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The Primary Cilia, a "Rab"-id Transit System for Hedgehog Signaling

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Summary

Intense focus has been centered around how the primary cilia transduces the hedgehog signal from Smoothened to the Gli transcription factors. New data indicates that ligand and signaling lipids help regulate small GTPase-dependent accumulation and activity of signaling components.

Introduction

Across the metazoan phyla, hedgehog (Hh) signaling plays a crucial role in organogenesis as it promotes proliferation and migration of stem cells in the body. Inappropriate Hh signaling results in a panoply of developmental defects and cancers and is implicated in the induction, maintenance and/or metastasis of up to 25% of human tumors [1]. While the pathway has been studied intensively, particular focus has been centered recently around how the serpentine membrane receptor smoothened (Smo) interprets the Hh signal to the Gli/Cubitus interruptus (Ci) zinc finger family of transcription factors, as this interpretation dictates the magnitude and quality of the resultant Hh-dependent target gene induction. Ongoing pathway studies in *Drosophila* have identified conserved components and pathway logic. However, studies of the pathway in higher vertebrates resulted in a major paradigm shift in our understanding of the pathway with the discovery that Hh signaling in normal development requires components of the primary cilia. Genetic studies in mice and humans uncovered a set of mutants resembling hedgehog mutants, but which contain mutations in components of the small, antenna-shaped organelle [2–4]. Resulting questions have emerged as to how the cilium accomplishes it's signaling responsibilities, and whether all or part of hedgehog signal transduction requires an intact cilium. This review will detail recent genetic and biochemical studies of the hedgehog pathway that give a glimpse of the inner workings of this novel organelle.

Primary cilia are cellular sensory transducers

Primary cilia are small microtubule-based organelles that use intraflagellar transport (IFT) of membrane-bound cargo along microtubules for regulating cell division, signal processing and cellular movement [3,5–8]. The ciliium can be found on most vertebrate and many

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Conflicts of Interest
None Perceived

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lower eukaryotic cells from Chlamydomonas to human and emerges from the older of the two centrioles after mitosis. The best example of how cilia transduce environmental signals comes from adhesion-based gamete selection in *Chlamydomonas reinhardi*, where the tip of the cilium adheres to an appropriate gamete and induces tyrosine kinase-dependent cascades within the cilium [9]. These signals depend not on coated pits or caveolae, but on ciliary IFT to induce the resulting gamete fusion and zygote formation. Experiments using a temperature-sensitive mutant in Kinesin II, an integral component of IFT, show that IFT helps transmit the adhesion signal, although the molecular details of regulating gamete fusion and other cilia-dependent signaling events remains poorly understood.

The logic of the hedgehog pathway

In both the invertebrate and vertebrate pathways, Hh ligand binds and inhibits the Patched (Ptch) receptor, allowing Smo to tip the balance of activity of the Gli/Ci proteins from repression to activation [10]. Smo does this both by inhibiting Ci repressor and inducing the accumulation of the Ci activator. First, Smo blocks protein kinase-dependent Gli3/Ci repressor formation that occurs through the cleavage of full-length Gli3/Ci into a smaller transcriptional repressor. Blocking this cleavage allows the expression of derepressed Hh target genes. For higher levels of target gene induction, Smo also enhances the full-length, activator forms of Gli/Ci. In flies, the atypical kinesin protein costal2 (Cos2) is a key interpreter of the signal from Smo. It does so by scaffolding Ci on vesicles with cleavage-promoting kinases, resulting in Ci phosphorylation and subsequent repressor formation [11]. With the addition of Hh binding to Ptch, Smo relocalizes to the plasma membrane and forms a distinct signaling complex with Cos2 away from the kinases and preventing efficient Ci repressor formation. Cos2 plays an additional role in stimulating activator forms by scaffolding the kinase Fused, which acts positively to increase the transcriptional activity of full-length Ci [11,12]. How Cos2 tethers Smo in the cytoplasm and what regulates Smo movement has not been identified.

