repressor to a full-length activator, leading to derepression/activation of Hh target genes. Increasing evidence suggests that phosphorylation participates in almost every step in the signal relay from Smo to Ci/Gli, and that differential phosphorylation of several key pathway components may be crucial for translating the Hh morphogen gradient into graded pathway activities. In this review, we focus on the multifaceted roles that phosphorylation plays in Hh signal transduction, and discuss the conservation and difference between *Drosophila* and mammalian Hh signaling mechanisms.

***Keywords*:** Hedgehog; Smo; Gli; signal transduction; phosphorylation; development; cancer

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Introduction

The Hedgehog (Hh) signaling pathway plays crucial roles in the control of cell growth and patterning during embryonic development and adult tissue homeostasis [1, 2]. Malfunction of this pathway has been linked to numerous human disorders including cancer [1, 3]. *hh* was first identified as a segment polarity gene in *Drosophila* and the core pathway was delineated by fly geneticists [2, 4]. Vertebrates contain multiple *hh* family members; e.g., mammals contain three *hh* genes: *Sonic Hedgehog* (*Shh*), *Indian Hedgehog* (*Ihh*) and *Desert Hedgehog* (*Dhh*), with *Shh* playing a prevalent role [3]. Hh proteins are dual lipid modified and form soluble protein complexes that promote its long-range signaling [3, 5, 6]. Secretion of lipidated Hh is mediated by a twelve-transmembrane protein Dispatched (Disp) as well as a glycoprotein Scube/You (vertebrate only) [7-10], whereas Hh gradient formation is regulated by HSPGs and other cell surface molecules [6, 11].

The Hh signal is transduced by a conserved core signaling pathway that culminates in the activation of a latent Zn-finger transcription factor Cubitus interruptus (Ci)/Gli (Figure 1). Vertebrates contain three Gli proteins: Gli1, Gli2 and Gli3, with Gli2/3 as the primary mediators of Hh signaling and Gli1 as a target of the Hh pathway that acts in a positive feedback to reinforce the Gli activity [12]. In the absence of Hh, Ci/Gli (mainly Gli3 and to a lesser extent Gli2) is proteolytically processed into a truncated form (Ci^R/Gli^R) that functions as a transcriptional repressor to block the expression of a subset of Hh target genes. Hh signaling inhibits Ci/Gli processing and thus the production of Ci^R/Gli^R, and converts the accumulated full-length Ci/Gli (Ci^F/Gli^F) into an active form (Ci^A/Gli^A) that stimulates Hh target gene expression.

Hh signaling pathway: an overview

The core components of the Hh pathway were initially

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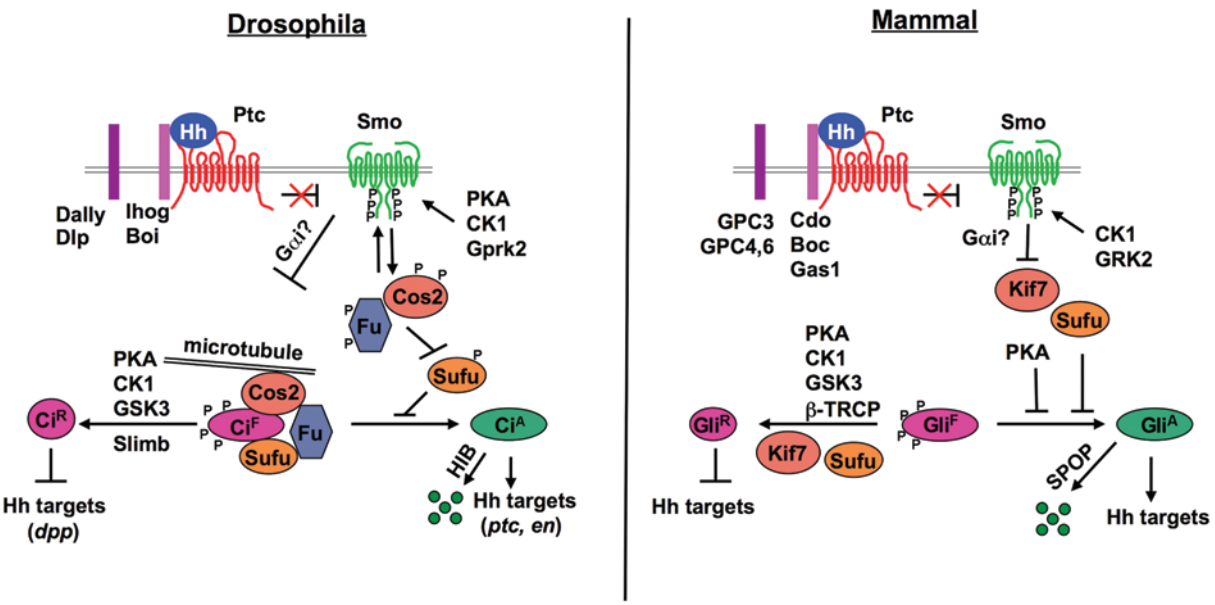

Figure 1 Hh signal transduction in *Drosophila* and mammalian systems. Hh protein is lipid-modified. *Drosophila* and mammalian HSPGs, Dally, Dally-like (Dlp), GPC3, GPC4 and GPC6 modulate Hh signaling. In the absence of Hh, Ptc inhibits Smo, allowing Ci^F/Gli^F to be phosphorylated by multiple kinases and targeted for Slimb/β-TRCP-mediated proteolysis to generate Ci^R/Gli^R. In *Drosophila*, the kinesin-like protein Cos2 acts as a molecular scaffold to bridge Ci to its kinases. In the presence of Hh, Ptc inhibition of Smo is released, which triggers Smo phosphorylation by PKA, CK1 and Gprk2/GRK2, leading to its cell surface accumulation and activation. Smo recruits Cos2-Fu complex to the cell surface, and dissociates Cos2-Ci-kinase complexes to inhibit Ci phosphorylation and processing. In the presence of high levels of Hh, Fu converts Ci^F into Ci^A by antagonizing Sufu inhibition. Ci^A is unstable and degraded by the HIB-mediated Ub/proteasome pathway. Fu-Cos2 also regulates Smo phosphorylation in a feedback loop mechanism. In mammals, Hh induces Smo phosphorylation by CK1 and GRK2, leading to its ciliary accumulation (not shown here) and activation. Sufu is a major whereas Kif7 a minor inhibitor of Gli proteins. The HIB homolog SPOP is responsible for degrading Gli proteins in the absence of Sufu. Ci^F/Gli^F: full length Ci/Gli; Ci^A/Gli^A: activator form of Ci/Gli; Ci^R/Gli^R: repressor form of Ci/Gli. Adapted from reference [1].

identified in *Drosophila* and are conserved in vertebrates. These include Patched (Ptc), a twelve-transmembrane protein that is structurally related to the bacterial RND family of proton-driven transporters [13], and Smoothened (Smo), a seven-transmembrane protein that is structurally related to GPCRs [14-16]. Ptc functions as the Hh receptor, whereas Smo functions as a signal transducer [16, 17]. Unlike most signal transduction pathways where receptors function as positive regulators of the pathways and activate downstream signaling components upon ligand binding, Ptc acts as a negative regulator of the Hh pathway by inhibiting Smo in the absence of the ligand [18]. Binding of Hh to Ptc, which is facilitated by the Ihog/Cdo family of coreceptors [19], alleviates its inhibition of Smo, leading to Smo phosphorylation and activation [20] (Figure 1).

