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## Hedgehog signaling from the primary cilium to the nucleus: an emerging picture of ciliary localization, trafficking and transduction

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### Abstract

The unexpected connection between cilia and signaling is one of the most exciting developments in cell biology in the past decade. In particular, the Hedgehog (Hh) signaling pathway relies on the primary cilium to regulate tissue patterning and homeostasis in vertebrates. A central question is how ciliary localization and trafficking of Hh pathway components lead to pathway activation and regulation. In this review, we discuss recent studies that reveal the roles of ciliary regulators, components and structures in controlling the movement and signaling of Hh players. These findings significantly increase our mechanistic understanding of how the primary cilium facilitates Hh signal transduction and form the basis for further investigations to define the function of cilia in other signaling processes.

### Introduction

The Hedgehog (Hh) signaling pathway (Figure 1) plays a key role in tissue patterning and homeostasis in diverse species [1–8]. A striking feature of Hh signaling is its reliance on the primary cilium for signal transduction [9,10]. The antenna-like primary cilium is present in most mammalian cells and approximately 800 ciliary proteins have been identified [11,12]. Ciliary assembly begins when a pair of centrioles docks at the apical plasma membrane in quiescent or interphase (G1 phase) cells [13–16]. The mother centriole is converted to the basal body to initiate the formation of the 9+0 axoneme, typical of the nonmotile primary cilium (Figure 2). The axoneme is built from nine peripheral microtubule doublets in a cylindrical array through intraflagellar transport (IFT) that involves kinesin-based anterograde and dynein-powered retrograde transport [17–19]. Ciliary protein entry and exit is believed to be regulated by a barrier or gate at the ciliary base that encompasses the transition zone at which the triplet microtubules of the basal body transition to the doublet microtubules of the ciliary axoneme [20,21] (Figure 2). The ciliary membrane of the transition zone likely corresponds to the ciliary necklace, a region of multiple rows of intramembranous particles where the plasma membrane meets the ciliary membrane [22–24] (Figure 2). Many other regulatory processes, including the trafficking of membrane and soluble proteins, likely interact with this specialized zone. It is proposed that the unique microenvironment maintained within the primary cilium enables efficient molecular interactions and thus facilitates the process of signal transduction initiated by external

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stimuli. Many regulatory or structural proteins of the cilium are expected to modulate or transduce the signals received from the extracellular environment.

Among the major signaling pathways, the relationship between Hh signaling and the primary cilium is best understood [4,25]. The Hh signaling pathway initiates a signal transduction cascade upon Hh ligand binding to its twelve-pass transmembrane receptor Patched (Ptc/Ptch/Ptch1), relieving Ptch1 inhibition on another multi-pass membrane protein Smoothed (Smo). Hh/Ptch1 interactions lead to Ptch1 internalization [26] and reduced intensity on the cilium [27]. Concomitant with these changes, mammalian Smo accumulates on the primary cilium [28]. Increased ciliary Smo levels are associated with activation of Gli transcription factors and expression of nuclear Hh target genes (Figure 1). Suppressor of fused (Sufu) and kinesin Kif7 are two key regulators of mammalian Hh signaling and mediate signal transduction between membrane receptors and transcriptional activators [29–33]. Both Sufu and Kif7 localize to the primary cilium in a dynamic manner [31,33,34], consistent with their proposed functions on the cilium. Interestingly, Fused (Fu), a putative serine-threonine kinase initially identified as a key player of fly Hh signaling, turns out to be dispensable for mammalian Hh signaling and instead is required for ciliogenesis of 9+2 motile cilia [35]. This highlights the divergence of Hh pathway design in different species [36]. Investigating the relationship between co-option of the cilium in vertebrate Hh signaling and rewiring of the Hh circuitry during evolution will shed light on how essential cellular processes acquire new properties. Importantly, mutations that disrupt the structure of the cilium or centrosome often result in dysregulation of the Hh pathway and underlie a subset of cilia-related human pathologies or ciliopathies [37,38]. Elucidating the molecular mechanisms by which the primary cilium controls Hh signaling will provide a better understanding of disease mechanisms and offer potential targets for therapies.