Distinct Regulatory Pathways within the Cilia

Increasing data suggests the primary cilium serves an analogous function as Cos2 in higher vertebrates in forming repressor and activating transcriptional activators. Ci transcriptional activator and repressor functions have been subsumed by the activator functions of Gli1 and Gli2 while the repressor functions are conferred on Gli3 and to a lesser extent Gli2. In contrast, Cos2 homologs KIF7 and KIF27 do not have a strict hedgehog phenotype, suggesting greater functional redundancy than exists in Drosophila [13,14]. Initial observations of murine primary cilia mutants indicate that they phenocopy aspects of the Sonic hedgehog (Shh) mutant phenotype in the neural tube [2], have an impaired ability to cleave Gli3 into a repressor, and fail to stimulate Gli2 into an activator. Further support for a direct role of the organelle in processing comes from the colocalization of all three Gli proteins and Smo with acetylated tubulin, a cilium marker [15,16]. While suggestive of a role for the cilium in regulating the pathway, the critical question remained how pathway components were regulated.

Two new studies indicate that Ptch and Smo entry and exit from the cilium is a critical step in pathway regulation [17]. Previous studies performed with overexpressed proteins at single time points lacked an accurate appreciation of the kinetics of signaling [15]. Using newly made antibodies that detected endogeous Ptch and Smo, the authors found that Ptch accumulates in a skirt-like distribution at the base of the cilia, near the ciliary necklace [18]. By contrast, Smo is excluded from the cilia. Fluorescently-labeled Shh added to cultured cells accumulates at the primary cilium with Ptch. Surprisingly, Shh addition caused Ptch levels to decrease in the skirt area while Smo levels accumulate as signaling begins. These

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observations support the idea that the cilium senses Shh levels and that Ptch-containing vesicles indirectly inhibit the movement and or accumulation of Smo in the cilia.

New genetic mutations in ciliary components also now separate the processes of Gli activator and repressor creation in the cilium. Strong cilia mutations that affect core IFT functions result in both the inability of the Gli3 repressor and the Gli1 and Gli2 activators to be formed at high levels and fail to provide additional insight into the individual pathways. A recent mouse mutation hennin, also identified by a forward genetic screen, encodes a small GTPase (see below). Hennin mutants are weaker than other mutations previously identified in the same screen and retain partial cilium structure [19]. Interestingly, Gli3 repressor functions of hennin-mutant cilia are retained, while accumulation of the Gli2 activator is defective. These data argue that the cilium contains two distinct hedgehog-dependent signaling processes, one for hennin-dependent activator formation and one for repressor formation. Given that pathway members assemble on vesicles, this data argues for the existence of two separable populations of Gli-associated vesicles within the cilia.

A “Rab-id” Transit System controls membrane shape and movement

What controls the precise trafficking and accumulation of Smo and Gli proteins in the cilium? Kinetic studies demonstrate that intraflagellar transport (IFT) maintains ciliary structure and coordinates rapid, bidirectional transport of vesicles between the cytoplasm and the distal tip of the cilium. While the role of these proteins is well-documented and has been the subject of many reviews [2–4], how the bidirectional transport regulates distinct signaling complexes remains unknown. Moreover, due to a lack of a conditional IFT mutant in hedgehog-responsive cells, direct evidence for how IFT regulates Gli activator and repressor accumulation remains elusive.

However, several recent studies suggest movement of signaling components within the cilium is critical and that Gli protein modification is accomplished by an army of small GTPases that move and tether distinct populations of vesicles. Small GTPases are related to the oncogene Ras and subdivided into several subfamilies including the Rab, ADP-Ribosylation (Arf) and Arf-like (Arl) groups. These proteins function as molecular switches that toggle between GTP (on) and GDP (off) states through the actions of GEFs (on) and GAPs (off). They perform the work in moving or tethering a vesicle from one subcompartment to another through the assembly of large compartment-specific effector complexes onto cytoplasmic motors [20,21].

Recent studies now bring the total to seven the small GTPases that play a role in the structure and/or function of the organelle in hedgehog signaling. That the number and phenotypes of these mutants come from this class of proteins speaks strongly for the important role for small GTPase both in the general function of the cilia and more specifically in regulating components of Hh signaling.