Several conserved pathway components function to link Smo to Ci/Gli, including the kinesin-like protein Costal2 (Cos2)/Kif7 and Sufu, as well as several Ser/Thr kinases including PKA, GSK3 and CK1 (Figure 1). In *Drosophila*, Cos2 forms a complex with the Ser/Thr kinase Fused (Fu) and Ci to impede Ci nuclear translocation and promote Ci processing into Ci^R [21-26]. Sufu also forms a complex with Ci to impede its nuclear translocation and block Ci^A activity [23, 27, 28]. In the absence of Sufu, Ci is still converted into Ci^R, and Ci^F is sequestered in the cytoplasm by Cos2 so that *Sufu* mutation does not cause discernable activation of the Hh pathway [28]. In contrast, *Sufu* knockout (KO) in mice leads to ectopic Hh pathway activation similar to loss of Ptc [29, 30], suggesting that Sufu plays a major role in restricting Gli activity downstream of Smo in the mammalian system. One possible reason is that in the mammalian Hh pathway, Sufu is required not only for inhibiting Gli^A formation but also for the production of Gli^R [31].

In *Drosophila*, the Fu kinase converts Ci into an active but labile form (Ci^A) that is degraded via a Cul3-based E3 ubiquitin ligase containing the BTB family protein HIB (also called Rdx) [32-34]. Interestingly, *hib* expression is induced by Hh signaling in both *Drosophila*

embryos and imaginal discs, thus forming a negative feedback loop to attenuate Hh pathway activity [32, 33]. SPOP, which is the vertebrate homolog of HIB, may play an analogous role in fine-tuning Hh signaling by degrading Gli2/3 proteins [30, 35, 36]. Unexpectedly, the mammalian Fu homolog (mFu) is not required for Hh signaling because $mFu^{-/-}$ mice do not exhibit any phenotypes indicative of the Hh pathway activity defect [37, 38].

Another major difference between the mammalian and *Drosophila* pathways is that mammalian but not *Drosophila* Hh signaling depends on the primary cilium, a microtubule-based membrane protrusion and antenna-like cellular structure [39]. Genetic screens in mice identified multiple intraflagellar transport proteins (IFTs) critical for appropriate Hh signaling [40, 41]. Major Hh signaling pathway components including Ptc, Smo, Kif7, Sufu, PKA and Gli proteins are present in the primary cilium with some exhibiting a dynamic pattern [30, 42-46]. For example, Hh promotes ciliary exit of Ptc but ciliary accumulation of Smo [42, 44]. Hh also stimulates the accumulation of Gli proteins at the cilium tip, a step likely reflecting Gli^A formation. Disruption of primary cilia impedes the formation of both Gli^R and Gli^A [40, 43, 47]. Thus, the primary cilium may function as a signaling center to orchestrate the molecular events leading to Gli processing in the absence of Hh and Gli activation in response to Hh, although the exact biochemical mechanisms remain poorly understood.

Regulation of Ci/Gli proteolysis: a story of three kinases

Ci/Gli processing is responsible for the production of Ci^R/Gli^R that plays important roles in preventing inappropriate Hh pathway activation in many developmental contexts, including *Drosophila* and vertebrate limb development [1, 12]. Unraveling the mechanism underlying Ci/Gli processing has been facilitated by genetic studies in *Drosophila* that identified two key components in this process: PKA and an F-box protein Slimb/β-TRCP that functions as a substrate recognition component of the SCF family of E3 ubiquitin ligases [48-51]. An important feature of the SCF family of E3s is that they only recognize substrates after the substrates are phosphorylated, thus providing a link between kinase-mediated phosphorylation and ubiquitin/proteasome-mediated proteolysis [52, 53]. Indeed, PKA can directly phosphorylate multiple Ser/Thr residues in the C-terminal half of Ci (Figure 2), and mutating these PKA sites abolishes Ci processing [54, 55]. However, phosphorylation of Ci by PKA alone does not confer recognition by Slimb, implying that additional phosphorylation events are required for Ci processing. Further genetic studies identified Shaggy (Sgg), the *Drosophila* GSK3, and CK1 as two kinases that act in conjunction with PKA to promote Ci processing [56-58]. These three kinases phosphorylate Ci sequentially at three clusters of sites, with PKA serving as the priming kinase for GSK3 and CK1 (Figure 2), and these phosphorylation events create the docking sites for SCF^{Slimb} [57, 59]. Strikingly, mutating any of the three phosphorylation clusters diminished the production of Ci^R *in vivo*, suggesting that they act cooperatively to regulate Ci processing [56, 57]. A more recent study suggested that an extended phosphorylation cluster primed by PKA sites 1 and 2 (SpTpYYGSp), which closely resembles the Slimb/β-TRCP binding site consensus $DSpGX_{2\text{-}4}Sp$, provides the primary contact site for Slimb (Figure 2), and that multiple phosphorylation events may lead to the recruitment of two copies of SCF^{Slimb} complex that bind Ci simultaneously [60]. Thus, efficient Slimb binding and Ci processing require phosphorylation at multiple sites by three kinases, rendering Ci proteolysis very sensitive to Hh regulation. Indeed, low levels of Hh signaling are sufficient to block the production of Ci^R [61].

Similar phosphorylation events mediated through PKA, GSK3 and CK1 regulate proteolysis of Gli2 and Gli3 by recruiting β-TRCP [36, 62-66]. In contrast to Gli3 where β-TRCP-mediated proteolysis leads to partial degradation and therefore the production of Gli^R, Gli2 proteolysis often leads to complete degradation of the protein, consistent with the genetic studies suggesting that Gli3 is the major contributor of Gli^R [12]. Taking advantage of this difference, Pan *et al*. [67] identified a processing determinant domain (PDD) that is responsible for the differentially regulated limited proteolysis of Gli2/3. Several sequence and structural motifs are also required for proper Ci processing [68, 69], and removal of a Ci processing motif renders complete degradation of Ci [60].