## Multiple machineries that regulate localization and accumulation of Hh pathway components on the primary cilium

Several lines of evidence suggest that protein distribution on the cilium is controlled by multiple mechanisms. Proteins involved in various aspects of ciliary construction and function have been shown to affect the movement and accumulation of Hh pathway components. For instance, the machinery that regulates membrane or soluble protein trafficking could influence ciliary distribution of Hh components. In addition, the base of the cilium anchors a ciliary gate above the basal body that mediates the exchange of molecules in and out of the cilium. The transition zone makes up most of the gate and a large number of proteins and complexes in this region have overlapping and distinct functions in determining the rate and destination of proteins traversing this region (Figure 2). These transition zone-mediated events will contribute in a major way to maintaining the unique microenvironment of the cilium. A key challenge is to identify the list of proteins controlled by each mechanism and elucidate the functional connections between these systems.

Distribution of ciliary membrane proteins is regulated partly through a diffusion barrier at the base of the cilium [39]. The septin family of guanosine triphosphatases (GTPases) located at the ciliary base confers important properties of the barrier. Loss of Septin2 limits Smo accumulation in the primary cilium and inhibits Hh signal transduction [39]. It is unclear whether other Hh components are regulated in a similar manner. Surprisingly, studies have also identified signals that allow plasma membrane proteins to anchor to the cortical actin cytoskeleton underlying the plasma membrane, thus excluding them from the periciliary membrane [40]. This finding suggests that complementary mechanisms may be utilized for transporting different classes of proteins or redundant mechanisms could be used for the same proteins [41].

Proteins encoded by genes found to be associated with human nephronophthisis (NPHP), Joubert (JBTS), and Meckel-Gruber (MKS) syndrome form a few large complexes. They are involved in apical organization and cilia integrity consistent with their distinct subcellular distributions. Some of the complexes localize to the transition zone and mutations in homologs of these components result in alterations in ciliary protein composition, suggesting their role in imparting ciliary gate function [42–44]. Interestingly, MKS proteins physically interact with the Tectonic family of proteins (Tectonic 1–3) and this complex co-localizes at the transition zone [42,45]. Recent studies [42,45] demonstrated that Tectonic 1 and 2 (*Tctn1*, *Tctn2*) are required for proper ciliogenesis in some tissues and the maintenance of the ciliary membrane. Mouse knockouts of *Tctn1* or *Tctn2* exhibit tissue-specific defects in Hh signaling, which are associated with reduced ciliary localization of Smo and Arl13b [46]. Perhaps Smo interactions with the transition zone facilitate its entry into the cilium. Endeavors to define the molecular process of Smo trafficking will uncover new protein-protein interactions that contribute to communications between the cilium and other subcellular compartments or membrane domains.

The BBSome is comprised of seven highly conserved Bardet-Biedl syndrome (BBS) proteins and one novel protein, which function as a coat complex to transport membrane proteins between the plasma and ciliary membrane. Notably, overexpressed Smo or Ptch1 physically interacts with several BBS proteins with BBS1 showing the strongest binding [47]. Loss of the BBSome components leads to increased accumulation of Smo and Ptch1 in cilia although Hh response is reduced in assays conducted in mouse embryonic fibroblasts [47,48]. This phenotype is reminiscent of that observed in *Dync2h1* mutants [49] and raises the possibility that Smo may fail to exit the cilium efficiently in the absence of the BBSome. Alternatively, the BBSome may participate in the exit of Ptch1 from the cilium or in the conversion of Smo into an active state. A genetic interaction between the BBSome and IFT proteins in mammalian cilia is revealed by more embryonic phenotypes in *Ift88/Bbs7* double mutants than single mutants [47]. By contrast, no physical interactions are detected between the BBSome and the NPHP/JBTS/MKS complex [42]. Biochemical assays and cell biological studies are required to reveal how the BBSome regulates Smo/Ptch1 translocation between the plasma membrane and ciliary membrane and how the BBSome functionally interacts with other protein complexes in this process. Since mice lacking the BBS genes do not exhibit the classical Hh defects, the BBSome likely modulates the transport of Hh components in conjunction with other machineries.