Previous studies examining the components of the primary cilia had noted several key structural components that were small GTPases[3,4,22]. Joining other GTPases Arl 6 /BBS3 [23], Rablike5 / IFTA-2 [24], and Rablike 4/IFT27 [25] in the general control of cilia membrane biogenesis is Rab8. Previous studies with Rab8 support its role in directing the trafficking of post-golgi vesicle to polarized apical membranes [26,27]. Biochemical analysis found that the eight core proteins of the Bardet-Biedl Syndrome (BBS) formed a tight complex called the BBsome [28]. Previous genetic analysis had implicated these proteins in this pleiotropic syndrome with patterning and polarity defects, obesity, and photoreceptor abnormalities not unlike those seen in primary cilia and Shh mutations [29–31]. In complex with the core BBsome was Rab8 and the Rab8GEF, Rabin8, which were shown [28], and confirmed independently [32], to be required for ciliary structure. These

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studies link the proteins of the BBsome with general effectors of ciliary function and suggests that Rabin8 stabilizes the Rab8GTP form to direct vesicular movement from the peripheral satellites into the cilia. In vivo results suggest some functional redundancy exists. While overexpressed GDP-locked Rab8 has a mild BBS-like phenotype in the retina [28], Rab8A-deficient mice do not display the classic polarity phenotypes seen in IFT mutants [27]. Additional experimentation will be required to determine if Rab8 isoforms or other small GTPases such as Rab17 [32] that also functions in ciliary formation, may compensate for Rab8A.

More specific to the regulation of the Hh signaling pathway are two small GTPases, Rab23 and Arl13B, that appear to balance the levels of Gli2 activator. Rab23, identified by the Anderson lab with other cilia mutants affecting Shh signaling, renders the Shh pathway constitutively active when lost [33,34]. Consistent with this mouse phenotype, human Rab23 mutations have also been found in Carpenter syndrome that causes craniosynostosis, polysyndactyly, obesity, and cardiac defects [35]. Shh pathway analysis demonstrates that Rab23 plays a minor role in promoting Gli3 cleavage to form Gli3 repressor but has a critical role in preventing the accumulation of the Gli1 and Gli2 activators [33].

Mutant Arl13b has a complementary phenotype to that of Rab23 mutants [19]. Arl, or Arf-like small GTPases function, in part, to regulate the tethering of endosome-derived transport vesicles [36]. Mutants of the hennin locus that encodes mouse Arl13b, have low levels of Gli2 activator function as indicated by the low Shh target gene induction in the ventral and middle of mutant neural tubes. In contrast, biochemical analysis supports the fact that like Rab23 mutants, Gli3 repressor can still form in Arl13b mutants. With its localization to the base of the cilia, Arl13B may function to antagonize Rab23-dependent inhibition of Gli2 activator accumulation. An intriguing possibility is that Arl13B effector complexes assemble, while Rab23 effector complexes dissolve, the Gli2 activator complex. Of further interest is the fact that newly identified mutations Fantom, a protein associated with a GTPase regulator [37], and two novel transmembrane proteins Evc [38], and Tectonic [39], each localize to the cilia and affect Shh signaling. The phenotypes of these mutants suggest that these the three proteins may function with Arl13b and in opposition to Rab23-dependent inhibition of Gli2 activator accumulation.

Signaling Lipids and Ligands Direct Traffic

While movement and targeting of membrane-associated signaling complexes seems likely to constitute the key step in determining the strength and duration of signaling, the intriguing question remains what regulates the traffic flow of the vesicles to their particular compartments? Studies over the past few decades have demonstrated that the distribution and composition of lipids possessing signaling properties accumulate differentially in different subcellular compartments [40]. The best known signaling lipid is inositol phosphate, whose metabolites play a well-established role in regulating vesicle trafficking during endocytosis through promotion of lipid -protein interactions [41,42]. Interestingly, the BBS5 component of the BBsome, contains two pleckstrin homology domains and binds to specific phospoinositides [28], supporting a role for ciliary vesicle trafficking.

Several clues implicate other lipid signaling molecules and ligands as traffic directors through Ptch in regulating Smo activity. Ptch is a member of the 12 pass transmembrane proteins containing sterol sensing domains (SSD). The best characterized member of the family is the Sterol response element binding protein (SREBP)-cleavage activating protein (SCAP) which controls the subcellular localization and activity of SREBP. High levels of cholesterol bind to the SSD-containing SCAP and allow the trafficking of SREBP from the endoplasmic reticulum to the golgi where golgi-specific enzymes release SREBP to enter

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the nuclease and downregulate cholesterol biosynthetic genes [43,44]. Screening of lipid ligands led to the discovery that oxysterols such as 25-hydroxycholesterol could activate Shh signaling in the absence of Shh [45,46]. With antibodies to endogenous Smo and Ptch it was also demonstrated that oxysterols could also allow accumulation of Smo in the cilia [17]. However, unlike Shh that moves Ptch out of the cilia, oxysterols function to allow Smo trafficking despite the presence of the inhibitory effects of Ptch. This argues that oxysterols induce a conformational change in Smo to make it Ptch-resistant. The observation that oxysterols do not bind to Smo but regulate Smo trafficking much like that of the synthetic Smo agonist SAG [45] suggest that oxysterols affect Smo function either by altering membrane dynamics or act indirectly through an as yet unidentified oxysterol-binding protein.