A recent study demonstrated that PKA is not only required for Gli3 processing but also has a critical role in restricting the activator activity of Gli2 [45]. PKA-deficient mice exhibited phenotypes similar to $Ptc^{-/-}$ or $Sufu^{-/-}$ mice with a full-blown ectopic activation of the Hh pathway and ventralized neural tubes. Removal of Gli2 from PKA mutant mice suppressed the ectopic Hh pathway activation. Furthermore, Gli2 was accumulated at the tips of primary cilia in PKA-null MEF cells in the absence of upstream signal, consistent with its being constitutively active [45]. A previous study revealed that PKA also regulates both the activator and repressor forms of Ci because loss of PKA not only leads to ectopic expression of *dpp*, which is repressed by Ci^R, but also *ptc*, which is activated by Ci^A, whereas loss of Slimb

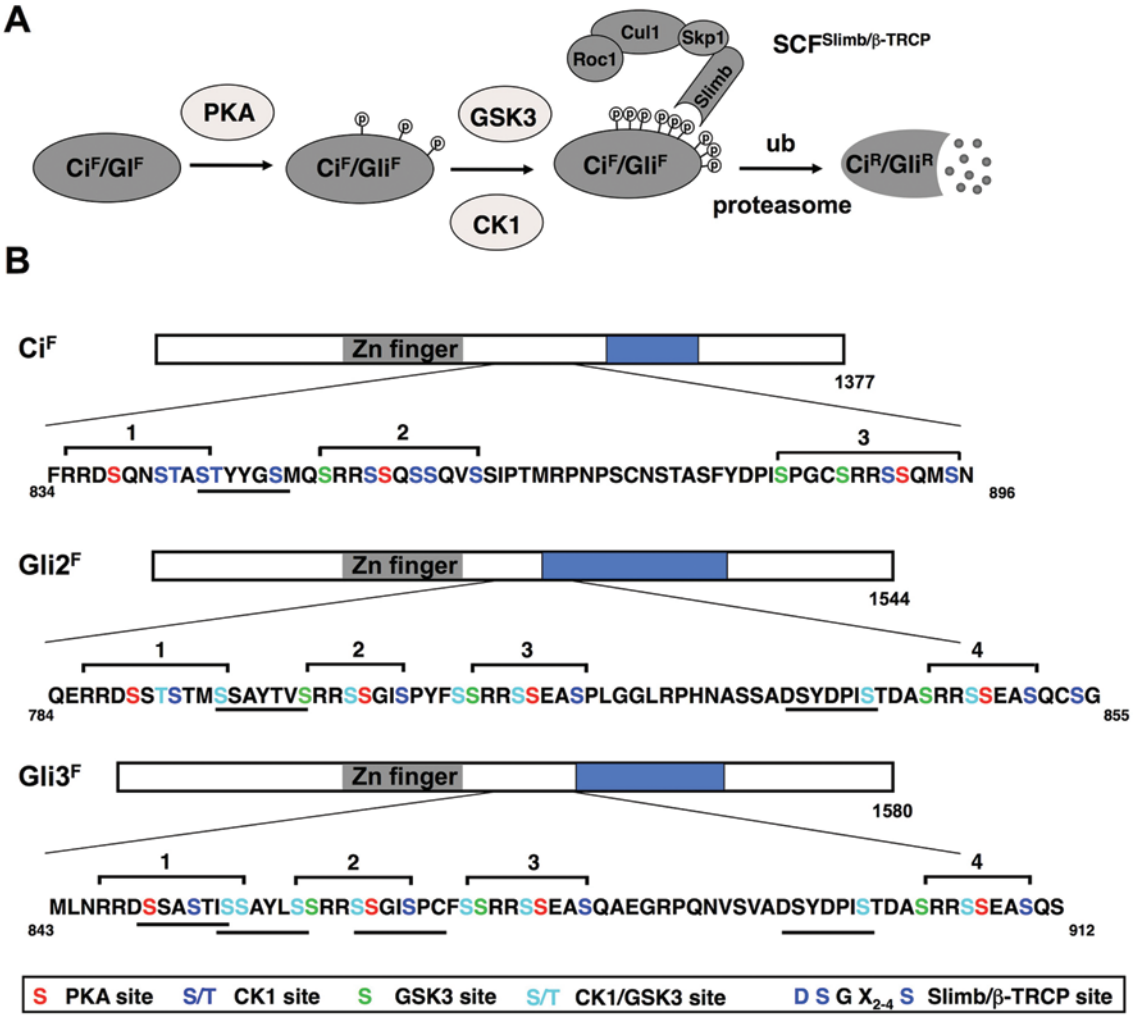

Figure 2 Ci/Gli phosphorylation and proteolysis. **(A)** In the absence of Hh, Ci^F/Gli^F is sequentially phosphorylated by PKA, GSK3 and CK1, and targeted to Ub/proteasome-mediated proteolysis through Slimb/β-TRCP to generate a truncated repressor Ci^R/Gli^R. **(B)** Diagrams of Ci, mouse Gli2 and human Gli3 showing the PKA/GSK3/CK1 phosphorylation clusters in Ci/Gli and the Slimb/β-TRCP binding consensus site. Putative Slimb/β-TRCP binding sites in Ci/Gli are underlined. Grey and blue boxes denote Zn-finger DNA binding domain and transactivation domain, respectively. ub: ubiquitin.

only leads to ectopic expression of *dpp* but not *ptc* [49, 55, 68]. Furthermore, a proteolysis-resistant form of Ci is still inhibited by PKA [70], suggesting that PKA can restrict Ci^A activity through a proteolysis-independent mechanism. It remains to be determined how PKA inhibits the activity of Ci^A/Gli^A.

GSK3, CK1 and Slimb/β-TRCP are also involved in the Wingless(Wg)/Wnt pathway by regulating the proteolysis of β-catenin [49, 71, 72], suggesting that these two developmental pathways employ a similar mechanism for signal transduction. These observations have also raised an important question of how pathway specificity is achieved. In the Wnt pathway, GSK3 and CK1 form a complex with β-catenin that is organized by the scaffolding proteins Axin and APC, and this processing complex is regulated by the specific interaction between the Wnt receptor complex and Axin [73]. An analogous mechanism has been proposed for the *Drosophila* Hh pathway in which Cos2 functions as a molecular scaffold to bridge Ci to its kinases, and Hh signaling impedes the formation of Cos2-Ci-kinase complexes [26]. Thus, Hh and Wnt may regulate distinct pools of GSK3 and CK1 to achieve pathway specificity.