Similarities between the ciliary localization signal (CLS) and the classic nuclear localization signal (NLS) support the idea that analogous mechanisms could regulate nuclear and ciliary import [50]. In this model, the cilium contains a selective transport system at its base similar to that in the nuclear pore. This notion is reinforced by the discovery that manipulation of Ran GTPase activity, known to control the NLS-importin complex, also affects Kif17 translocation to the cilium [50]. Moreover, both nucleoporins and importins that control nuclear entry localize to the ciliary base, and inhibition of these proteins blocks the ciliary entry of kinesin-2 and Kif17 motor proteins (Figure 2) [51]. Whether this system regulates transport of Hh pathway components has not been reported. Likewise, elucidating the connection between this system and others such as IFT and the transition zone will provide new insight into protein trafficking on the cilium.

## Trafficking of Hh pathway components on the primary cilium

A major event associated with active Hh signaling is the increased levels of various Hh components along the primary cilium or in a ciliary subdomain. Hh ligand binding to its membrane receptor Ptch1 results in a dramatic reduction in Ptch1 intensity on the cilium [27] and a concomitant increase of ciliary Smo levels [28]. This is consistent with a model in

which exit of Hh-bound Ptch1 from the cilium allows Smo entry to the cilium. As discussed in this review, studies that manipulate retrograde ciliary transport or other machinery revealed increased Smo levels on the cilium without Hh ligand stimulation [52,53]. This suggests the presence of dynamic movements of Ptch1 and Smo in the cilium even in the absence of active Hh signaling. Our knowledge of this important process remains limited due to the inability to follow the movement of Hh components in real time and at physiological levels. Moreover, reagents currently available to detect ciliary distribution of Hh components do not yield the spatial resolution required to define their distributions in subdomains of the cilium. A key unresolved issue is how to design assays to probe the functional consequences of trafficking of Hh components on the cilium. Despite these deficiencies, recent studies of ciliary regulators and components have uncovered multiple mechanisms that control the movement of Hh players.

The axoneme is assembled via IFT that consists of kinesin-mediated anterograde and dynein-powered retrograde transport. It is proposed that disruption of IFT perturbs cilia architecture that subsequently leads to perturbed signaling [49,54]. Inactivation of retrograde transport, for instance, by mutating the *cytoplasmic dynein-2 motor heavy chain protein 1*, *Dync2h1*, swells the cilium due to an increased amount of ciliary proteins. Indeed, Smo, Ptch1 and Gli2 levels are also elevated in the cilium. Retention of Hh signal transducers on the cilium caused by defective retrograde transport could impair Hh signal transduction beyond the cilium and result in reduced Hh signaling [49]. Unexpectedly, defective retrograde transport originating from mutations in either *Ift122* or *Thm1 (Ift139)* also results in accumulation of Hh components on the cilium but Hh signaling is enhanced, implying a different underlying mechanism [55,56]. It is possible that in *Ift122* or *Thm1* mutant cilia, amassed Hh components may have undergone constitutive activation at a particular ciliary subdomain due to aberrant retrograde transport. A role of IFT in transporting Hh components is also revealed through analysis *Ift25* mutant mice. Loss of *Ift25* does not affect ciliary assembly but leads to increased Ptch1 and Smo levels on the cilium [57]. This leads to the hypothesis that IFT25 is required not only for ciliary export of Smo during the inactive state but also for Ptch1 export during the active state. Since Hh components contain multiple positive and negative effectors, defective IFT could produce a complex output depending on the extent to which individual Hh component is affected by a specific combination of genetic perturbations.

## Hh signal relay from the primary cilium to the nucleus

Several Hh pathway components function downstream of Smo to transduce the Hh signal in the cilium, cytoplasm and the nucleus. They include Sufu, Kif7, protein kinase A (PKA) and three Gli transcription factors (Gli1–3). These Hh players exhibit a complex interaction to generate graded Hh responses through a combination of positive and negative regulations. Uncovering the biochemical mechanisms that underlie the interactions of Hh components and identifying the location and sequence of actions will provide new insight into how Hh signaling controls key biological processes.