In summary, intense study of how the primary cilia transduces signals from Smo to the Gli transcription factors have led to enormous insight into the organization and regulation of vesicle-based signaling. The overall pathway logic has survived the divergence between Drosophila and higher vertebrates, but the use of the primary cilia small GTPase transit system to partition signaling complexes seems to afford greater control and potential cross talk opportunities with other signaling pathways. Uncovering how the Rab and Arl effector complexes partition signaling in response to ligands and signaling lipids are likely to uncover additional ways to intervene therapeutically as well.

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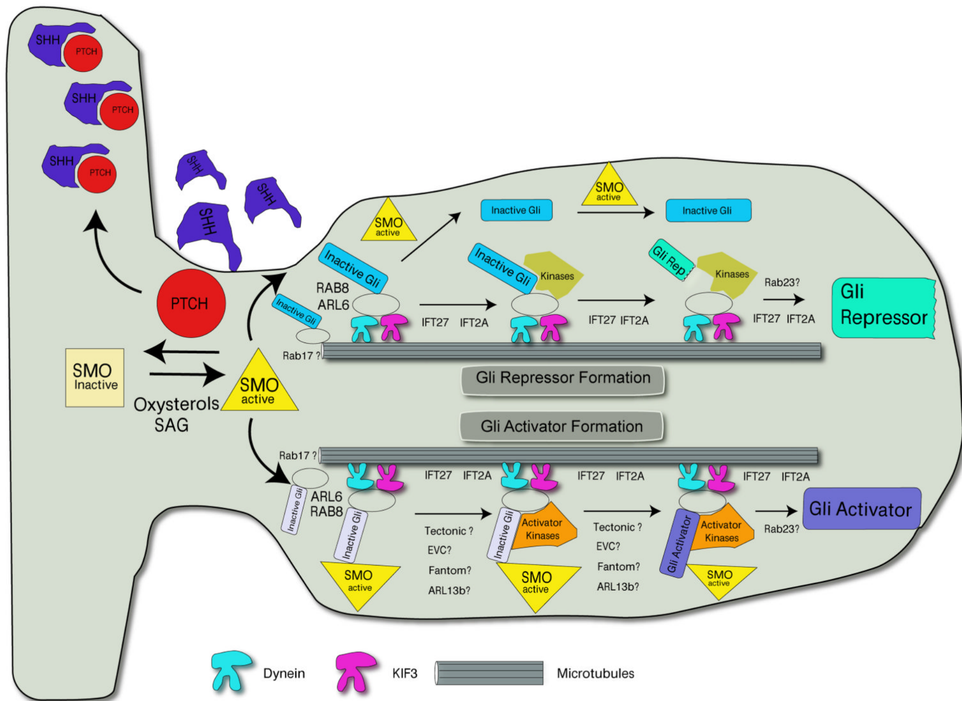

Figure 1. Trafficking Shh signaling components

Model for the movement of Shh signaling components in the primary cilia. In the absence of Shh, Ptch blocks Smo trafficking to the cilia. Shh binding to Ptch moves Ptch out of the cilia, allowing Smo to become active in the cilia. Oxysterols and the synthetic Smo agonist SAG alter Smo to become Ptch-insensitive and enter the cilia. Distinct Gli dependent protein complexes appear to assemble with the help of small GTPases to allow activator and repressors to form.

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Small GTPase	GAP/GEF	Mutation	General Function	Effector Complex
Rab23	EVI5Like GAP [29]	Openbrain [31] Carpenter's Syndrome [32]	Inhibition of Gli activators	
Arl13b		Hennin [17]	Production and sequestration of Gli activator	
Rab8	Rabin8 GEF [25,29]	Microvillus Inclusion Disease? [24]	Vesicle Trafficking to apical membranes	BBsome?
Rab17	TBC1D7 GAP [29]			
IFT27			Intraflagellar Transport	
IFT-2A			Intraflagellar Transport	
Arl6		Bardet-Biedl Syndrome [20]	Ciliary Membrane Trafficking	BBsome?

Figure 2.
Identified small GTPases and their function in primary cilia

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