In the vertebrate Hh pathway, primary cilia may have an important role in the regulation of Gli phosphorylation and proteolysis. For example, PKA is localized at the cilia base, suggesting that Gli proteins are phosphorylated during cilia transit [45]. This observation may

explain why Gli processing is impeded in the absence of primary cilia. Both Sufu and Kif7 are required for Gli3 processing [30, 31, 35, 74-77], raising a possibility that they may also regulate Gli3 phosphorylation. Indeed, Sufu can simultaneously bind GSK3 and Gli3, and thus recruit GSK3 to phosphorylate Gli3 [74]. Shh signaling may inhibit Gli phosphorylation by dissociating the Sufu-Gli-kinase complex [31, 46, 74].

Smoothened phosphorylation code

Smo belongs to the GPCR family and is an obligatory signal transducer of the canonical Hh signaling pathway. Oncogenic mutations in *Smo* contribute to basal cell carcinoma (BCC) and medulloblastoma [1, 78], and Smo has emerged as a prominent target for cancer therapeutics [79]. For example, vismodegib, a potent synthetic oral Smo inhibitor, has been approved by FDA for the treatment of advanced BCC. Thus, a complete understanding of how Smo is regulated and how it functions should not only provide important insights into the Hh signal transduction mechanisms but also may guide cancer drug development and treatment.

Smo activation in flies: dual roles of PKA and CK1

In *Drosophila*, Hh induces cell surface accumulation and phosphorylation of Smo [20]. Identification of Smo kinases came from the unexpected findings that PKA also plays a positive role in Hh signaling in both embryos and imaginal discs [80, 81]. Gain of PKA function promotes Smo accumulation and Hh pathway activation, whereas loss of PKA function blocks Hh-induced Smo accumulation as well as high levels of Hh signaling [81]. Similarly, inactivation of CK1α/ε by RNAi blocks Hh-induced Smo accumulation and high levels of Hh signaling activity, suggesting that CK1 also plays dual roles in Hh signaling [81]. Interestingly, CK1 plays dual roles in Wnt/Wg signaling [72, 82], extending the similarity between these two pathways.

Biochemical studies demonstrated that PKA and CK1 sequentially phosphorylate three clusters of Ser/Thr residues in the Smo C-terminal cytoplasmic tail (C-tail), with PKA serving as the priming kinase for CK1 phosphorylation [81, 83, 84] (Figure 3A). Phospho-deficient Smo variants exhibit reduced cell surface expression and diminished Hh signaling activity, whereas phospho-mimetic Smo variants exhibit increased cell surface levels and constitutive activity [81, 83, 84]. Interestingly, increasing the number of phospho-mimetic mutations in Smo results in a progressive elevation of Smo activity, suggesting that graded Hh signals may induce different levels of Smo activity through differential phosphorylation [81]. Indeed, a recent study using a phospho-specific antibody to monitor Smo phosphorylation has provided evidence that Hh induces Smo phosphorylation in a dose-dependent manner [85].

Phospho-regulation of Smo trafficking and conformation

Two recent studies suggested that phosphorylation promotes Smo cell surface expression by inhibiting ubiquitination-mediated endocytosis and degradation [86, 87]. In the absence of Hh, Smo is both mono- and polyubiquitinated at multiple Lys residues in its C-tail, leading to its endocytosis and degradation by both lysosome- and proteasome-dependant mechanisms. Smo endocytosis is also promoted by Kurtz (Krz), the *Drosophila* non-visual arrestin [87, 88]. Hh inhibits Smo ubiquitination and attenuates Smo/Krz interaction, thereby stabilizing Smo on the cell surface [86, 87]. The E3 ligase(s) that catalyzes Smo ubiquitination has remained elusive but a deubiquitinating enzyme, UBPY/USP8, is required for Hh-induced deubiquitination of Smo [86, 87]. Interestingly, UBPY/USP8 is also required for the deubiquitination of the Wnt receptor, Fz [89], thus providing another link between the Hh and Wnt pathways.

In addition to the regulation of Smo trafficking, phosphorylation also controls Smo conformation. Using FRET analysis, Zhao *et al*. [90] provided the first evidence that phosphorylation activates Smo by inducing a conformational switch of Smo C-tail, and it does so by antagonizing multiple Arg clusters in an autoinhibitory domain (SAID) located in the Smo C-tail (Figures 3A and 3B). The Arg clusters maintain the Smo C-tail in a closed conformation through intramolecular electrostatic interaction; Hh-induced phosphorylation abrogates this interaction and promotes an open conformation and clustering of Smo C-tails (Figure 3B). An interesting feature of the SAID domain is that it contains multiple regulatory modules each of which consists of an Arg cluster linked to a phosphorylation cluster (Figure 3A). The pairing of positive and negative regulatory elements offers a more precise mode of regulation in response to graded Hh signals, as increasing levels of phosphorylation may gradually neutralize the negative effect of multi-Arg clusters, leading to a progressive change in Smo cell surface accumulation, conformation and activity [90]. Indeed, decreasing the number of functional Arg motifs has the same effect as increasing the number of phospho-mimetic mutations, both leading to a progressive increase of Smo activity [81, 90].

Regulation of Smo by additional kinases

Phospho-mimetic mutations in the three PKA/CK1 phosphorylation clusters render Smo constitutively ac-