In the absence of Smo activation, the majority of Gli3 undergoes limited proteolysis mediated by the proteasome to produce a transcriptional repressor (GliR) that blocks Hh target gene expression in the nucleus. Upon Hh pathway activation, GliR formation is inhibited and Gli activators (GliA) derived from the full-length Gli proteins (primarily Gli2) are produced and stimulate Hh target gene expression in the nucleus (Figure 1). Smo accumulation on the primary cilia is accompanied by increased Gli protein localization to cilia tips and the nucleus [53,58].

### Smo trafficking and activation on the primary cilium

Besides the ciliary gate that limits Smo diffusion, another distinct domain on the cilium has been identified recently that orchestrates Smo activity and movement. In Hh-responsive cells, the ciliary proteins Evc and Evc2 localize to a region lying above the transition zone and act downstream of Smo to relay the Hh signal to a subsequent step that involves Sufu/PKA [59–62]. Importantly, the exogenous Evc/Evc2 complex was found to co-localize with Smo within the EvC-expressing zone and can also physically associate with Smo (at least with crosslinker) in response to Hh pathway activation. It is postulated that activation and conformational change of Smo takes place in this distinct Evc/Evc2(+) domain on the cilium [59]. Consistent with this model, the Evc complex is disrupted in human Ellis-van-Creveld syndrome and Weyers acrocardial dystosis, two ciliopathies characterized by skeletal dysplasia [63]. However, the limited tissue expression of Evc/Evc2 and the mild phenotypes associated with *Evc/Evc2* mutations in mammals raise the question of whether Evc/Evc2 merely represent a tissue-specific mode of Hh regulation.

### PKA controls Gli protein function via the primary cilium

cAMP-dependent protein kinase A (PKA) is a major negative regulator of Hh signaling in various overexpression studies *in vitro* and *in vivo*. PKA localizes to the base of the primary cilium, adjacent to the centrosome marker [64,65] and in proximity to the proteasome and the Skp1-Cul1-F-box (SCF) protein ligase complex [66,67]. New insight into how PKA controls Gli2 and Gli3 activity in relation to the cilium was garnered by the production of mice in which *PKA* is genetically ablated. Embryos lacking PKA activity exhibit full activation of Hh signaling [65], placing PKA in the same category as Ptc1 based on their ability to repress Hh signaling. This system also offers a pertinent genetic setting to examine the molecular mechanisms by which PKA controls Hh signaling. Analysis of neural tube patterning in *PKA*-deficient mice reveals a major function of PKA in repressing Gli2 activator production. Moreover, when the primary cilium is disrupted in a *PKA*-deficient background, Hh signaling fails to be activated, suggesting that inhibition of Hh signaling by PKA is dependent on primary cilia [65]. While PKA does not affect Smo ciliary localization, Gli2 is enriched in *PKA*-deficient cilia tips even in the absence of Hh stimulation [65]. These results argue that modification of Gli2 and Gli3 by PKA at the cilium base is a crucial event in controlling Gli activity and trafficking. The exact site and sequence of action cannot be deduced from the current knowledge and would require a better molecular characterization of the biogenesis and movement of the different forms of Gli. Interestingly, identification of a ciliary G-protein-coupled receptor, Gpr161, which can increase cAMP levels and Gli3 processing, provides a link between Hh signaling and PKA activity [68].

### Multiple roles of Sufu in Hh signaling

Suppressor of Fused (Sufu) is another major negative regulator of mammalian Hh signaling. Genetic studies have shown that the Hh hyperactivation in *Sufu* knockout neural tube depends on the enhanced activator activity of Gli1/2 and to a lesser extent decreased levels of Gli3 repressor [69–71]. Furthermore, cell-based studies have unveiled a positive role of Sufu in Hh signaling, likely due to the involvement of Sufu in maintaining sufficient Gli protein levels that are necessary for activator production [72]. This idea is further strengthened by genetic analysis in the mouse neural tube. Removal of *Sufu* in *Gli1* mutants results in failure to specify the ventral-most cell types in the spinal cord (such as the floor plate and V3 interneurons) that require maximal Hh signaling activity [70]. By contrast, *Gli1* mutant mice are viable with no apparent neural tube defects [73].