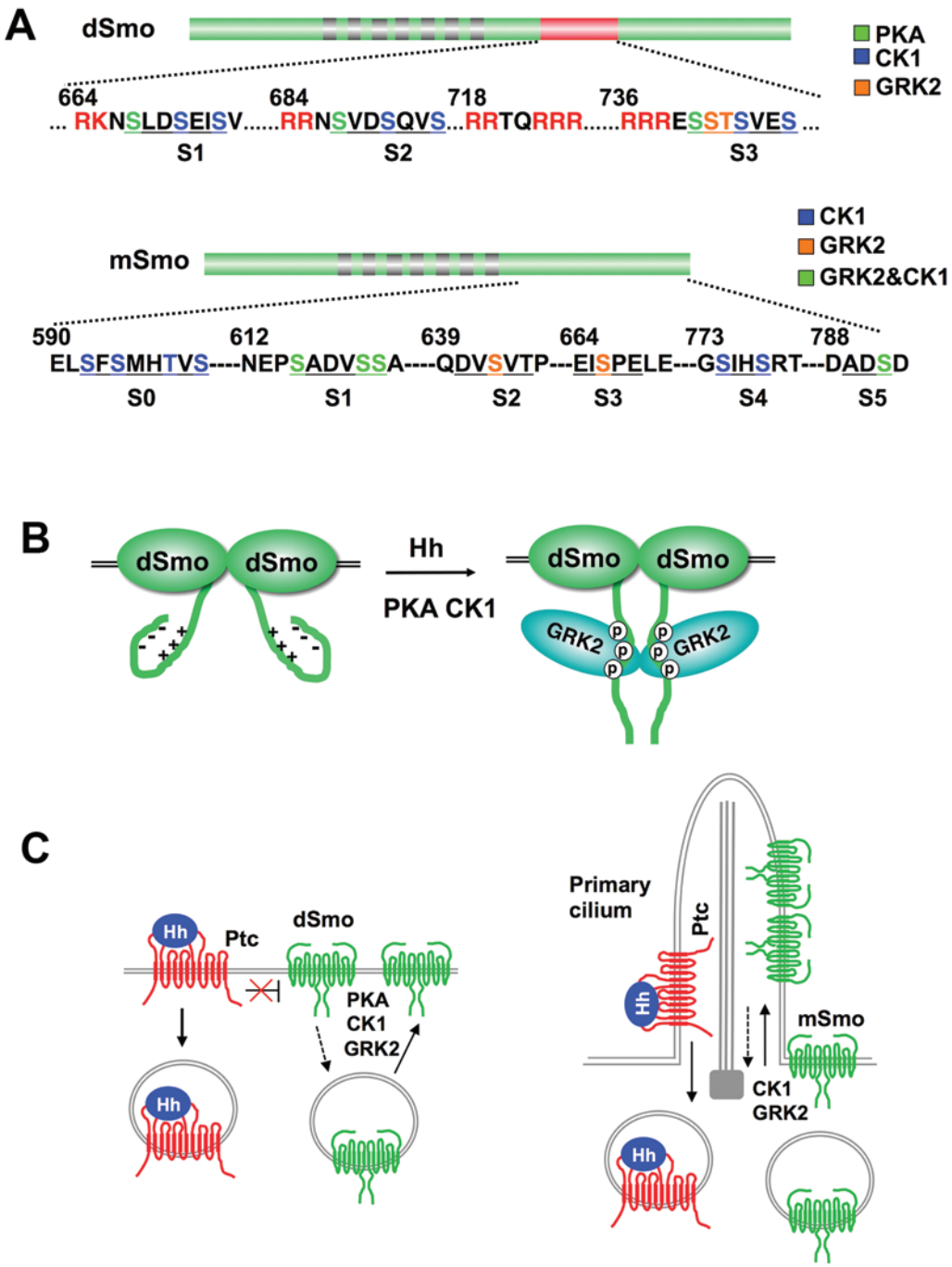

Figure 3 Phosphorylation of Smo regulates its subcellular localization and conformation in both *Drosophila* and mammalian systems. **(A)** Diagrams showing the phosphorylation sites in *Drosophila* (top) and mammalian Smo (bottom). The Arg motifs adjacent to phosphorylation clusters in *Drosophila* Smo are indicated in red. The red box in *Drosophila* Smo C-tail denotes the SAID domain. **(B)** Diagrams of closed inactive (left) and open active (right) conformations of *Drosophila* Smo. The closed conformation is maintained by intramolecular electrostatic interactions between multiple Arg motifs in the SAID domain and multiple acidic clusters in the C-terminal region of Smo. Hh-induced phosphorylation by PKA and CK1 neutralizes the positive charges and disrupts the intramolecular electrostatic interactions, leading to the opening and dimerization of Smo C-tail. Binding of GRK2 to Smo C-tail stabilizes its dimerization. **(C)** In *Drosophila*, Smo phosphorylation by PKA and CK1 leads to its cell surface accumulation and active conformation whereas in mammals, Smo phosphorylation by CK1 and GRK2 leads to its ciliary accumulation and active conformation. SAID: Smo autoinhibitory domain. Adapted from the references [1, 92, 100].

tive but fail to confer full activity [90], suggesting that full Smo activation may involve additional mechanisms. Such mechanisms may include additional phosphorylation events, non-phosphorylation events or both. Indeed, a mass spectrometry analysis identified many other phosphorylation sites in Smo C-tail, besides the three PKA/CK1 phosphorylation clusters [84]. Genetic modifier screens identified casein kinase 2 (CK2) and G-protein-coupled receptor kinase 2 (Gprk2/GRK2) as positive regulators of Hh signaling [91, 92]. Previous studies also revealed a positive role of GRK2 in the mammalian Hh pathway [93-95]. A recent study showed that inhibition of CK2 downregulated the Hh/Gli signaling in human lung cancer cell lines, suggesting a conserved positive role of CK2 in the mammalian Hh pathway [96]. In *Drosophila*, CK2 promotes Hh signaling by regulating the stability of both Smo and Ci [91]. *In vitro* kinase assay indicated that CK2 directly phosphorylated multiple Ser residues in the Smo C-tail [91], some of which were also phosphorylated in cultured cells in the presence of Hh [84].

In *Drosophila*, GRK2 promotes high-level Hh signaling through both kinase-dependent and kinase-independent mechanisms [92, 97]. GRK2 phosphorylates Smo C-terminal tail at Ser741/Thr742, which is facilitated by PKA/CK1-mediated phosphorylation at adjacent Ser residues [92] (Figure 3A). In addition, GRK2 forms a dimer and binds Smo to stabilize its open conformation, and promote the dimerization of Smo C-tail [92] (Figure 3B). Interestingly, GRK2 expression is induced by Hh signaling in wing imaginal discs, thus forming a positive feedback loop to facilitate high-level Hh signaling [92, 97].

A conserved Smo activation mechanism in mammals

Mammalian Smo (mSmo) diverges significantly from *Drosophila* Smo (dSmo) in the primary sequence of its C-tail and does not contain the three PKA/CK1 phosphorylation clusters found in dSmo C-tail (Figure 3A). In addition, mSmo traffics through the primary cilia to transduce the Hh signal (Figure 3C). These and other differences have led to the proposal that mSmo and dSmo are regulated by fundamentally distinct mechanisms [98, 99]. However, a recent study has revealed unexpected similarities between dSmo and mSmo activation mechanisms: both dSmo and mSmo are regulated by multi-site phosphorylation in a dose-dependent manner, and phosphorylation regulates both their subcellular localization and conformation [100] (Figure 3C).