Upon Hh pathway activation, Sufu accumulates in the primary cilium, a process that relies on the presence of ciliary Gli2/3 [74,75]. Treatment with forskolin, which activates PKA by

raising cellular cAMP levels, abolishes ciliary localization of Gli2/3 and Sufu [75]. Surprisingly, forskolin can also block constitutive accumulation of Gli2 at the cilium tips in *PKA*-deficient cells [65]. This suggests that the negative effects of forskolin on ciliary translocation of Gli2/3 and Sufu and Hh signaling could be independent of PKA activity. For instance, forskolin may affect the cilium environment through the action of cAMP-gated channels unrelated to PKA [76,77]. Moreover, forskolin-induced Smo translocation to the cilium base [78] does not occur in *PKA*-deficient cells. These findings underscore the importance of genetic analysis to complement cell-based assays [65].

Sufu inhibits Hh signaling in the cytoplasm by preventing the conversion of Gli3 into a transcriptional activator [58,75] and by sequestering Gli2 [79,80]. Recent work also suggests that Hh signaling triggers dissociation of Sufu from full-length Gli2/Gli3 [58,75]. This would lead to the production of a labile form of active Gli proteins that accumulate in the nucleus to stimulate Hh target gene expression. In *Kif3a*<sup>-/-</sup> cells, in which the primary cilium fails to form, Sufu does not dissociate from Gli2/3 upon treatment of a Smo agonist (SAG). This observation bolsters the claim that dissociation of Sufu and Gli2/3 upon Hh activation depends on the primary cilium and that Sufu/Gli dissociation may take place in the primary cilium [58,75]. Since the absence of Sufu/Gli dissociation without the cilium could be due to lack of Smo activation, the exact location and relevance of Sufu-Gli dissociation during Hh signal transduction cannot be unambiguously assessed. This issue is further complicated by the finding that Sufu still exerts its full effects on Gli proteins in the absence of primary cilia [69,72]. A recent study reported impaired Sufu dissociation from Gli2 in *Kif7*-deficient keratinocytes but this results in a relatively mild Hh phenotype [79]. All of these point to the importance of testing the functional consequences of Sufu-Gli association and dissociation.

### **Kif7's dual function on Hh signaling depends on the primary cilium**

*Kif7* plays both positive and negative roles in Hh signaling. *Kif7* primarily localizes to the base of the primary cilium in the absence of Hh signaling, but is enriched at the ciliary tip when the Hh pathway is activated [31,33]. Consistent with its localization, *Kif7* activity depends on the presence of an intact primary cilium [31], similar to the requirement of the primary cilium for PKA activity [65], but is distinct from the unaltered *Sufu* phenotype without cilia [69,72]. *Kif7*-deficient embryos exhibit defects related to decreased levels of Gli3 repressor and enhanced activity of Gli2 activator, although the phenotype is mild [31–33] in comparison with that in *PKA*- [65] or *Sufu*-deficient embryos [29,30]. A reduction of Gli3 repressor levels in *Kif7* mutants [31–33] suggests that *Kif7* controls Gli3 repressor formation. Since PKA, the proteasome, the SCF E3 ligase components and Sufu all participate in Gli3 repressor formation [58,81], it is possible that they act in concert to control the production of Gli3 repressor. One can speculate that Sufu-Gli3 may be modified in the cilium through *Kif7*, rendering Gli3 a better substrate for partial proteolysis. This allows subsequent PKA phosphorylation of Gli3 at the ciliary base and targets Gli3 for ubiquitination and processing by the proteasome [65]. The molecular basis of cilium control of Gli3 repressors requires additional studies.

*Kif7*'s positive effect on Hh signaling contributes to floor plate patterning, which needs maximal Hh signaling activity. Removal of *Kif7* in a *Sufu*-deficient background has no consequences on ectopic formation of floor plate observed in *Sufu* mutant embryos [82]. This could be interpreted by a model in which *Kif7* executes its positive function by inhibiting Sufu. Alternatively, *Kif7* and Sufu could act in parallel to control Hh signaling. In this case, removal of *Kif7*'s positive function fails to counteract global Hh activation due to loss of *Sufu*. In *Kif7* mutant chondrocytes, ciliary localization of Gli2/3 and Sufu is increased. This phenotype resembles that in *PKA* mutants and suggests that *Kif7* may

restrain Gli2/3 from translocating to cilia tips [83]. Similarly, the molecular mechanisms by which Kif7 controls Gli2/3 trafficking and the functional consequences remain unclear.