Although PKA is not involved in mSmo activation, CK1α and GRK2 are required for Hh-induced mSmo phosphorylation and pathway activation [93, 94, 100, 101]. CK1α and GRK2 bind mSmo in response to Hh stimulation and phosphorylate mSmo C-tail at six Ser/Thr clusters (S0-S5; Figure 3A) [100]. Functional study using both cultured mammalian cells and chick neural tubes suggested that multiple CK1/GRK2 phosphorylation sites regulate mSmo activity in a dose-dependent manner, with the two membrane-proximal clusters (S0 and S1) playing a major role [100]. As is the case for dSmo [81, 85], mSmo phosphorylation is regulated in a dose-dependent manner with increasing amounts of Hh inducing a progressive increase of mSmo phosphorylation, suggesting that Hh gradient is translated into an mSmo phosphorylation and activity gradient [100].

A prevalent view is that Hh activates mSmo by inducing its ciliary localization [42, 44] (Figure 3C). Indeed, CK1/GRK2-mediated phosphorylation promotes mSmo ciliary localization by recruiting β-arrestins that link mSmo to the anterograde kinesin-II motor [100, 102]. However, ciliary localization of mSmo is insufficient for its activation because the Hh pathway inhibitor cyclopamine promotes rather than blocks ciliary localization of mSmo [103-105]. In addition to regulating mSmo ciliary localization, Hh induces a conformational switch that results in the dimerization/oligomerization of mSmo C-tail [90]. A similar conformational change is also induced by oncogenic Smo mutation (A1/M2) and Smo agonist SAG but blocked by cyclopamine, suggesting that Hh-induced mSmo conformational switch represents an additional and critical step for mSmo activation [90]. Hh-induced conformational switch is governed by CK1/GRK2-mediated multi-site phosphorylation of mSmo C-tail [100]. Cyclopamine traps ciliary-localized mSmo in an unphosphorylated form that adopts an inactive conformation, whereas ciliary-localized mSmo in response to Hh or Smo agonists is phosphorylated and thus adopts an active conformation [100]. Thus, Smo phosphorylation is a more faithful readout for pathway activation than Smo ciliary localization, and can serve as a biomarker for cancers caused by deregulated Hh pathway activation.

Hh stimulates the binding of both CK1α and GRK2 to mSmo, which likely contributes to the signal-induced mSmo phosphorylation [100]. In the absence of Hh, the kinase-binding pockets in mSmo are masked when mSmo C-tail adopts a closed conformation. Shh stimulates CK1α binding to a juxtamembrane site, likely by inducing a local conformational change, to initiate the phosphorylation and conformational change of mSmo C-tail. This further increases the binding of CK1α/GRK2 to mSmo C-tail, forming a positive feedback loop to increase or sustain mSmo phosphorylation [100]. Interestingly, CK1α is accumulated in the primary cilia in response to Hh stimulation, which may explain, at least in

part, why phosphorylation of mSmo is more effective in primary cilia [100].

Hh signal transduction: from Smo to Ci/Gli

Smo can function as a GPCR to directly activate Gαi in frog melanophores as well as in *Drosophila* and mammalian cultured cells [106-108]. In *Drosophila* wing discs, Gαi is required for the expression of Hh target gene *dpp* [108]; however, a physiological role for Gαi in Shh signaling has not been demonstrated [109]. On the other hand, Smo can act via a G-protein-independent mechanism to relay the Hh signal to downstream signaling components [1, 110]. In addition, downstream signaling components, including Cos2, Fu, Sufu and perhaps Ci/Gli, are phosphorylated in response to Hh stimulation, although the kinases involved and the physiological relevance of these phosphorylation events are not fully understood.

Cos2 phosphorylation and regulation of Hh signaling complexes

Several studies demonstrated that Smo interacts with a Cos2-Fu complex through its C-tail [108, 111-115]. The interaction between Smo and Cos2/Fu can occur even in the absence of Hh but there is evidence that Smo-Cos2-Fu complex formation is facilitated by Hh signaling in both *Drosophila* embryos and cultured cells [114, 116, 117]. How the dynamic interaction between Smo and Cos2-Fu complex is regulated remains poorly understood, but it is possible that Smo and Cos2-Fu form distinct complexes depending on the phosphorylation status of individual components, and pathway activation may rely on changes in the location, composition and conformation of the complexes. Indeed, deletion analyses identified two distinct regions of Smo C-tail capable of binding to Cos2: a membrane proximal domain between aa 651-686 and a C-terminal domain between aa 818-1035 [111, 112]. The membrane proximal domain appears to mediate an inhibition of Smo phosphorylation by Cos2 that forms a complex with PP4, and Cos2/PP4-mediated inhibition of Smo phosphorylation is alleviated by Fu upon Hh signaling through a positive feedback loop [118-120]. In contrast, the C-terminal Cos2-binding domain plays a positive role in Smo activity [111], and its association with Cos2 is stimulated by Hh [116, 117].

The interaction between Cos2 and different Smo domains appears to be regulated by Cos2 phosphorylation. In response to Hh, Fu phosphorylates Cos2 at Ser572 and Ser931 [121-123]. Using phospho-specific antibodies that recognize pSer572 or pSer931, Ranier *et al*. [123] provided evidence that Ser572 phosphorylation can be induced by low levels of Hh, whereas Ser931 phosphorylation only occurs in the presence of high levels of Hh. In addition, they showed that Ser931 phosphorylation correlated with increased Smo/Cos2 association and pS931 signal was mainly detected at the plasma membrane. Previous studies showed that Ser572 phosphorylation attenuated the association between Cos2 and Smo, and that a phospho-mimetic form of Cos2 (Cos2S572D) exhibited reduced binding to the membrane proximal domain of Smo C-tail [119, 122]. Taken together, these results suggest that differential phosphorylation of Cos2 may regulate the formation of distinct Smo-Cos2-Fu complexes: Ser572 phosphorylation disrupts the inactive complex in which Cos2 binds to the membrane proximal region of Smo C-tail, whereas Ser931 phosphorylation favors the formation of an active complex in which Cos2 binds to the C-terminal region of Smo. However, whether Ser931 phosphorylation is the cause or consequence of stable Smo-Cos2-Fu complex formation has not been firmly established.