The dual function of Kif7 is reminiscent of its *Drosophila* ortholog Costal 2 (Cos2). Cos2 promotes repressor formation of Ci (Gli ortholog) by scaffolding kinases for Ci phosphorylation in the absence of the Hh ligand; Cos2 also exerts a positive function through its association with active Smo to relay the Hh signal [7]. The primary cilium may have assumed the scaffolding role of ancient Cos2. Whether the requirement of Cos2/Smo association to transduce the Hh signal is substituted by other proteins in mammals awaits further investigation.

## Conclusion

Hedgehog signaling is a central developmental pathway uniquely tied to primary cilium function. Future studies that focus on live imaging, functional assays and phenotypic analysis will provide insight into how the primary cilium controls trafficking and activity of Hh components and discern the direct effects of cilia in these processes.

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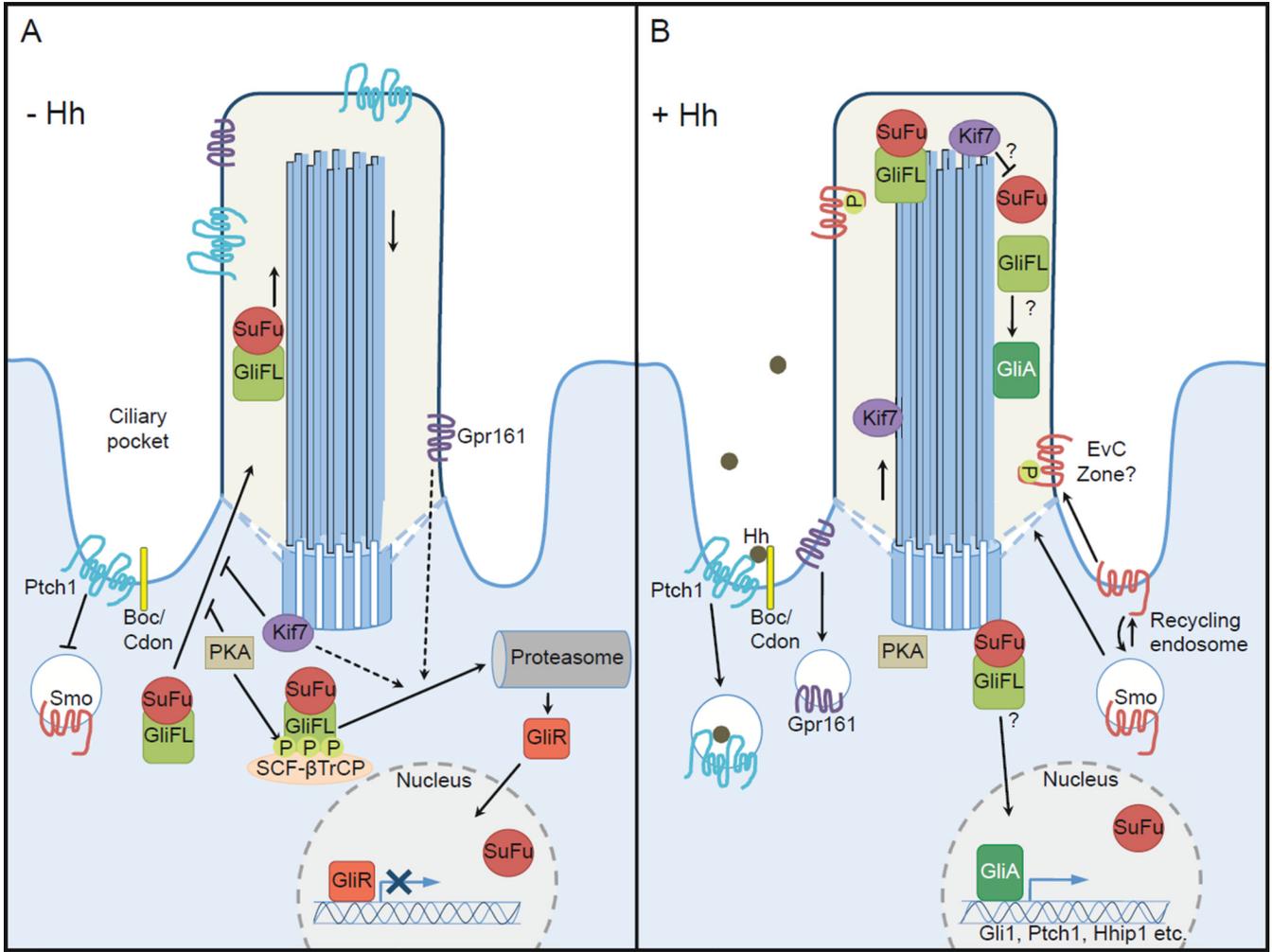
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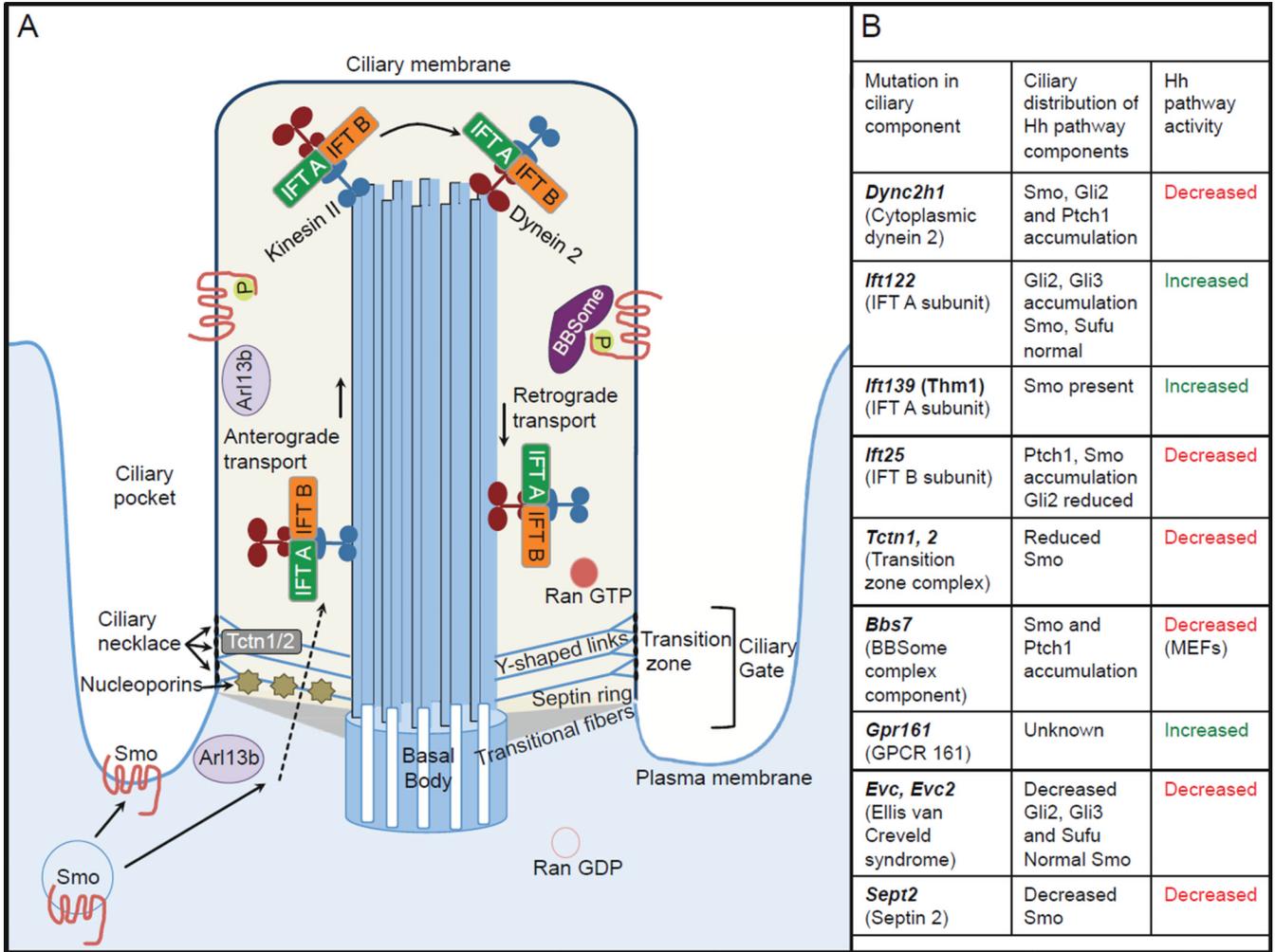
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**Figure 1. The mammalian Hh signaling pathway**