Fu phosphorylation and action

The Ser/Thr kinase Fu is a positive regulator of Hh signaling in *Drosophila* as well as in zebrafish [124, 125]. Fu contains an amino-terminal catalytic domain (Fu-KD) and a C-terminal regulatory domain (Fu-RD) that mediates Fu interaction with other proteins including Cos2 and Sufu [22, 126]. In addition, Fu-RD can interact with Fu-KD, and this intramolecular interaction may provide a mechanism for Fu autoinhibition [127]. In response to Hh or activated Smo, Fu is hyperphosphorylated in a manner dependant on Fu kinase activity and CK1 [112, 116, 128, 129], suggesting that signal-stimulated Fu phosphorylation may involve both autophosphorylation and phosphorylation by other kinases. Many kinases can be activated by dimerization-induced transphosphorylation of their kinase domains within the activation loops [130]. Indeed, FRET analyses demonstrated that Hh induces dimerization of the Fu kinase domain in a dose-dependent manner [116, 117]. Fu dimerization is mediated by a conformational switch and dimerization of the Smo C-tail, and depends on its association with Cos2 [116, 117] (Figure 4). Importantly, forced dimerization of Fu via heterologous dimerization domains can induce its autophosphorylation and Hh pathway activation [116, 117]. The requirement of Cos2 for Fu dimerization and activation may explain the positive role of Cos2 in Hh signaling observed previously [23, 24]. Indeed, expression of a dimerized Fu variant can restore high levels of Hh signaling in *cos2* mutants [116].

The activation loop (AL) of Fu-KD contains four conserved Ser/Thr residues: Thr151, Thr154, Thr158 and

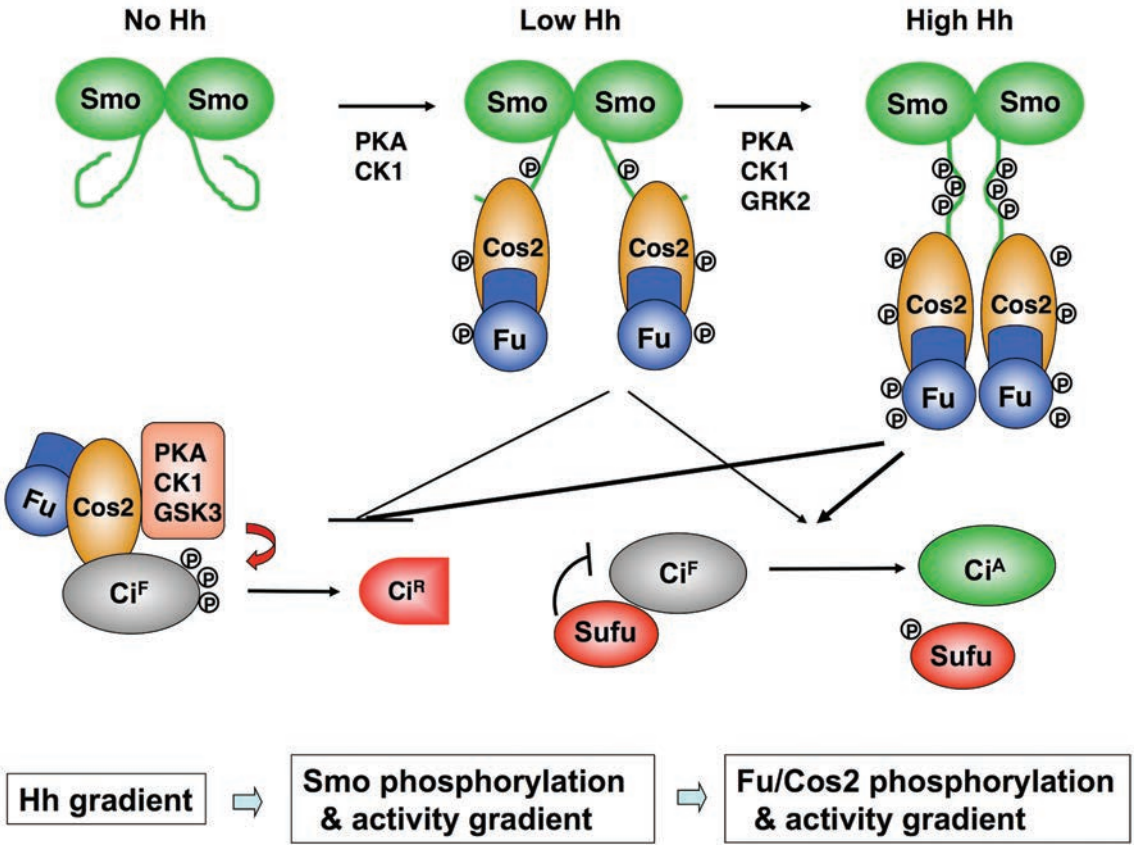

Figure 4 A model for Smo phosphorylation and conformational switch leading to Hh pathway activation in *Drosophila*. Hh morphogen gradient is translated into Smo phosphorylation and activity gradients, which are in turn translated into Fu/Cos2 phosphorylation and activity gradients. Smo phosphorylation induces an open conformation of Smo C-tail, which facilitates the recruitment of Cos2/Fu. In addition, dimerization of Smo C-tail leads to clustering of the bound Cos2/Fu, resulting in Fu phosphorylation and activation. Activated Fu inhibits Ci^R production by dissociating Cos2-Ci-kinase complexes and stimulates Ci^A formation likely by dissociating Sufu from Ci. Adapted from the reference [116].

Ser159 [116, 129]. Mutagenesis experiments indicated that Ser159 is absolutely essential for Fu activity, whereas phosphorylation at the Thr residues is required for optimal activity; overall, there appears to be a correlation between the level of AL phosphorylation and the level of Fu activity [116, 129]. Using phospho-specific antibodies that recognize dual phospho-residues, pThr151/pThr154 or pThr158/pSer159, Shi *et al.* [116] provided evidence that phosphorylation of these two clusters increased progressively in response to increasing levels of Hh or Smo phosphorylation, suggesting that Hh/Smo activity gradient is translated into a Fu phosphorylation and activity gradient (Figure 4).

Fu-RD is phosphorylated both by Fu-KD and CK1, and these phosphorylation events may also regulate Fu kinase activity [116, 129]. Indeed, mutating several CK1 consensus sites in the Fu regulatory domain (such as Ser485 and Thr486) reduces the activity of a constitutively active form of Fu [129]. It is possible that CK1 may phosphorylate additional sites in the Fu-RD or even in the Fu-KD to regulate Fu activity. It will be important to determine how phosphorylation of the Fu-RD contributes to Fu activation. One possibility is that phosphorylation of the Fu-RD may attenuate Fu autoinhibition.