**A.** In the absence of the Hh ligand, the Hh receptor Patched (Ptch1) inhibits the accumulation of the signal transducer, Smoothened (Smo), on the ciliary membrane. As a result, at the base of the primary cilium, PKA and Kif7 promote proteolytic processing of the transcription factor Gli3 by the proteasome into a repressor form (GliR) that suppresses Hh target gene expression in the nucleus. In addition, SuFu stabilizes the Gli proteins and inhibits the transcriptional activity of Gli2, while PKA prohibits the accumulation of full-length Gli2 (GliFL) in the cilium. All of these events ensure silencing of the Hh pathway without the ligand.

**B.** The Hh ligand binds to its receptor Ptch1 and co-receptors Boc/Cdon. Ptch1 is internalized with Hh, relieving the inhibition on Smo. Smo accumulates in the ciliary membrane through both lateral transport and the secretory pathways. Phosphorylation of Smo, for instance, at the EvC zone in osteoblasts leads to its dimerization and activation. This in turn abrogates PKA function and promotes the movement of SuFu-Gli2/3 complexes and Kif7 to the ciliary tip and perhaps dissociation of Gli2/3 from SuFu in this process. Kif7 also facilitates the trafficking of Gli2/3 into the cilium (for example, in chondrocytes). Accumulation of Gli2/3 at the ciliary tip is associated with the production of Gli activators (GliA), which are derived from the full-length Gli proteins. Accumulation of GliA to the nucleus enables activation of Hh target genes such as Ptch1, Gli1 and Hhip1.



**Figure 2. Control of trafficking of Hh components on the primary cilium**

**A.** The primary cilium is anchored at the plasma membrane through transitional fibers emanating from the basal body. The ciliary membrane maintains its unique membrane composition through multiple mechanisms including a diffusion barrier surrounding the ciliary base. Ciliary proteins pass through the ciliary gate to enter and exit the cilium. The ciliary gate is mostly made up of the transition zone. This specialized region is characterized by Y-shaped links that demarcate the ciliary neck and connect the axoneme to the ciliary membrane. Septin ring and a nuclear pore-like structure also contribute to the ciliary diffusion barrier at its base.

The transition zone contains several protein complex networks that function in ciliary assembly and sorting. For instance, transition zone proteins Tectonic 1 and 2 participate in targeting Smo and Arl13b to the primary cilium in some cell types. Septin appears to confer important barrier properties at the ciliary base and is known to control Smo trafficking. An intraflagellar transport (IFT) system is responsible for ciliary construction and maintenance. It consists of the IFT particles (A and B complexes) that are moved along the axoneme through their interactions with the anterograde (kinesin II) and retrograde (dynein 2) motors. IFT is known to be involved in Hh signal transduction. Several components of the IFT complexes have been shown to influence the distribution of Hh components on the cilium and affect Hh signaling in a complex manner. For instance, disruption of IFT139 (THM1) in IFT A complex or IFT122/25 in IFT B complex both leads to accumulation of Hh

components on the cilium but Hh signaling is enhanced in *Thm1/Ift122* mutants while it is reduced in *Ift25* mutants. The coat-like BBSome complex is involved in the trafficking of molecules between the plasma and ciliary membrane and may contribute to the dynamic ciliary distribution of Smo and Ptch1. Taken together, these studies highlight the fact that multiple mechanisms regulate ciliary protein distribution and understanding how they interact to control Hh component distribution and signaling on the cilium is a key unresolved issue.

**B.** Summary of changes in ciliary distribution of Hh pathway components and Hh pathway activity in various mutants that affect ciliary structure and function.