A conventional view regarding its role in Hh signaling is that Fu is only required for transducing high levels of Hh signaling by converting Ci^F into Ci^A [28, 124]; however, the full spectrum of Fu function could have been underestimated due to the lack of a null mutation [131], and/or the existence of paralleled mechanisms such as Gαi activation [108]. Using a gain-of-function approach, two recent studies demonstrated that activated forms of Fu can block Ci processing into Ci^R in addition to converting Ci^F into Ci^A [116, 129]. Furthermore, overexpression of a dominant negative form of Fu blocks Ci accumulation and *dpp* expression [131]. These new findings

suggest that Fu may participate in the regulation of both Ci^R and Ci^A. Mechanistically, activated Fu can phosphorylate Cos2, leading to the disassembly of the Cos2-Ci-kinase complexes required for Ci phosphorylation and processing (Figure 4) [116, 122, 123, 129]. Activated forms of Fu can also phosphorylate Sufu and dissociate Sufu from Ci, leading to Ci activation [116, 117] (Figure 4).

Signaling downstream of mammalian Smo

In mice, Sufu is a major negative regulator of Shh, signaling downstream of Smo [29, 132]. Sufu forms complexes with Gli proteins to inhibit their nuclear localization and transcriptional activity [133-136]. Recent studies using mammalian cultured cells revealed that Shh signaling induces dissociation of full-length Gli proteins from Sufu [31, 46], suggesting that inhibition of Sufu-Ci/Gli complex formation could be a conserved mechanism for Ci/Gli activation. Shh induces phosphorylation of full-length Gli3 that correlates with its nuclear localization and activation [31]. Although mammalian Fu is not required for Shh signaling, several kinases have been implicated in the regulation of Gli activity. For example, a kinome siRNA screen identified Cdc2l1 as a positive regulator of Shh signaling that acts downstream of Smo and upstream of Gli proteins [101]. Interestingly, Cdc2l1 can associate with Sufu to antagonize its repression of Gli in cultured cells and activate Shh pathway when overexpressed in zebrafish [101]. Furthermore, a Fu-related kinase, Ulk3, can phosphorylate Gli proteins *in vitro* and promote Gli1 transcriptional activities in cultured cells [137].

In addition to regulating Sufu/Gli association, Shh can also regulate Sufu stability through the ubiquitin/proteasome pathway in certain contexts, and thus indirectly regulates Sufu/Gli complex formation [138]. Interestingly, Sufu stability is regulated via dual phosphorylation at Ser342/Ser346 by PKA and GSK3, and blocking Sufu phosphorylation either by mutating Ser346 to Ala or by treating cultured cells with PKA inhibitors attenuates Sufu ciliary accumulation, whereas phospho-mimetic forms of Sufu exhibits increased ciliary localization [139]. The requirement of PKA-mediated phosphorylation for Sufu stabilization may account, at least in part, for the constitutive activation of Gli in PKA mutants [45].

As is the case for Cos2 in *Drosophila* Hh signaling, Kif7 also plays both positive and negative roles in mammalian Hh signaling [75-77]. A recent study suggested that Kif7 positively regulates Hh signaling in chondrocytes by destabilizing Sufu and restricting its ciliary accumulation [140]. In chondrocytes, Kif7 also inhibits Gli transcriptional activity independent of Sufu [77, 140]. Interestingly, a recent study provided evidence that Kif7 can form a complex with activated forms of Smo, and that the association requires the presence of two ciliary proteins, Evc and Evc2 [141], the products of genes responsible for the human disease Ellis-van Creveld syndrome [142, 143]. $Evc^{-/-}$ mice exhibit bone phenotypes indicative of Ihh signaling defects that mimic the human syndrome [142]. In cultured cells, Evc/Evc2 acts downstream of Smo but upstream of Gli and Sufu [141, 144]. Interestingly, Hh stimulates the binding of Evc/Evc2 to Smo depending on phosphorylation of the Smo C-tail and the primary cilia, suggesting that Evc/Evc2 may link activated Smo to downstream signaling components in the primary cilia [141, 144].

Conclusion and future prospect

Studies in the past decade have identified many kinases and numerous phosphorylation events that regulate Hh signal transduction. Biochemical and functional analyses of these kinases and phosphorylation events have provided critical insights into the Hh signal transduction mechanisms, and begun to address important biological questions such as how different levels of Hh signal are transduced. Given the power of new development in technologies such as mass spectrometry and genome-wide *in vivo* RNAi screen, it is conceivable that additional kinases and phosphorylation events will be uncovered. Despite these achievements, insights into the regulation of many key phosphorylation events are still lacking. The opposing roles of PKA in the regulation of Smo and Ci have posed a unique challenge for understanding how phosphorylation by PKA is regulated. Hh signaling could in principle downregulate PKA through a Smo-Gαi-cAMP axis [108]; however, direct evidence for a PKA activity change in response to Hh is still lacking [50]. Furthermore, downregulation of PKA does not explain how Hh stimulates Smo phosphorylation. Nevertheless, the involvement of PKA in Hh signaling does allow the pathway activity to be regulated by other GPCRs, as has been implicated by several recent studies [145, 146]. The question of how Smo phosphorylation is regulated is intimately linked to the question of how Ptc inhibits Smo. A prevalent view is that Ptc functions as a transporter, regulating the subcellular distribution of lipophilic small molecules that act as either Smo agonists or antagonists [147]. Promising candidates for endogenous Smo agonists include oxysterols that influence Hh signaling by directly binding to Smo and promoting Smo phosphorylation [100, 148]. It remains to be determined whether Ptc regulates oxysterols, and how binding of oxysterols to Smo influences its phosphorylation and activity. A re-

cent study in *Drosophila* suggests that the phospholipid PI4P acts downstream of Ptc to regulate Smo cell surface expression and activity [149]. It awaits to be determined whether PI4P acts at a step upstream or downstream of Smo phosphorylation. At the other end of the pathway, the full spectrum of Ci/Gli phosphorylation has not been explored. Although PKA-mediated phosphorylation of Ci/Gli inhibits Hh signaling, Ci/Gli may undergo other phosphorylation events resulting in pathway activation. In this regard, it is interesting to note that other pathways such as the mTOR/S6K pathway can promote Hh pathway activity by phosphorylating Gli1 in cancer cells [150]. One important future avenue would be to determine whether phosphorylation of Ci/Gli is responsible for the conversion of Ci^F/Gli^F into Ci^A/Gli^A in response to Hh stimulation.

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